Effect of Type of Dietary Polyunsaturated Fatty Acid Supplement (Corn Oil or Fish Oil) on Immune Responses in Healthy Horses

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The objective of this study was to compare effects of dietary polyunsaturated fatty acid supplementation (corn oil or fish oil) on selected immune responses in normal horses. Two groups of horses (n = 5) were randomly assigned a dietary supplement with either 3.0% corn oil or fish oil for a period of 14 weeks. Plasma fatty acid profiles were monitored to ensure uptake of dietary fatty acids. Cell-mediated immunity was assessed by a delayed-type hypersensitivity (DTH) skin test to keyhole limpet hemocyanin (KLH), and humoral immunity was assessed by measuring antibody titers to KLH. Production of prostaglandin E2 (PGE2), expression of tumor necrosis factor-α (TNF-α), and phagocytosis of latex beads by bronchoalveolar lavage fluid (BALF) cells were also assessed. Lipopolysaccharide (LPS)-stimulated BALF cells from horses fed corn oil showed a higher production of PGE2 compared with those from horses fed fish oil at 6 and 12 weeks. Production of TNF-α by LPS-stimulated BALF cells was higher in both groups of horses at 6, 8, and 12 weeks compared with pretrial values, and phagocytic activity of BALF cells was higher at 8 and 12 weeks, however, there were no differences between the 2 groups of horses. The DTH skin test and antibody titers to KLH revealed no differences between horses fed corn or fish oil. Based on these studies, dietary polyunsaturated fatty acids modulate the inflammatory response of horses. Both fatty acid supplements increased production of the proinflammatory cytokine TNF-α, whereas only corn oil increased production of the proinflammatory eicosanoid PGE2, by LPS-stimulated BALF cells. It is possible that fish oil, because it did not increase production of PGE2, could have value in the treatment of equine recurrent airway obstruction or other equine inflammatory diseases.

**Key words:** Bronchoalveolar lavage fluid; (n-3) Fatty acids; PGE2; Phagocytosis; Tumor necrosis factor-α.

Fish oils contain the long chain (n-3) polyunsaturated fatty acids (PUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Corn oil is a rich source of linoleic acid, an (n-6) PUFAs. Increasing the amount of dietary (n-3) PUFA relative to (n-6) PUFA allows (n-3) fatty acids to be preferentially incorporated into cell membrane phospholipids. The type of eicosanoids that cells produce, and consequently communication between cells of the immune system, can potentially be modulated through dietary supplementation of PUFA.

Consumption of fish oils also reduces cell-mediated immune responses. For example, there is decreased lymphocyte proliferation, T cell-mediated cytotoxicity, natural killer cell activity, macrophage-mediated cytotoxicity, monocyte and neutrophil chemotaxis, major histocompatibility class II expression and antigen presentation, and adhesion molecule expression. Dietary supplementation with fish oil can also affect production of certain cytokines.

Cytokines mediate the immune response by altering lymphocyte proliferation, differentiation, and activation. There is decreased production of the proinflammatory cytokines interleukin (IL)-1, IL-6, and tumor necrosis factor-α (TNF-α) when a diet is supplemented with (n-3) PUFA. Also, it has been shown that dietary consumption of PUFA affects other immune functions such as phagocytosis and antibody production. The (n-3) PUFA may work by altering signal transduction, gene expression, or both within inflammatory and immune cells.

The goal of this investigation was to compare the effect of diets rich in (n-3) fatty acids on selected immune responses of healthy horses. Evaluated variables included immune functions of bronchoalveolar lavage fluid (BALF) cells (phagocytosis of latex beads, production of TNF-α, and PGE2 production), systemic antibody production to keyhole limpet hemocyanin (KLH), and a delayed-type hypersensitivity (DTH) skin test to KLH. Of particular interest in this study was the effect of dietary (n-3) fatty acids on TNF-α and PGE2 production. Our goal was to determine whether dietary supplementation with fish oil reduced expression of the proinflammatory eicosanoid PGE2, proinflammatory cytokine TNF-α, or both in BALF cells from normal horses.

