Effects of feeding sunflower oil or seal blubber oil to horses with recurrent airway obstruction

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Abstract

A crossover feeding trial was performed with 9 horses suffering from recurrent airway obstruction (RAO). The study aimed to determine whether ingestion of sunflower oil (SFO), rich in linoleic acid, or seal blubber oil (SBO), a source of long-chain omega-3 polyunsaturated fatty acids (LC n-3 PUFAs), changes the fatty acid (FA) ratios in plasma and leukocyte membrane phospholipids (PLs) or the leukocyte numbers or proportions of cell types in the airways. We also investigated diet-related changes in respiratory rate, maximum change in pleural pressure (ΔPpl_{max}), dynamic compliance (C_{dyn}), and pulmonary resistance (R_L). Each animal was fed hay and oats supplemented with 320 mg/kg body weight (BW) of either SFO or SBO for 10 wk. Before and after the feeding periods, we performed FA analyses, cytologic testing of the pulmonary epithelial lining fluid (PELF), clinical scoring, and pulmonary function testing. The results demonstrated that supplementary FAs were readily ingested and incorporated into leukocyte cell membranes. The n-6:n-3 FA ratios in plasma and leukocyte PLs were reduced after SBO supplementation, as were the PELF leukocyte counts ($P < 0.05$). On the other hand, pulmonary function and clinical signs were not markedly changed by the different dietary FAs. These results indicate a possible influence of dietary n-3 PUFAs on the pulmonary inflammation of horses with RAO. Further studies are warranted to address effects on inflammatory mediators and clinical outcome.

Résumé

Une expérience d'alimentation en croisée a été effectuée chez neuf chevaux souffrant d'obstruction respiratoire chronique (RAO). L'étude visait à déterminer si l'ingestion d'huile de tournesol (SFO), riche en acide linoléique, ou d'huile de gras de phoque (SBO), une source d'acides gras poly-insaturés à longue chaîne omega-3 (LC n-3 PUFAs), changeait les ratios d'acides gras (FA) dans le plasma et les phospholipides membranaires des leucocytes (PLs) ainsi que le nombre ou les proportions des leucocytes dans les voies respiratoires. Une évaluation a également été faite de l'effet de la diète sur des changements dans le rythme respiratoire, le changement maximal de la pression pleurale (ΔPpl_{max}), la compliance dynamique (C_{dyn}) et la résistance pulmonaire (R_L). Chaque animal a été nourri avec du foin et de l'avoine supplémentés avec une quantité de 320 mg/kg de poids corporel (BW) soit de SFO ou de SBO pendant 10 semaines. Avant et après les périodes d'alimentation, des analyses de FA ont été effectuées, une évaluation cytologique du liquide bordant l'épithélium pulmonaire (PELF) a été faite, et un pointage clinique et une évaluation de la fonction pulmonaire ont été effectuées. Les résultats ont démontré que les FA supplémentaires étaient rapidement ingérés et incorporés dans les membranes cellulaires des leucocytes. Bien que les fonctions respiratoires et les signes cliniques étaient peu modifiés par les différents FA alimentaires, les ratios n-6:n-3 dans le plasma et les PLs des leucocytes étaient réduits après ajout de SBO, tout comme les comptes de leucocytes du PELF ($P < 0,05$). Cette dernière observation indique une influence possible des PUFAs n-3 alimentaires sur l'inflammation pulmonaire des chevaux avec RAO et des études additionnelles sont nécessaires pour évaluer les effets sur les médiateurs de l'inflammation et le devenir clinique des animaux.

(Traduit par Docteur Serge Messier)

Introduction

Recurrent airway obstruction (RAO) is a reversible lower-airway condition that is often diagnosed in horses. The clinical signs are triggered by environmental factors such as antigens found in hay and straw (1). Human asthma has many similarities to RAO, being characterized by inflammation, hypersensitivity, and obstruction of the airways (2). Neutrophils play a central role in RAO exacerbations, owing to their pulmonary recruitment after natural challenge and their potential to cause lung injury (1,3). Therefore, although neutrophils might not be essential for the development of airway obstruction (4), an important objective of RAO therapy is to control neutrophilic inflammation (3).

Omega-3 (n-3) polyunsaturated fatty acids (PUFAs) have been shown to exert anti-inflammatory effects in laboratory animals and humans (5), as well as in horses (6–8). They influence the balance among eicosanoids, which can be potent inflammatory mediators. Prostaglandins (PGs) and thromboxanes (TXs) of the 3-series and leukotrienes (LTs) of the 5-series that are produced after n-3 PUFA ingestion are less proinflammatory than their 2- and 4-series analogs

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and may even be anti-inflammatory (5). These FAs also change the membrane fluidity of neutrophils (9) and reduce the production of proinflammatory cytokines (10), thus potentially alleviating symptoms of many inflammatory conditions and allergic diseases, including asthma in humans and animals.

