Dietary Flaxseed Supplementation Ameliorates Inflammation and Oxidative Tissue Damage in Experimental Models of Acute Lung Injury in Mice[1]

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ABSTRACT Flaxseed (FS) is a nutritional supplement with high concentrations of (n-3) fatty acids and lignans that have anti-inflammatory and antioxidant properties. The use of FS in the prevention or treatment of acute lung disease is unknown. In this study, we evaluated diets with high FS content in experimental murine models of acute lung injury and inflammation. The kinetics of lignan accumulation in blood, following 10% FS supplementation, was determined using liquid chromatography tandem mass spectrometry. Mice were fed isocaloric control and 10% FS-supplemented diets for at least 3 wk and challenged by hyperoxia (80% oxygen), intratracheal instillation of lipopolysacharide, or acid aspiration. Bronchoalveolar lavage was evaluated for white blood cells, neutrophils, and proteins after a 24 h postintratracheal challenge of hydrochloric acid or lipopolysacharide, or after 6 d of hyperoxia. Lung lipid peroxidation was assessed by tissue malondialdehyde concentrations. The plasma concentrations of the FS lignans, enterodiol and enterolactone, were stable after mice had eaten the diets for 2 wk. Following hyperoxia and acid aspiration, bronchoalevolar lavage neutrophils decreased in FS-supplemented mice ($P = 0.012$ and $P = 0.027$, respectively), whereas overall alveolar white blood cell influx tended to be lower ($P = 0.11$). In contrast, neither lung injury nor inflammation was ameliorated by FS following lipopolysacharide instillation. Lung malondialdehyde levels were lower in hyperoxic mice than in unchallenged mice ($P = 0.0001$), and decreased with FS treatment following acid aspiration ($P = 0.011$). Dietary FS decreased lung inflammation and lipid peroxidation, suggesting a protective role against pro-oxidant-induced tissue damage in vivo. J. Nutr. 136: 1545–1551, 2006.

KEY WORDS: • *flaxseed* • *lignans* • *acute lung injury* • *oxidant stress*

The lung is a major target of oxidant stress. Several pulmonary diseases, including adult respiratory distress syndrome (ARDS)[3] and asthma, have been associated with an imbalance in the pro-oxidant vs. antioxidant state of the lung tissue itself (1). Although major progress has been achieved in deciphering the mechanisms of oxidative tissue injury, safe therapeutic antioxidant therapies have yet to be validated. Several antioxidant approaches, such as systemic or targeted delivery of antioxidant enzymes, have been evaluated in the context of lung disease with variable outcomes (2). Because dietary intervention with antioxidant micronutrients such as vitamins, minerals, and plant-derived biomolecules have shown some promise in the context of cardiovascular disease (3,4), further evaluation in the context of lung disease could be a fruitful area for additional study.

To date, certain antioxidant nutrients have been implicated in the protection against lung diseases, such as chronic obstructive pulmonary disease (5), ARDS, and asthma (6–8). ARDS patients were shown to have significantly lower plasma levels of vitamin E and vitamin C, and of β-carotene and selenium (9), which was thought to be an indication that the antioxidative system in these patients was compromised. Gadek et al. (10) investigated the role of dietary supplementation of antioxidant nutrients in patients with sepsis-induced ARDS and showed that enteral feeding of antioxidants (and oil supplements) improved lung microvascular permeability, oxygenation, and cardiopulmonary function. Furthermore, this treatment reduced proinflammatory eicosanoids (cyclooxygenase and lipooxygenase metabolites of arachidonic acid) and augmented the antioxidant profile of the patients.

We hypothesized that flaxseed (FS) could be another useful antioxidant nutrient. FS, which is used as a nutritional supplement, has a high concentration of (n-3) fatty acids and lignans. It has gained popularity due to its potential use in the reduction of cardiovascular diseases (11). (n-3) Fatty acids help to reduce inflammation and may be helpful in treating a variety of cardiovascular and autoimmune diseases (12–14). Lignans,

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[3] Abbreviations used: ALI, acute lung injury; ARDS, adult respiratory distress syndrome; BAL, bronchoalveolar lavage; ED, enterodiol; EL, enterolactone; FS, flaxseed; LC/MS/MS, liquid chromatography tandem mass spectrometry; LPS, lipopolysacharide; MDA, malondialdehyde; PMN, polymorphonuclear leukocyte; SDG, secoisolariciresinol diglucoside; SECO, secoisolariciresinol; WBC, white blood cells.