The horses used in his study were the same horses described in a companion paper. In that paper, we measured leukotriene B (LTB4), and LTB4 production in calcium ionophore–stimulated peripheral blood neutrophils. In this paper, the focus is on immune responses of cells isolated from airways of these horses, as well as on in vivo tests to assess cell-mediated and humoral immunity.

**Materials and Methods**

**Horses and Diets**

Horses and dietary treatments used for this study were the same as those previously described. Briefly, 10 mature, nonpregnant, light-breed mares ranging in age from 5 to 20 years and weighing between...
429 and 553 kg (mean 502 kg) were fed hay-based diets with a supplement that differed only in its source of oil, either corn oil (low n-3) or fish oil (high n-3). Horses were determined to be healthy based on repeated physical examinations, complete blood counts, and serum biochemical evaluations.

**Protocol**

Horses were randomly assigned to 1 of 2 feeding groups (n = 5) and fed oil-supplemented diets for a period of 14 weeks following the diet acclimation period, as previously described.19 Jugular venous blood was collected into tubes with ethylenediamine tetraacetic acid at 0, 6, 8, and 12 weeks. BALF was collected at 0, 6, 8, and 12 weeks. At 8 weeks horses were inoculated with KLH suspension. A second KLH “booster” inoculation was given at week 10, 2 weeks after the first inoculation. Cell-mediated immune response was evaluated using a DTH skin test the day after the second KLH inoculation, at 10 weeks and 1 day. At 12 weeks, the KLH antibody titer was determined. The experimental protocol was reviewed and approved by the Oregon State University Animal Care and Use Committee according to principles outlined by the National Institutes of Health.20

**Bronchoalveolar Lavage**

BALF cells were isolated to evaluate phagocytic activity and for stimulation to assess PGE, and TNF-α production. Horses were sedated with 5 mg of detomidine given IV for the procedure. The bronchus was infused 3 times with physiological saline (100-ml aliquots) and fluid was immediately aspirated after each infusion. Approximately 200 ± 30 mL of BALF was obtained from each horse. The BALF was kept on ice until centrifuged for 20 minutes at 400 x g. Supernatants were discarded and cell pellets were combined and resuspended in 2 mL of Hank's balanced salt solution. Cell concentrations were determined using a Coulter ZB1 Counter. Cell differential counts were determined by a certified medical technologist by way of microscopic examination of a Wright-Giemsa stained aliquot of these cells.

**Phagocytosis of Latex Beads**

Phagocytic activity of BALF cells was assessed using latex beads.21 Briefly, for horses, cells were incubated on ice with either a monoclonal antibody (mouse, anti-equine, pangranulocyte/monocyte marker, cell-line DH59B)22 or an isotype negative control (a purified mouse immunoglobulin G [IgG] 1 isotype standard) for 5 minutes in the dark. Medium was also supplemented with 100,000 U/L penicillin, 100 mg/L streptomycin, 2 mmol/L-glutamine, and 5% fetal calf serum. Cells were incubated in tissue culture flasks for 40 hours at 37°C in a humidified atmosphere of 5% CO₂. After incubation, cells were centrifuged, and supernatants were filtered through a 0.45-micron filter and stored at ~70°C until assayed.

**Statistical Analysis**

The trial was a completely randomized design. Data were analyzed using the mixed procedure of Statistical Analysis Systems for repeated measures. Main effects were diet, week, and their interaction analyzed over time. For each dependent variable, a horse nested in treatment (diet) was subjected to differing covariance structures. The best model fit was determined by lowest parameter values for covariance structure. For main effects found to be significant, mean differences were determined by pairwise differences or probability values for differences of the least-squares means (PDFF) for preplanned comparisons.23 Data are reported as least squares means ± SEM unless otherwise indicated. A two-sample t-test was used to compare data not meeting repeated measures criteria. Data for the KLH antibody titer required a log transformation before comparing groups. Values were considered significant at P ≤ .05 unless otherwise indicated.
Fig 1. Effect of feeding horses diets supplemented with oils that differed in the (n-6) and (n-3) fatty acid content on phagocytic activity of bronchoalveolar lavage fluid (BALF) cells. Horses fed a corn oil supplement (white bars) received a low level of (n-3) fatty acids, whereas horses fed a fish oil supplement (black bars) received a high level of (n-3) fatty acids. Each bar represents the percentage of phagocytosis (population of cells engulfing 1 or more fluorescent beads / total population of cells × 100%) after the horses had consumed their respective diets for 0, 6, 8, and 12 weeks. Values are mean ± SEM, n = 4 or 5. Different letters above bars indicate when mean values differed significantly (P ≤ .05) across time of study.