Major n-3 FAs are α-linolenic acid (ALA, C18:3 [n-3]), which is found in some vegetable oils, and long-chain (LC) PUFAs from marine sources, mainly eicosapentaenoic acid (EPA, C20:5 [n-3]), docosapentaenoic acid (DPA, C22:5 [n-3]), and docosahexaenoic acid (DHA, C22:6 [n-3]). The LC n-3 PUFAs can be effectively integrated into phospholipids (PLs) without the need for chain elongation or desaturation, in contrast to those derived from vegetable sources, which require both chain elongation and desaturation (11). Seal blubber oil (SBO), a marine oil source of LC n-3 PUFAs, has a higher concentration of DPA than fish oils (12). Furthermore, in both the oil and the chylomicrons formed after the intestinal absorption of SBO, the n-3 FAs are bound mainly to the 1st and 3rd positions of the glycerol molecule. This increases their availability to lipoprotein lipase, which is *sn*-1,3-specific. In contrast, the n-3 FAs of fish oils are attached mainly at the *sn*-2 position in triacylglycerols in both the oil and the resultant chylomicrons (12).

Dietary LC n-3 PUFA supplementation for 10 mo reduced respiratory symptoms and the airway hyperresponsiveness to acetylcholine in children with bronchial asthma (13). Similarly, elevated n-3 PUFA ingestion reduced methacholine-induced airway hyperresponsiveness in humans with asthma, likely because of modulated LT production (14). There is epidemiologic evidence that a high intake of fish, and thus n-3 FAs, reduces the risk of atopy (15). Still, others have found no beneficial effects of n-3 FA ingestion in humans with asthma (16) or no relation between plasma FA patterns and the risk of asthma (17).

Although n-3 FA may also have anti-inflammatory effects in horses, this has rarely been tested. There are a few reports of studies of LC n-3 PUFA dietary supplementation in horses (18–21) and 2 reports on dietary supplementation with vegetable oils as sources of ALA in horses (6,7). A diet containing 8% linseed oil (rich in ALA) reduced endotoxin-induced production of tumor necrosis factor alpha (TNFα) by peritoneal macrophages (7) and in vitro production of TXB_2 but not LTB_4 (6). An intravenous infusion of a 20% lipid emulsion containing n-3 PUFA (mainly ALA) resulted in reduced in vitro production of TXB_2, TXB_3, and TNFα by isolated monocytes, compared with baseline values and those after n-6 FA infusion (8).

Although use of LC n-3 PUFAs, especially from an SBO source, might be effective in the treatment of horses with RAO, this had not been investigated. Therefore, the 1st goal of this study was to ascertain the effects of dietary SBO and sunflower oil (SFO) on the palatability of feed and on the plasma and cell membrane lipids in RAO-affected horses. The 2nd goal was to describe the potential clinical impact of the oil supplementation in terms of lung function, cytologic findings in pulmonary epithelial lining fluid (PELF), and clinical signs.

Materials and methods

Horses

Nine horses, 5 geldings and 4 mares, of mean age 15.3 (4 to 26) y and body weight (BW) 474 (407 to 529) kg, all with a history of RAO, were included in the study. Before the trial, they underwent a histamine challenge test (22) and baseline measurements of pulmonary function and PELF neutrophil percentage. For inclusion in the study, the horses had to meet at least 2 of the following 3 criteria (23): a maximum change in intrapleural pressure (ΔPpl_{max}) greater than 15 cm H_2O, a PELF neutrophil proportion greater than 25%, and either an increase of at least 75% in pulmonary resistance (R_L) or a decrease of at least 35% in dynamic compliance (C_{dyn}) after a histamine dose of less than 6 mg/mL. The in vivo experimental procedures of the trial were approved by the Animal Welfare Board of the Austrian Federal Ministry for Education, Science, and Culture.

Housing and diet

The animals were kept at the University of Veterinary Medicine, Vienna, Austria, in paddocks during the day and in single stables with dust-free wood shavings at night. Two horses with severe RAO were kept outside both day and night; shelter was offered in the form of open-front stables.

The horses were fed a common horse ration calculated to meet their individual requirements (24). The basic ration consisted of grass hay (1st cut in bloom, 8 kg per horse and day) and oats (1 to 3 kg per horse and day), oil supplements, and mineral feed (Equivital;[a] MFE, Marienfelde, Germany; 40 g per horse and day). The calculated average energy and nutrient content of this diet was 9.6 MJ of digestible energy, 85 g of crude protein, 306 g of crude fiber, 45 g of ether extract, and 86 g of crude ash per kilogram of dry matter. The horses lost about 0.3% of their initial BW per week during the 1st trial period; therefore, we elevated the energy content of the feed by adding sugar beet pulp to the ration (0 to 3 kg per horse and day, depending on the requirements of the individual animal). The diet then provided an average of 10.8 MJ of digestible energy, 89 g of crude protein, 256 g of crude fiber, 38 g of ether extract, and 71 g of crude ash per kilogram of dry matter. The concentrations, in colony-forming units, of molds and aerobic bacteria, respectively, were less than 2×10^3 and 2×10^6 per gram of hay and less than 3×10^3 and 3.5×10^6 per gram of oats.