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widely occurring plant compounds closely related to lignin (which forms the woody component of trees and other plants), reportedly possess antioxidant properties (15). Secoisolariciresinol diglucoside (SDG), isolated from FS, is metabolized in the mammalian intestine to the lignans enterodiol (ED) and enterolactone (EL). The oxygen radical scavenging properties of the FS lignans were shown in vitro by either direct hydroxyl radical scavenging activity (16,17) or inhibiting lipid peroxidation (18). In addition, because of the ability of lignans to act as platelet-activating-factor antagonists (19), the lignan SDG may exert antioxidant activity by inhibiting reactive oxygen species production by white blood cells (WBC). The antioxidant properties of FS lignans were also verified in models of endotoxic shock in dogs (19), diabetes in rats (20), and in carbon tetrachloride-induced oxidative stress in rats (21). However, although reactive oxygen species production and oxidant stress have been implicated in the etiology of acute and chronic lung injury, the therapeutic or preventive use of dietary FS, or FS-derived lignans, have never been evaluated in lung disease.

Accordingly, in this study we evaluated the efficacy of dietary FS supplementation in antioxidant protection of the lung in clinically relevant murine models of pulmonary oxidant stress. The models include hyperoxia, lipopolysaccharide (LPS), and acid aspiration–induced lung damage.

MATERIALS AND METHODS

Animals

Female C57BL/6 mice, aged 6–8 wk (Charles River), were used throughout the study. All animals were cared for, handled, and housed at the Children's Hospital of Philadelphia (CHOP) animal facility. All protocols were performed in accordance with NIH guidelines and with the approval of the CHOP and the University of Pennsylvania Animal Use Committee (IACUC).

Diets and dietary treatments

A semipurified AIN-93G diet was used as the base diet (22), which, as established from previously published reports (23), was supplemented with 5 and 10% (w:w) FS, prepared by Purina Mills (TestDiet). Control and experimental diets were isocaloric and equivalent in terms of the percentage of protein, carbohydrate, and fat. The physiological fuel value for all 3 diets was kept the same, at 7.08 kJ/g (for details see **Table 1**). Whole ground yellow FS (Lot 1012338) was kindly provided by Dr. Jack Carter (North Dakota State University, Fargo, ND, and the North Dakota Disease Council). Diets were prepared using tocopherol-stripped oils, and vitamin E was removed from the AIN-93G vitamin mix. Because FS naturally contains small amounts of vitamin E (~3.35 mg/kg in the 10% FS diet and 1.68 mg/kg in the 5% FS diet), a constant amount of 3.35 mg/kg vitamin E was maintained in all 3 diets by adding the appropriate amount of *dl*-α-tocopheryl acetate as the source of vitamin E (see Table 1). To avoid potential oxidative degradation, the diets were stored at 4°C, and the cage receptacles were changed completely on a weekly basis.

Evaluation of toxicity of the FS-supplemented diet

To determine how well mice would tolerate a 10% FS supplemented diet, compared with an isocaloric control diet (0% FS), weight gain and lung tissue peroxidation were evaluated after 1, 2, 3, 4, and 10 wk of feeding ($n = 5$ mice/time). Mice were killed at each time point, weighed, and lung MDA levels were determined from lung homogenates. In addition, in a separate pilot experiment, hematoxylin and eosin-stained sections were evaluated from the formalin-fixed, paraffin embedded livers of mice fed 0, 5, and 10% FS ($n = 6$ mice/diet). Slides of tissue after 10 wk of dietary FS supplementation were coded and evaluated in a blinded fashion by a pathologist (C.C.-S.).