Fig 2. Effect of feeding horses diets supplemented with oils that differed in the (n-6) and (n-3) fatty acid content on TNF-α production by lipopolysaccharide-stimulated bronchoalveolar lavage fluid (BALF) cells. Horses fed a corn oil supplement (white bars) received a low level of (n-3) fatty acids, whereas horses fed a fish oil supplement (black bars) received a high level of (n-3) fatty acids. Each bar represents the percentage of lysis of L929 cells by TNF-α after the horses had consumed their respective diets for 0, 6, 8, and 12 weeks. Values are mean ± SEM, n = 4 or 5. Bars with * above them denote a significant difference (P ≤ .05) from week 0 within a group of horses. Sample week as a main effect also influenced mean production of tumor necrosis factor-α activity by BALF cells. Different letters above bars indicate when mean values for both groups of horses differed significantly (P ≤ .05) across time of study. Dietary means were different only at week 6.

Results

Horses and Diets

As reported in a companion paper, dietary treatment resulted in an altered plasma fatty acid profile, which is consistent with the supplement's fatty acid profile. Horses consuming fish oil experienced an elevation of (n-3) fatty acids, predominately EPA and DHA (but also arachidonic acid; AA), starting at 6 weeks and continuing through 12 weeks of the study.

BALF Cytologies

Differential cell counts on BALF were not influenced by dietary treatment, but the percentage of macrophages (P = .001), lymphocytes (P = .0001), and other cells (P = .02, including eosinophils, mast cells, and epithelial cells) were altered by collection week. Neutrophil percent was not different across diet or time periods, ranging between 1.4 and 3.2%. The percentage of macrophages increased over the study collection periods from 87.9 ± 2.0% to 94.4 ± 0.9%. The mean percentage of macrophages at weeks 8 and 12 were greater than those in the pretrial period. The percentage of lymphocytes started at 4.0 ± 1.2%, declined to 0.4 ± 0.3% at week 8, and then increased to 2.7 ± 1.0% at week 12. The percentage of other cells was highest during the pretrial period (6.4 ± 1.8%) and declined over time (1.3 ± 0.3% at week 12).

Phagocytosis

The pangranulocyte/monocyte marker did not distinguish between macrophages and granulocytes. Because the predominant BALF cell type was the macrophage, phagocytosis results were interpreted as macrophage activity. Percentage of BALF cells engulfing latex beads increased with week of study. Phagocytic activity was highest at week 12 compared with all other test times for horses fed both diets. Although the percentage of macrophages engulfing latex beads was numerically higher in corn oil–fed than in fish oil–fed horses at all time points, differences between the 2 groups of horses were not significant at any time point during the feeding period (Fig 1). Both dietary treatments showed similar changes over time.