The oil supplements were shipped and stored at 4°C in a nitrogen atmosphere in their original packing and were added to the oats twice daily, just before feeding. With SBO (Arctic Omega-3; Atlantis Marine, St. John's, Newfoundland), we used 320 mg/kg BW/d, analogous to 70 to 75 mg/kg BW/d of LC n-3 PUFA, containing 0.4% tocopherols (a mixture of α, β, and γ). Depending on the energy content of the diet, 4.0% to 7.1% of the daily energy intake was provided by the oil. The same amount of SFO (Osana; VFI Vereinigte Fettwaren Industrie, Wels, Austria) was used. The SBO or SFO and the mineral feed were top-dressed on the oats. Table I shows the FA composition of the oils and feed components. For the horses receiving SFO, the

[a] Contents per kilogram of mineral feed: 160 g of calcium, 40 g of phosphorus, 90 g of sodium, 20 g of magnesium, 500 000 IU of vitamin A, 50 000 IU of vitamin D_3, 4000 mg of vitamin E, 60 mg of vitamin K_3, 2000 mg of vitamin C, 260 mg of vitamin B_1, 200 mg of vitamin B_2, 170 mg of vitamin B_6, 1250 μg of vitamin B_{12}, 500 mg of pantothenic acid, 1100 mg of nicotinic acid, 20 mg of folic acid, 5000 μg of biotin, 6000 mg of choline chloride, 2500 mg of iron, 4500 mg of manganese, 4500 mg of zinc, 850 mg of copper, 75 mg of iodine, 15 mg cobalt, and 14 mg of selenium.

Table I. Major fatty acids (FAs) of sunflower oil (SFO), seal blubber oil (SBO), and feed components

	% of total FA				
FAs	SFO	SBO	Oats	Hay	Beet pulp
C16:0	5.23	7.72	19.4	24.0	22.1
C16:1	0.06	17.4	0.21	1.74	NI
C18:0	4.32	0.98	2.18	4.88	NI
C18:1	24.9	24.3	38.8	13.1	13.8
C18:2 (n-6)	62.7	2.07	34.1	24.0	51.9
C18:3 (n-3)	0.17	0.62	1.19	23.4	7.3
C20:4 (n-6)	NI	0.48	NI	0.44	NI
C20:5 (n-3)	0.08	7.87	0.10	0.15	NI
C22:5 (n-3)	NI	5.52	NI	NI	NI
C22:6 (n-3)	0.22	7.37	0.13	NI	NI
Total MUFA	25.8	60.5	41.7	16.3	13.8
Total n-3 PUFA	0.47	21.4	1.42	23.6	7.3
Total n-6 PUFA	62.7	2.55	34.1	24.5	51.9
Total n-6:total n-3	133	0.12	24.4	1.04	7.1

NI — not identified; MUFA — monounsaturated FA; PUFA — polyunsaturated FA.

total n-6 and n-3 FA intake per day averaged about 140 to 150 g and 34 to 37 g, respectively, depending on the energy content of the diet. Thus, the n-6:n-3 ratio was 4.2 to 4.3. For the horses receiving SBO, the corresponding FA amounts were 51 to 58 g and 65 to 68 g, and the n-6:n-3 ratio was 0.8 to 0.9. The total amount of fat consumed per horse each day ranged between 356 and 472 g. Palatability of the feed and oils was monitored routinely during and after feeding.

Experimental design

Before the start of the treatment periods, symptoms were recorded and the horses underwent bronchoalveolar lavage (BAL), the same pulmonary function testing as in the selection process, and blood sampling. Thereafter, the animals were randomly assigned to 1 of 2 groups. One group was first treated with SFO, and the other group was first treated with SBO. After 10 wk, a crossover of treatments took place. At the end of each 10-wk treatment period, all sampling procedures were repeated.

Data collection and analysis

The clinical examination was always carried out by the same experienced clinician, who was blinded as to the pulmonary function data and used fixed clinical protocols and scoring systems. Dyspnea and the results of auscultation at rest and with a rebreathing bag were scored as 0 (no dyspnea and normal sounds on auscultation), 1 (mild dyspnea and increased but normal sounds on auscultation), 2 (moderate dyspnea and adventitious sounds on auscultation), or 3 (severe dyspnea and marked adventitious noise on auscultation). Cough was scored as 0 when absent, 1 when not spontaneous but present after laryngeal compression, or 2 when spontaneous during half an hour of observation.

For BAL, the respiratory mucosa was locally anesthetized with 40 mL of 0.4% lidocaine (Xylanest purum; Gebro Pharma, Fieberbrunn, Austria) in 0.9% NaCl solution (Mayrhofer Pharmazeutika, Linz, Austria). A videobronchoscope (Dr. Fritz, Tuttlingen, Germany) 175 cm long, with an internal diameter of 11.8 mm, was placed into a large bronchus, and 500 mL of a sterile 0.9% NaCl solution at body temperature was administered and immediately aspirated with a pump (Aerosan; Laborex-Sanesco, Vienna, Austria). For cytologic examination, 10 mL of the BAL fluid (BALF) was centrifuged at 378 × *g*, and 4 smears were dried and stained with Haemafix (Biomed, Munich, Germany) for cell differentiation. Total cell counts were performed with a hemocytometer (ADVIA 120 System; Bayer Healthcare, Tarrytown, New York, USA). Urea concentrations in BALF supernatant were determined by an enzymatic assay (Urea/BUN Roche Kit 148 93 64; Roche Diagnostics, Vienna, Austria) with use of the Hitachi 911 analyzer (Roche Diagnostics). The dilution of the PELF from which the cells originated was calculated from the BALF and blood urea concentrations according to the method of McGorum and colleagues (25), and the cell counts were corrected appropriately. The median of the dilution of PELF in BALF was 46 (upper quartile 85, lower quartile 28, minimum dilution 13, and maximum dilution 964).