TABLE 1

Composition of study diets[1]

Nutrient profile	Control	5% Flaxseed	10% Flaxseed
		g/kg	
Cornstarch	375.4	344.1	312.8
Casein-vitamin free	200.0	200.0	200.0
Dextrin	132.0	132.0	132
Sucrose	100.0	100.0	100.0
Corn oil (tocopherol stripped)	92.0	73.0	54.7
Flaxseed	NA	50.0	100.0
Powdered cellulose	50.0	50.0	50.0
AIN93G mineral mix	35.0	35.0	35.0
AIN-93 vitamin mix[2] (no vitamin E)	10.0	10.0	10.0
L-Cystine	3.0	3.0	3.0
Choline bitartrate	2.5	2.5	2.5
dl-α-Tocopheryl acetate[3] (source of vitamin E)	0.00335	0.001675	NA
% Energy[4]			
Protein	19.34	20.19	21.03
Fat	21.68	22.76	23.87
Carbohydrate	58.97	57.04	55.09
Physiological fuel value, *kJ/g*	7.08	7.08	7.08

[1] The basic diet against which FS diets were compared was based on the AIN-93G standard formula (22) using corn oil instead of soybean oil.

[2] The AIN-93G vitamin mix used for both diets was vitamin E-free and the oils used were tocopherol-stripped to eliminate an antioxidant contribution to tissue protection, other than from FS.

[3] Because FS naturally contains small amounts of vitamin E (~3.35 mg/kg in the 10% FS and 1.68 mg/kg in the 5% FS diet), a constant amount of 3.35 mg/kg vitamin E was maintained in all 3 diets by addition of appropriate amounts of *dl*-α-tocopheryl acetate as the source of vitamin E.

[4] The levels of all ingredients were adjusted so as to make all the diets isocaloric.

Analytical evaluation of lignan concentrations in murine plasma samples

Eno Labs was contracted to determine the chemical structures of secoisolariciresinol (SECO), ED, and EL by liquid chromatography tandem mass spectrometry (LC/MS/MS) using commercially available standards of 95% purity (Chromadex) and to determine their concentration in mouse plasma samples (shown in **Figs. 1**, **3**). Plasma lignan levels were evaluated in 5 randomly selected mice at each time point (0, 1, 2, 3, 4, and 10 wk).

Chromatographic conditions. Lignans were separated by HPLC using gradient elution with 0.1% acetic acid in acetonitrile (Fisher) and 0.1% acetic acid in HPLC-grade water (Fisher). The flow rate was 0.5 mL/min over an endcapped C_{18} column (Phenomenex Aqua, 150 × 4.6 × 10 mm, 5-μm particle size, from Phenomenex) . The samples were stored at room temperature in the autosampler and the column was heated to 40°C. Standards of ED and EL were obtained in 95% purity from Chromadex. Standard solutions were prepared in acetonitrile and stored at −4°C. Retention times for ED and EL were 7.8 and 9.5 min, respectively.

Mass spectrometric conditions. Lignans were monitored by negative ion electrospray tandem mass spectrometry. The MRM transitions monitored were as follows (parent ion > daughter ion): SECO 361 > 361; ED 301 > 301; EL 297 > 297. Capillary voltage, cone voltage, and collision energy was optimized at 2.5 kV, 40 V, and 15 V, respectively, with a collision cell gas pressure of 0.22 Pa.

Extraction and sample preparation. EDTA-stabilized mouse plasma was purchased from Valley Biomedical and stored at −20°C prior to use. Standards and controls were prepared by adding 20 μL of the ED/EL standard in acetonitrile to 150 μL of EDTA-stabilized

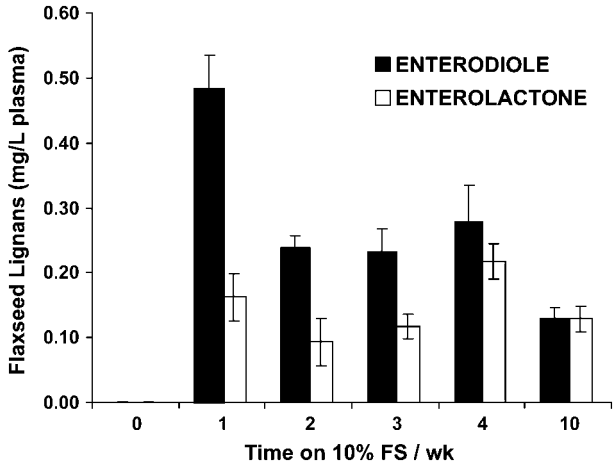

FIGURE 1 Plasma ED and EL concentrations in C57/Bl6 mice fed a 10% FS-supplemented diet for 0, 1, 2, 3, 4, and 10 wk. Results are shown as means ± SEM, $n = 4$. Plasma concentrations did not change from 2–10 wk, $P > 0.05$.