Tumor Necrosis Factor-α Production

There was an observed interaction between dietary treatment and sample period for mean production of TNF-α activity by BALF cells (Fig 2). Sample week as a main effect influenced mean production of TNF-α activity. For both groups of horses, all sample periods had higher TNF-α activity compared with that of week 0. Weeks 6 and 12 had the highest mean values, whereas week 8 activity was greater than that of week 0, but lower compared with that of weeks 6 and 12. Diet did not influence TNF-α activity; however, corn oil–fed horses had higher (88.2 versus 83.9% lysis of L929 cells) activity than fish oil–fed horses. Spontaneous production of TNF-α activity by BALF cells incubated in media without LPS was 0, 8, 15, and 18% of LPS-stimulated production at 0, 6, 8, and 12 weeks, respectively.
Production of PGE$_2$ by BALF cells was influenced by diet and sample week, but not by diet by week interaction (Fig 3). Overall mean PGE$_2$ production was greater for horses fed corn oil (9,600 pg/mL) compared with those fed fish oil (4,020 pg/mL) over the 12 weeks of study. Production of PGE$_2$, for corn oil–fed horses was greatest at week 12, whereas there was no difference across time periods for fish oil–fed horses. Within sample weeks, PGE$_2$ production for corn oil–fed horses was greater than those fed fish oil at 6 weeks (8,340 versus 2,770 pg/mL) and 12 weeks (12,540 versus 3,730 pg/mL). Spontaneous production of PGE$_2$ by BALF cells incubated in media without LPS was 0% of LPS-stimulated production at 0, 6, 8, and 12 weeks (Overall diet effect, $P < .01$). Values are means ± SEM, $n = 4$ or 5. Bars with $P$ values above them indicate times when values between groups differed significantly. Different letters over bars within corn oil treatment indicate when mean values are different ($P \leq .01$).

**Prostaglandin E$_2$ Quantification**

The ratio of EPA to AA in the plasma was correlated with PGE$_2$ production by stimulated BALF cells. Across all weeks and dietary treatments, there was a negative correlation (correlation coefficient = $-0.46$; $P = .01$) between EPA: AA ratio and PGE$_2$ concentration. Across both dietary treatments, EPA: AA ratio was correlated (correlation coefficient = $-0.69$; $P = .04$) with PGE$_2$ production at week 12.

**Delayed-Type Hypersensitivity Skin Tests**

Both groups of horses had large reactions on the neck in response to the KLH intradermal injection: there was a central ring of induration surrounded by a larger area of swelling. Wheal diameter was influenced by time, but not diet, although there was a tendency ($P = .08$) for an interaction between diet and time. Overall, wheal diameter increased from 0.5 hour (16.9 ± 0.9 mm, corn oil; 15.7 ± 0.9 mm, fish oil) to 24 hours (69.7 ± 5.7 mm, corn oil; 89.0 ± 6.3 mm, fish oil), then declined over time. In horses fed corn oil, the reaction area remained greater at 48 hours (52.1 ± 9.4 mm) and 72 hours (37.0 ± 9.4 mm) than at 0.5 hour. The reaction area was not different between 0.5 and 96 hours (15.5 ± 4.3 mm). In horses fed fish oil, the reaction area at 48 hours (38.8 ± 9.4 mm) was greater than it was at 0.5 hour, but less than it was at 24 hours. Reaction areas at 72 hours (19.1 ± 9.4 mm) and 96 hours (21.9 ± 4.3 mm) were not different from those of 0.5 or 48 hours.

The area of DTH reaction was also measured at 2 independent points on the ear (base and tip). Reaction area at the ear tip was influenced by time, and diet by time interaction, but not by diet. Across dietary treatments, the reaction area was greatest at all time points compared with 0.5 hour. Comparisons between time points were difficult to interpret because of large variation in mean values at 72 and 96 hours. Numerically, mean areas increased at each successive time up to 72 hours, and they remained high at 96 hours. In corn oil–fed horses, reaction area at 24 hours (52.6 ± 8.1 mm$^2$) was the only time point different from 0.5 hour (28.8 ± 4.5 mm$^2$). In contrast, fish oil–fed horses had a constantly increasing reaction area whereby all time points were greater than they were at 0.5 hour. No differences were found between time points for 48, 72, and 96 hours.

Reaction area at the ear base was influenced by time, but not by diet or diet by time interaction. Across both treatments, reaction area was increased at all time points compared with 0.5 hour, but none of the time points were different from each other.

**Keyhole Limpet Hemocyanin Antibody Titer**

The humoral immune response to KLH was measured at 12 weeks; 2 weeks after horses received the second inoculation with KLH protein. There were no significant differences in antibody titers between the 2 groups of horses. The log titer was 3.75 ± 0.17 in fish oil–fed horses and 3.97 ± 0.17 in fish oil–fed horses. This was a significant response following sensitization to a foreign protein in both groups of horses.