Avoiding circadian variation, we performed the pulmonary function tests between 6 and 12 am. The horses were sedated with 0.01 mg/kg detomidine hydrochloride (Dormosedan; Pfizer, Vienna, Austria) and 0.01 mg/kg butorphanol (Butomidor; Richter-Pharma, Wels, Austria). Intrapleural pressure was measured with an esophageal balloon mounted over the distal tip of a polypropylene catheter (length 2.4 m and inner diameter 4.5 mm), which was connected to a pressure transducer (DP45-28; Validyne Engineering, Northridge, California, USA). Simultaneously, the gas flow was measured with the use of a face mask attached to a pneumotachograph (Fleisch no. 5; OEM Medical, Richmond, Virginia, USA) and connected to a pressure transducer (DP45-14; Validyne Engineering). The data were processed by pulmonary function test software (XA BioSystem; Buxco Electronics, Wilmington, North Carolina, USA) that calculated the respiratory frequency, ΔPpl_{max}, C_{dyn}, and R_L.

Blood was obtained by puncture of the jugular vein with the use of serum and ethylene diamine tetraacetic acid Vacutainer systems (Vacuette; Greiner bio-one, Kremsmuenster, Austria) for serum urea determination and FA analysis, respectively. Serum urea concentrations were determined as described for BALF. Plasma for the FA analysis was stabilized with 100 mmol/L of butylhydroxytoluene (Sigma, St. Louis, Missouri, USA) and stored at −80°C until analysis. For the isolation of leukocytes, 12 mL of blood was allowed to sediment for 30 min at room temperature. The leukocyte-rich plasma was carefully layered over 14 mL of a density gradient medium (Percoll; Sigma) adjusted to a density of 1.085 g/mL, and centrifuged at 440 × *g* for 35 min at 15°C. The buffy coat was washed 3 times with 5 mL of phosphate-buffered saline (Gibco, Paisley, Scotland) and, after the addition of 100 mmol/L of butylhydroxytoluene, stored at −80°C for subsequent FA analysis. The FAs of the oils and feedstuffs were extracted by a modification of the method of Bligh and Dyer (26). Plasma FA samples were directly esterified as described by Lepage and Roy (27), with slight modifications. For the extraction of leukocyte PLs, we used a modification of the method of Folch and collaborators (28). The PLs were separated from other lipids by sequential elution of solid-phase extraction columns (Isolute NH_2, 100 mg/3 mL; Separtis, Grenzach-Wyhlen, Germany), as described by Kaluzny and associates (29), with a few modifications. After

Table II. Differences in FA composition of plasma and leukocyte membrane phospholipids after 10 wk of dietary supplementation with SFO or SBO in 9 horses with recurrent airway obstruction

	% of total FA; mean (and standard error)					
	Plasma			Leukocyte membrane phospholipids		
FAs	SFO	SBO	*P*	SFO	SBO	*P*
C16:0	11.1 (0.28)	12.6 (0.33)	< 0.001	18.8 (1.19)	19.3 (1.03)	0.784
C16:1	1.94 (0.06)	4.40 (0.21)	< 0.001	1.28 (0.12)	1.12 (0.12)	0.449
C18:0	16.2 (0.38)	15.1 (0.34)	0.004	21.7 (0.93)	23.3 (1.51)	0.368
C18:1	9.00 (0.24)	10.6 (0.22)	< 0.001	11.7 (0.80)	12.8 (1.11)	0.093
C18:2 (n-6)	53.1 (0.44)	36.4 (0.71)	< 0.001	26.4 (2.05)	16.7 (1.48)	0.001
C18:3 (n-3)	0.91 (0.11)	1.02 (0.13)	0.635	0.39 (0.02)	0.51 (0.05)	0.054
C20:4 (n-6)	1.10 (0.05)	2.06 (0.09)	< 0.001	4.17 (0.23)	2.57 (0.32)	0.004
C20:5 (n-3)	0.10 (0.01)	4.71 (0.28)	< 0.001	0.46 (0.07)	2.50 (0.28)	< 0.001
C22:5 (n-3)	0.15 (0.01)	1.28 (0.14)	< 0.001	1.30 (0.09)	1.46 (0.13)	0.289
C22:6 (n-3)	0.17 (0.03)	2.22 (0.18)	< 0.001	1.99 (0.21)	2.89 (0.45)	0.084
Total MUFA	12.1 (0.22)	18.3 (0.48)	< 0.001	19.6 (0.91)	21.9 (1.75)	0.230
Total n-3 PUFA	1.32 (0.10)	9.24 (0.54)	< 0.001	4.19 (0.26)	7.47 (0.77)	0.005
Total n-6 PUFA	54.3 (0.41)	38.5 (0.68)	< 0.001	31.4 (2.16)	19.9 (1.55)	0.001
Total n-6:total n-3	43.6 (4.23)	4.33 (0.33)	< 0.001	7.54 (0.39)	2.88 (0.32)	< 0.001

Table III. Results of cytologic study of the pulmonary epithelial lining fluid (PELF) and pulmonary function testing after the 10 wk of dietary supplementation with SFO or SBO