mouse plasma; they were vortexed and allowed to incubate for 20 min at room temperature. To the standards, controls, and 150 μL samples, we added 0.5 mL of 134 kU/L β-glucuronidase solution (Sigma) in sodium acetate buffer, pH 5.0; the samples were vortexed, covered, and incubated at 37 ± 2°C overnight (16 h). Buffered plasma was extracted 3 times with 2 mL ethyl acetate (Fisher) by vortex mixing for 2 min followed by centrifugation (2,000 × *g*; 5 min) to separate layers. The combined organic layers were evaporated under nitrogen at 40°C. The extracts were reconstituted to 100 μL with 30% acetonitrile/70% water for chromatography.

Mouse models of lung injury

Mice were fed FS-supplemented diet for 8 wk (Expt. series 1) or 3 wk (Expt. series 2 and 3) prior to lung challenge (as described in **Table 2**). Two of the 3 lung injury models tested (acid aspiration and LPS-induced injury) required the intratracheal administration of the proinflammatory agents (24). The exact concentrations and volumes of the inflammatory stimuli injected in the various models are described below.

Lipopolysacchride-induced lung injury. LPS (serotype 0111:B4, Sigma) was administered intratracheally (0.5 μg LPS in 100 μL of saline). Evaluation was performed 24 h after LPS administration because inflammation [as manifested by elevated protein, neutrophil, and total WBC levels in bronchoalveolar lavage (BAL) fluid] was shown to be maximal at this time (25).

Acid aspiration–induced lung injury. After mice were anesthetized, 40 μL of 0.1 mol/L HCl was administered to each mouse, as adapted from Goldman et al. (24).

Hyperoxic exposure. Mice were housed for 6 d in cages inside a hyperoxic chamber with a continuous flow of O_2 at 10 L/min, yielding O_2 concentrations of 80%, as determined by an oxygen analyzer (ESD model 600). Food (0 or 10% FS diets) and water were continuously available. After 6 d, lungs were evaluated for histopathology and BAL fluid was analyzed for protein concentrations and differential cell counts.

Measurement of lung malondialdehyde

Malondialdehyde (MDA), a reactive carbonyl compound formed upon the decomposition of polyunsaturated fatty acid peroxides, was

TABLE 2

Indicators of lung injury in mice fed 0 or 10% FS diets for 10 wk and subjected to AA, hyperoxia, or LPS instillation

ALI model and experimental series[2]	Dietary FS, %	BAL proteins, *g/L*	BAL WBC, *10^9cells/L*	BAL PMN, *10^9cells/L*	PMN, %
Acid aspiration					
1	0	1.41 ± 0.12	408 ± 51	136 ± 28	34 ± 9
	10	0.91 ± 0.26	296 ± 39	90 ± 33	28 ± 8
2	0	0.93 ± 0.31	710 ± 53	357 ± 103	59 ± 14
	10	1.0 ± 0.16	396 ± 18	68 ± 27	17 ± 6
3	0	1.43 ± 0.61	330 ± 38	109 ± 18	33 ± 4
	10	0.89 ± 0.08	277 ± 35	49 ± 16	22 ± 4
4	0	1.72 ± 0.6	316 ± 117	88 ± 29	38 ± 9
	10	1.55 ± 0.6	447 ± 112	79 ± 29	18 ± 5
		$P = 0.36$[3]	$P = 0.12$[3]	$P = 0.012$[3]	$P = 0.002$[3]
Hyperoxia					
1	0	5.10 ± 0.72	173 ± 31	72 ± 20	44 ± 15
	10	3.21 ± 0.97	158 ± 25	32 ± 13	24 ± 10
2	0	6.17 ± 1.85	519 ± 59	217 ± 101	39 ± 17
	10	4.53 ± 1.34	388 ± 59	43 ± 31	14 ± 11
3	0	NA[4]	NA[4]	NA[4]	NA[4]
	10	3.32 ± 0.74	119 ± 11	17 ± 9	12 ± 6
		$P = 0.15$[3]	$P = 0.10$[3]	$P = 0.027$[3]	$P = 0.07$[3]
LPS					
1	0	0.20 ± 0.02	210 ± 44	154 ± 43	69 ± 9
	10	0.30 ± 0.01	287 ± 49	206 ± 37	72 ± 6
2	0	0.35 ± 0.04	408 ± 25	302 ± 4	74 ± 4
	10	0.33 ± 0.04	457 ± 4.5	301 ± 20	66 ± 4
3	0	0.19 ± 0.03	442 ± 66	325 ± 57	71 ± 6
	10	0.26 ± 0.03	537 ± 67	441 ± 72	81 ± 4
		$P = 0.10$[3]	$P = 0.09$[3]	$P = 0.16$[3]	$P = 0.71$[3]