**Total White Blood Cell and Differential Counts**

There was an interaction between diet and week on mean white blood cell count. Mean white blood cell count was influenced by week of study, but not by diet (9,210 ± 390 cells/μL, corn oil; 8,590 ± 390 cells/μL, fish oil). Most of the weekly variation in white blood cell count resulted from weekly effects and from diet by week effects on neutrophil counts. No other blood cell type was influenced by diet, week, or their interaction. All horses showed an alternating pattern for white blood cell and neutrophil counts, in which lower counts were observed at 0 and 8 weeks and higher counts at 6 and 12 weeks. Even though there were significant weekly changes in white blood cell and neutrophil counts, all values remained within normal reference intervals for horses.
Discussion

Increased phagocytosis of latex beads by pulmonary alveolar macrophages from horses fed corn or fish oil for 12 weeks was similar to what has been observed in rats. Although the percentage of macrophages engulfing latex beads was higher in horses fed corn oil than in horses fed fish oil, and differences increased as the duration of the study progressed, there were no significant differences between groups of horses at any time point during the feeding period.

Membrane fluidity is important in determining macrophage adhesion and phagocytic activity (eg, degree of unsaturation of fatty acids in culture media of macrophages affects subsequent phagocytic activity). Phagocytic activity of macrophages increases after they are cultured with highly polyunsaturated fatty acids, yet macrophages cultured with EPA and DHA have lower phagocytic activity than AA-cultured cells. In our study, horses fed fish oil had higher plasma levels of EPA and DHA, but numerically lower phagocytic activity by pulmonary alveolar macrophages than horses fed corn oil, the latter having higher plasma levels of linoleic acid.

The horses’ response to a DTH skin test following KLH administration after consuming corn or fish oil for 12 weeks was unexpected. In dogs, a high dietary intake of (n-3) PUFA suppresses the DTH response to intradermal injection of KLH antigen. The diameter of induration was significantly smaller at 24 and 48 hours in dogs supplemented with a high level of (n-3) PUFA compared with dogs that consumed medium or low levels of an (n-3) PUFA supplement. In this study, the DTH reactions on the neck following KLH administration were most pronounced at 24 hours in horses fed both corn oil and fish oil. At 48 and 72 hours they were reduced in size, with those from horses fed fish oil being smaller. This was not considered a typical DTH response. These results were more consistent with an inflammatory response to KLH. Reactions at the ear base were not different between horses fed corn or fish oil, although wheal diameter and reaction area in both groups of horses was greatest at 72 hours, which is a more typical DTH reaction. Reactions at the ear tip in general persisted longer in fish oil–fed horses for elevation, wheal diameter, and reaction area. At this location, fish oil–fed horses had a more pronounced increase in reaction area with time.

The KLH antigen has been used previously to evaluate DTH responses in horses, with the horses’ reactions to KLH starting early (4 hours) and subsiding after 24 hours. The DTH skin response of horses to KLH might be a combination of both humoral and cellular mechanisms. In our study, horses’ humoral immune response was evaluated by measuring the production of antibodies to KLH. IgG antibody titers were not significantly different between groups of horses in our study. A more definitive test of humoral immunity would involve measuring additional classes of immunoglobulin. In particular, measurement of IgE antibody titers would help define whether horses’ reaction to KLH was the result of an immediate hypersensitivity reaction. Yamada et al have shown that unsaturated fatty acids can inhibit production of IgG, IgM, and IgA by cultured lymphocytes from rat mesenteric lymph nodes, while stimulating production of IgE. Those researchers concluded that this was, in part, related to production of oxidation products from fatty acids containing higher numbers of carbon atoms, double bonds, or both.

In the study reported here, supplementation with either corn oil or fish oil enhanced production of TNF-α by LPS-stimulated BALF cells. Compared with pretrial levels, BALF cells from horses fed fish oil produced significantly higher levels of TNF-α activity at 6, 8, and 12 weeks, whereas BALF cells from horses fed corn oil produced significantly higher TNF-α activity at 6 and 12 weeks. In contrast, a decrease (approximately 10-fold) in TNF-α production by LPS-stimulated peritoneal macrophages was noted in another study when horses were fed a diet enriched in α-linolenic acid for 8 weeks. It is interesting that in the study by Morris et al, when cells were cultured in media without LPS, cells from horses consuming the α-linolenic acid diet produced significantly more TNF-α than cells obtained pretrial, before dietary intervention. Thus, it is difficult to interpret the effects of dietary α-linolenic acid supplementation on TNF-α production in that study.