Variable	SFO	SBO
PELF cell counts[a] ($\times 10^3/\mu L$)	Median (and range)	
Total cells[b]	25.1 (10.4–596.4)	20.1 (5.5–65.6)
Neutrophils	12.8 (2.9–225.6)	5.8 (0.5–48.0)
Mononuclear cells	14.0 (6.8–471.2)	16.1 (3.8–28.4)
Pulmonary function	Mean (and standard error)	
Respirations/min	12.38 (1.40)	10.95 (1.09)
ΔPpl_{max} (cm H_2O)	14.35 (3.03)	15.57 (3.19)
C_{dyn} (L/cm H_2O)	2.63 (0.42)	2.16 (0.32)
R_L (cm H_2O/L·s)	0.91 (0.12)	1.00 (0.16)

ΔPpl_{max} — maximum change in intrapleural pressure; C_{dyn} — dynamic compliance; R_L — pulmonary resistance.

[a] Normal values, average (and range): 21.7 (12.5–36.8), 0.3 (0.0–1.5), and 19.6 (7.1–40.8), respectively (25).

[b] Significant difference between the treatment groups, by univariate analysis of variance ($P < 0.05$).

evaporation, the samples were subjected to direct transesterification (27). We determined FA patterns by means of capillary gas chromatography, with use of the Hewlett Packard 5890A (Agilent Technologies, Karlsruhe, Germany), the Supelcowax 10, a 0.25-μm column 30 m × 0.32 mm (Supelco, Bellefonte, Pennsylvania, USA), and Millennium 2.10 software (Waters Corporation, Milford, Massachusetts, USA). The oven temperature was set to 140°C for 5 min and elevated stepwise to 220°C, then was kept constant for 10 min. The detector temperature was 240°C.

Discrete values of the clinical scoring system were examined by methods of descriptive statistics. Cytologic data for PELF were logarithmically transformed and subsequently treated like the pulmonary function and FA data, which were all found to be normally distributed. Univariate analysis of variance was performed with SPSS 12.0 for Windows (SPSS, Munich, Germany) to compare the SBO and SFO treatment groups, with diet, sampling time, and treatment order as the fixed factors and the individual horse as the random factor. Significance was set at a *P*-value of less than 0.05. Treatment-by-period interactions were not examined. Some selected data were also subjected to Pearson's correlation analysis.

Results

Both oil supplements were willingly and completely ingested by all the horses at all times. The horses lost 0.3% of their BW per week during the initial phase of the study, the mean BW declining from 474 (standard deviation 43) to 459 (29) kg at the time of treatment crossover. At this point the energy content of the diet was increased.

The animals then gained weight and finished the trial at their initial mean BW of 474 (32) kg.

The FA composition of the plasma and the leukocyte PLs after dietary supplementation reflected the different FA sources, changes being more pronounced in plasma than in leukocyte PLs (Table II). The proportion of linoleic acid (LA, C18:2 [n-6]) was significantly higher ($P < 0.001$) in both plasma and leukocyte PLs after SFO supplementation than after SBO supplementation. The proportions of EPA (C20:5 [n-3]), DPA (C22:5 [n-3]), and DHA (C22:6 [n-3]) were significantly higher ($P < 0.001$) in plasma after SBO supplementation than after SFO supplementation; this effect was also observed for EPA in the leukocyte PLs ($P < 0.001$), and a similar trend was observed for DHA ($P < 0.1$). The proportion of arachidonic acid (AA, C20:4 [n-6]) was significantly higher ($P < 0.001$) in plasma but lower ($P < 0.01$) in leukocyte PLs after SBO supplementation than after SFO supplementation. The n-6:n-3 ratio in both plasma and leukocyte PLs was lower ($P < 0.001$) after SBO supplementation than after SFO supplementation. The sampling time, but not the treatment order, influenced the proportion of some n-6 FAs in the leukocyte PLs; namely, LA ($P = 0.01$) and total n-6 FA ($P = 0.01$). However, the effect was smaller than with diet ($P = 0.001$). Of the cytologic results, only the total cell count in the PELF was influenced by treatment order ($P = 0.036$).

Dietary supplementation had no effect on the clinical scores (data not shown) or the results of pulmonary function testing but did affect the cytologic findings in the PELF (Table III). The total cell count in the PELF was significantly lower ($P < 0.05$) after SBO supplementation than after SFO supplementation, but the order of treatment influenced this count. There was a tendency towards a weak positive correlation ($r = 0.442$; $P = 0.066$) between the AA content in the leukocyte PLs and the PELF total cell count and towards a weak negative correlation between the plasma DHA content and the PELF total cell count ($r = -0.449$; $P = 0.061$). The median PELF neutrophil count was lower after SBO supplementation than after SFO supplementation but not significantly so. The neutrophil count showed a strong correlation with the PELF total cell count ($r = 0.866$; $P < 0.001$) and a tendency towards a weak negative correlation with the plasma AA and DHA content ($r = -0.431$ and -0.417, respectively; $P < 0.1$). The PELF mononuclear cell counts did not change in the course of the study.

Discussion

The feed supplemented with SFO or SBO was well accepted by all the horses, in agreement with observations of others (18,20). A washout period, which is commonly recommended for crossover studies, could not be used in our study since the risk of seasonal influences and variations in feed quality necessitated as short a trial as possible. Because there were no statistically significant differences in FA pattern in either plasma or leukocyte PLs between the 2 treatment orders, the study design was considered appropriate for the aims.

Although a report about the impact of dietary fish oil on the plasma FA content in horses has been published recently (18), the effect on cell membranes had not yet been studied. In our trial, major changes in plasma and leukocyte PL FAs occurred. Feeding different oils affected the n-3 and n-6 PUFA content and even the monounsaturated FA content in plasma and, to a lesser extent, leukocyte PLs, whereas the content of saturated FAs was more or less unchanged, as was also observed by Hall and coworkers (18). This close relationship between dietary and tissue FA composition was described earlier (30). The levels of the supplemented n-3 PUFAs (EPA, DPA, and DHA) were significantly elevated in plasma. However, after 10 wk of SBO supplementation, only EPA was significantly incorporated into the leukocyte PLs. Although DPA was not incorporated, there was a trend towards incorporation of DHA. The fact that DPA can be converted to DHA in humans (31) might explain this finding in the horse.

The highest concentration of an n-6 FA was for LA. The reduced proportion of LA after the SBO diet and the elevated proportion after the SFO diet are typical of n-3 PUFA supplementation. The influence of sampling time on the LA content of the leukocyte PLs was not a carryover effect, because the animals that received SFO after SBO showed an even higher elevation of LA content in the leukocyte PLs after the SFO diet than was observed in the animals receiving SFO first; thus, this effect might be incidental. We know that LA plays an important role as a precursor of AA, which is important as a substrate for the enzymes cyclooxygenase (COX) and lipoxygenase (LOX); therefore, LA acts as the precursor of proinflammatory eicosanoids (5).

Since SBO contains about 0.5% AA, whereas SFO contains none, the elevated AA concentration in plasma after SBO supplementation, compared with SFO supplementation, could be expected. Such an effect was also observed in horses fed fish oil (18). The cellular AA content may be more relevant with respect to inflammatory mediators. We found lower AA amounts in leukocyte PLs than in plasma after the SBO diet compared with the SFO diet. Competition among fatty acids for a limited number of esterification sites and rapid endogenous conversion of a high proportion of LA to AA after incorporation into tissues and complex lipids have been described (11). Thus, the low amount of cellular AA after the SBO diet can be explained by such a competition between AA and EPA, just as the observed elevation of plasma AA can be explained by the continuously high plasma LA levels. Whereas Hall and coworkers (18) suggested that the horse might be unable to convert LA into AA, our findings clearly indicate that such conversion is possible. Thus, an equine diet high in LA can support the elevated AA content of the leukocyte cell membrane. It could also be that dietary LC n-3 PUFAs competitively replace some of the AA in leukocyte cell membranes of horses.

These results indicate that in equines the plasma FA content reflects the dietary FA content, whereas FAs extracted from membrane PLs may give a better estimate of long-term FA storage, providing insight into a possible role in physiological and pathological processes.

Accumulation of n-3 and reduction of n-6 PUFAs are known to result in various anti-inflammatory effects in many species (5,10), including horses (6–8,19,32). This could explain the diet-related reduction of the PELF total cell count after SBO supplementation compared with SFO supplementation. In addition, treatment order influenced the PELF total cell count. Because of the long duration of each feeding period, it is unlikely that this finding was due to a

carryover of the SBO effect towards the end of the SFO treatment. We observed a tendency towards a weakly positive correlation between the AA content of leukocyte PLs and the PELF total cell count and a weakly negative correlation between the plasma DHA content and both the total cell count and the neutrophil count in the PELF. Also, no carryover effects were observed in any FA pattern in the plasma or the leukocyte PLs.

The environment has a great impact on the PELF of horses affected by RAO (1). During a study time of 20 wk, environmental factors likely fluctuated, although there were no changes in pulmonary function over time. It is possible that the influence of time, environment, and diet interfered with each other and contributed to the lack of clinical or lung function changes due to diet. The observed influence of dietary order on the PELF total cell count was not the result of environmental influences, because all animals were subject to the same conditions at all times.

After SBO supplementation, 8 out of 9 horses had a PELF total cell count within or below the range reported for healthy horses (25); in contrast, after SFO supplementation, 4 out of 9 animals had an elevated PELF total cell count. Such effects of SBO could be due to reduced chemotaxis after n-3 FA ingestion or reduced synthesis of eicosanoids that have chemotactic properties, such as LTB4 (5). The dynamics of lipid mediator concentrations as well as cytokine expression as a result of these diets would be of great interest. Reduced production in vitro of PGE2 but not of TNFα activity by BALF cells was reported for healthy horses fed fish oil in comparison with a group fed corn oil (19). Such effects might bring about favorable changes related to RAO pathogenesis, but the results of studies of the role of these mediators in RAO and the effect of their inhibitors are inconclusive (33–36). The fact that LC n-3 PUFAs influence both COX and LOX pathways may mean that dietary supplementation with these FAs might prevent the assumed shunting from one pathway to the other by unilateral anti-inflammatory therapy (34). Hall and collaborators (19) did not find any diet-related changes in BALF cytologic results in healthy horses after supplementation with 3% fish oil. Possibly such changes can be observed in horses affected by RAO rather than in healthy animals. Neutrophils are the major inflammatory cells involved in RAO exacerbations (1,3); their percentage in BALF correlates with the severity of clinical signs (37). Although they might not be directly involved in causing airway obstruction (4), they are potential sources of inflammatory mediators such as eicosanoids in the lung (3). Therefore, it was expected that a reduction in PELF cell counts would result in an improvement in clinical signs and lung function, which was not observed.