In another study, horses were infused IV with 20% lipid emulsions enriched with (n-3) or (n-6) fatty acids. Fatty acid analysis of monocyte membrane phospholipids demonstrated changes in the fatty acid composition persisting for up to 7 days after infusion. In vitro production of TNF-α by peripheral blood monocytes was diminished by (n-3) lipid infusion and was unchanged or increased by (n-6) lipid infusion. Unstimulated monocytes had significantly lower TNF-α production after (n-3) infusion compared with that before (n-3) infusion. The inconsistencies between these two studies and ours may indicate a differential response to constituatively produced TNF-α and inducible TNF-α in the presence of (n-3) fatty acids.

Feeding dietary (n-3) PUFA has been shown to decrease PGE₂ production by peripheral blood mononuclear cells in dogs. Dogs consuming diets high in (n-3) fatty acids showed a significant decrease in PGE₂ production compared with dogs that consumed diets low in (n-3) PUFA. In the study reported here, LPS-stimulated BALF cells from horses fed fish oil produced lower levels of PGE₂ than those from horses fed corn oil.

It has been shown that high levels of PGE₂ can diminish TNF-α production by increasing cellular levels of cyclic adenosine monophosphate, whereas low levels of PGE₂ stimulate cyclic guanosine monophosphate production, which leads to increased TNF-α production. However, the results of our study are not directly in support of the relationship that high levels of PGE₂ decrease TNF-α production, because clearly, BALF cells from horses fed corn oil should have produced less TNF-α than those from horses that were fed fish oil.

In the current study, there was a significant, negative correlation between the ratio of EPA : AA in plasma fatty acids and the production of PGE₂ by LPS-stimulated BALF cells. Thus, as the EPA : AA ratio increased (eg, in the fish oil–fed horses), PGE₂ production decreased. We did not measure the fatty acid profiles of membrane phospholipids of BALF cells in our study. However, others have shown that equine monocytes have increased (n-3) fatty acid incorporation into their membrane phospholipids when fed (n-3)
enriched dietary supplements or given (n-3) enriched IV infusions.

The PGE2 monoclonal antibody used in this study had a 43% cross-reactivity with PGE3. Because PGE2 and PGE3 were not separated by high-performance liquid chromatography before quantification, it could be that actual PGE2 concentrations were even lower in the horses fed fish oil, because part of the recorded PGE2 may have represented increased PGE3, similar to the LTB4 and LTB5 findings in the accompanying paper by Hall et al.19

Even though there were significant weekly changes in white blood cell and neutrophil counts, all values remained within normal reference intervals. Differential cell counts on BALF showed that neutrophil percentages were not different across diet or time periods. The changes in the other cell types (macrophages, lymphocytes, and others) were statistically but not clinically significant.

In summary, dietary supplementation with either corn oil or fish oil modulated the inflammatory response of normal horses in several ways. Phagocytic activity of BALF cells was increased after dietary supplementation with either corn or fish oil compared with pretrial levels. Both fatty acid supplements increased production of the proinflammatory cytokine TNF-α after 6 weeks of supplementation. In addition, corn oil increased production of the proinflammatory eicosanoid PGE2 after 12 weeks of supplementation. Supplementation with fish oil appears to inhibit the LPS-induced increase in PGE2 production by BALF cells.

**Footnotes**

1. Dormosedan, SmithKline Beecham Animal Health, Exton, PA
2. Coulter Electronics, Inc., Hialeah, FL
3. VMRD Inc., Pullman, WA
4. Pharmingen International, San Diego, CA
5. Jackson ImmunoResearch Inc., West Grove, PA
6. Sigma Chemical Company, St. Louis, MO
7. UNIFLX-Plus, Scheicher and Schuell, Keene, NH
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