In vitro results indicating beneficial effects of dietary LC n-3 PUFAs (6,7) or of an FA infusion (8) are not easily reproduced in vivo with the use of feeding trials. Likely explanations are environmental influences or insufficient duration or dosage of oil application. In the present study, we observed a 30-fold elevation of the EPA level in the plasma and a 2.5-fold elevation in the leukocyte PLs. Immediately after infusion of a lipid emulsion containing 20% menhaden oil in horses, McCann and colleagues (8) found a monocyte PL n-3 proportion of 12.9% and an immediate reduction in the production of $TXB_{2/3}$ and TNFα by these cells in vitro. Considering these rapid changes, it seems unlikely that our study was too short or the dosage too low for changes in the production of inflammatory mediators.

Because of the multifactorial nature of RAO, LC n-3 PUFA supplementation alone might not be able to change clinical signs owing to the unidirectional mode of action. There is some evidence that airway neutrophils do not solely contribute to the development of airway obstruction; thus, a reduction in PELF cell counts alone might not be able to inhibit airway obstruction (4). The amount of oil fed per kilogram of BW was about double that reported in human studies, but it was not more than 10% of the total energy intake, which has been recommended as the safe limit for PUFAs (38). We cannot exclude the possibility that this amount of SBO had negative effects on surfactant composition (39) or on the antioxidative status of the horses (40), both of which could theoretically exacerbate RAO in horses.

In conclusion, LC n-3 PUFAs added to the daily feed were palatable and effectively incorporated in both plasma and leukocyte PLs of RAO-affected horses. The reduced PELF cell counts in the horses indicate that such oils from marine sources might be helpful. Evidence from this trial and previous work (18,19) indicates that long-term trials are necessary to evaluate the clinical efficacy as well as the molecular mechanisms of PUFAs in RAO-affected horses. Furthermore, the influence of these FAs on surfactant quality and on the antioxidative status of horses has to be elucidated.

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References

1. Robinson NE, Derksen FJ, Olszewski M, Buechner-Maxwell VA. The pathogenesis of chronic obstructive pulmonary disease of horses. Br Vet J 1996;152:283–306.
2. Bowles KS, Beadle RE, Mouch S, et al. A novel model for equine recurrent airway obstruction. Vet Immunol Immunopathol 2002; 87:385–389.
3. Marr KA, Lees P, Cunningham FM. Antigen challenge increases adherence of circulating neutrophils in horses with chronic obstructive pulmonary disease. Equine Vet J 2002;34:65–70.
4. Olszewski MA, Robinson NE, Zhu F-X, Zhang X-Y, Tithof PK. Mediators of anaphylaxis but not activated neutrophils augment cholinergic responses of equine small airways. Am J Physiol 1999;276:L522–L529.
5. Calder PC. Immunoregulatory and antiinflammatory effects of n-3 polyunsaturated fatty acids. Braz J Med Biol Res 1998;31: 467–490.
6. Henry MM, Moore JN, Feldman EB, Fischer JK, Russell B. Effect of dietary alpha-linolenic acid on equine monocyte procoagulant activity and eicosanoid synthesis. Circ Shock 1990;32:173–188.
7. Morris DD, Henry MM, Moore JN, Fischer JK. Effect of dietary α-linolenic acid on endotoxin-induced production of tumor necrosis factor by peritoneal macrophages in horses. Am J Vet Res 1991;52:528–531.

8. McCann ME, Moore JN, Carrick JB, Barton MH. Effect of intravenous infusion of omega-3 and omega-6 lipid emulsions on equine monocyte fatty acid composition and inflammatory mediator production *in vitro*. Shock 2000;14:222–228.
9. Sipka S, Dey I, Buda C, Csongor J, Szegedi G, Farkas T. The mechanism of inhibitory effect of eicosapentaenoic acid on phagocytic activity and chemotaxis of human neutrophil granulocytes. Clin Immunol Immunopathol 1996;79:224–228.
10. Blok WL, Katan MB, Van der Meer JWM. Modulation of inflammation and cytokine production by dietary (n-3) fatty acids. J Nutr 1996;126:1515–1533.
11. Nelson GJ. Effects of dietary fatty acids on lipid metabolism. In: Chow CK, ed. Fatty Acids in Foods and their Health Implications. New York: Marcel Dekker, 2000:481–516.
12. Christensen MS, Høy C-E. Effects of dietary triacylglycerol structure on triacylglycerols of resultant chylomicrons from fish oil- and seal oil-fed rats. Lipids 1996;31:341–344.
13. Nagakura T, Matsuda S, Shichijyo K, Sugimoto H, Hata K. Dietary supplementation with fish oil rich in ω-3 polyunsaturated fatty acids in children with bronchial asthma. Eur Respir J 2000;16:861–865.
14. Broughton KS, Johnson CS, Pace BK, Liebman M, Kleppinger KM. Reduced asthma symptoms with n-3 fatty acid ingestion are related to 5-series leukotriene production. Am J Clin Nutr 1997;65:1011–1017.
15. Kankaanpää P, Sütas Y, Salminen S, Lichtenstein A, Isolauri E. Dietary fatty acids and allergy. Ann Med 1999;31:282–287.
16. Hodge L, Salome CM, Hughes JM, et al. Effect of dietary intake of omega-3 and omega-6 fatty acids on severity of asthma in children. Eur Respir J 1998;11:361–365.
17. Woods RK, Raven JM, Walters EH, Abramson MJ, Thien FCK. Fatty acid levels and risk of asthma in young adults. Thorax 2004;59:105–110.
18. Hall JA, Van Saun RJ, Wander RC. Dietary (n-3) fatty acids from menhaden fish oil alter plasma fatty acids and leukotriene B synthesis in healthy horses. J Vet Intern Med 2004;18:871–879.
19. Hall JA, Van Saun RJ, Tornquist SJ, Gradin JL, Pearson EG, Wander RC. Effect of type of dietary polyunsaturated fatty acid supplement (corn oil or fish oil) on immune responses in healthy horses. J Vet Intern Med 2004;18:880–886.
20. O'Connor CI, Lawrence LM, Lawrence ACS, Janicki KM, Warren LK, Hayes S. The effect of dietary fish oil supplementation on exercising horses. J Anim Sci 2004;82:2978–2984.
21. Zeyner A, Behnisch C, Gropp J. Investigations on the palatability and digestibility of fish oil containing diets in adult riding horses. Proc 7th Conf Eur Soc Vet Comp Nutr 2003:75.
22. Doucet MY, Vrins AA, Ford-Hutchinson AW. Histamine inhalation challenge in normal horses and in horses with small airway disease. Can J Vet Res 1991;55:285–293.
23. Hoffman A. Does measurement of airway reactivity assist in population definition? In: International Workshop on Equine Chronic Airway Disease. Equine Vet J 2001;33:5–19.
24. Subcommittee on Horse Nutrition, Committee on Animal Nutrition, Board on Agriculture Committee, National Research Council: Nutrient Requirements of Horses, 5th rev ed. Washington, DC: National Academy Press, 1989.
25. McGorum BC, Dixon PM, Halliwell REW, Irving P. Evaluation of urea and albumen as endogenous markers of dilution of equine bronchoalveolar lavage fluid. Res Vet Sci 1993;55:52–56.
26. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959;37:911–917.
27. Lepage G, Roy CC. Direct transesterification of all classes of lipids in a one-step reaction. J Lipid Res 1986;27:114–120.
28. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 1957;226:497–509.
29. Kaluzny MA, Duncan LA, Merritt MV, Epps DE. Rapid separation of lipid classes in high yield and purity using bonded phase columns. J Lipid Res 1985;26:135–140.
30. Lands WE, Morris A, Libelt B. Quantitative effects of dietary polyunsaturated fats on the composition of fatty acids in rat tissues. Lipids 1990;25:505–516.
31. Pawlosky RJ, Hibbeln JR, Novotny JA, Salem N Jr. Physiological compartmental analysis of α-linolenic acid metabolism in adult humans. J Lipid Res 2001;42:1257–1265.
32. O'Neill W, McKee S, Clarke AF. Flaxseed *(Linum usitatissimum)* supplementation associated with reduced skin lesional area in horses with *Culicoides* hypersensitivity. Can J Vet Res 2002; 66:272–277.
33. Lindberg A, Naesman-Glaser B, Lindgren JA, Robinson NE. Evaluation of leukotriene biosynthetic capacity in lung tissues from horses with recurrent airway obstruction. Am J Vet Res 2002;63:794–798.
34. Gray PR, Derksen FJ, Robinson NE, Carpenter-Deyo LJ, Johnson HG, Roth RA. The role of cyclooxygenase products in the acute airway obstruction and airway hyperreactivity of ponies with heaves. Am Rev Respir Dis 1989;140:154–160.
35. Watson ED, Sweeney CR, Steensma, KA. Arachidonate metabolites in bronchoalveolar lavage fluid from horses with and without COPD. Equine Vet J 1992;24:379–381.
36. Marr KA, Lees P, Page CP, Cunningham FM. Inhaled leukotrienes cause bronchoconstriction and neutrophil accumulation in horses. Res Vet Sci 1998;64:219–224.
37. Naylor JM, Clark EG, Clayton HM. Chronic obstructive pulmonary disease: usefulness of clinical signs, bronchoalveolar lavage, and lung biopsy as diagnostic and prognostic aids. Can Vet J 1992;33:591–598.
38. Eritsland J. Safety considerations of polyunsaturated fatty acids. Am J Clin Nutr 2000;71(suppl):197–201.
39. Wolfe RR, Martini WZ, Irtun O, Hawkins HK, Barrow RE. Dietary fat composition alters pulmonary function in pigs. Nutrition 2002;18:647–653.
40. Deaton CM, Marlin DJ, Smith NC, et al. Pulmonary epithelial lining fluid and plasma ascorbic acid concentrations in horses affected by recurrent airway obstruction. Am J Vet Res 2004;65:80–87.