[1] Values are means ± SEM, $n = 4$–5.

[2] Mice in experiment series 1 were fed their respective FS diets for 8 wk and mice in experiment series 2, 3, and 4 were fed their diets for 3 wk prior to ALI challenge.

[3] 10% FS diet was compared with 0% FS-challenged control in each experimental series (experiments 1, 2, 3, and 4).

[4] Mice fed the control diet (0% FS) died within the experimental period (5 d), with maximal lung injury (macroscopic evaluation showing very dark, hemorrhagic lungs), and therefore no BAL samples could be collected for analysis.

measured as an indicator of oxidative stress (26). Lipid peroxidation in homogenized lung tissue samples was detected using a chromogenic reagent, N-methyl-2-phenylindole, which combines with MDA to yield a chromophore with maximum absorbance at 586 nm. The assay was conducted using a commercially available kit (OXIS International) according to the manufacturer's recommendations.

Evaluation of lung injury

The mice were killed at various time points after the induction of lung injury and BAL was performed as previously described (24). The concentration of total WBCs and neutrophils in the BAL fluid was determined from precipitates remaining after removal of the cell-free supernatant (24). The amount of protein in the BAL fluid was assayed using the BCA Protein Assay Kit (Pierce).

Statistical analysis

Results are presented as means ± SEM. Body weights, MDA levels in the lungs of unchallenged mice, and plasma lignan levels after 1, 2, 3, 4, and 10 wk of FS supplementation was analyzed by Student's *t* test. The effect of diet on BAL proteins, WBC, and polymorphonuclear leukocyte (PMN) percentages for each acute lung injury (ALI) model (i.e., acid aspiration, hyperoxia, and LPS) was assessed by 2-way ANOVA, controlling for Expt. series 1–4 (Table 2). Homogeneity of variances was evaluated using Levene's test, and data for PMN in the hyperoxia model underwent square root transformation to stabilize variance across groups. Differences in lung MDA levels among baseline, unchallenged mice, and each independent ALI model were evaluated using 2-way ANOVA (**Fig. 2**). Within each challenge group (acid aspiration, LPS, and hyperoxia), differences between 0 and 10% FS within models were evaluated using Student's *t*-test. Statistical analyses were conducted using R and Stata 7.0 (Stata Corporation). Differences were considered significant at $P < 0.05$.

RESULTS

Dietary FS supplementation is well tolerated over a long supplementation period. The body weights of mice fed the 10% FS-supplemented diet did not differ from those of mice fed the isocaloric control diet at any of the times examined ($P > 0.05$). Similarly, lung MDA concentrations of mice fed the control did not differ from those fed the FS-supplemented diets. In addition, histological evaluation did not show liver toxicity due to FS supplementation.

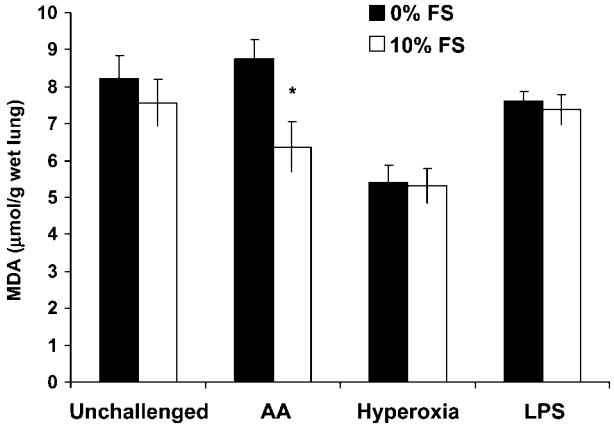

FIGURE 2 Effect of 10% flaxseed supplementation for 10 wk on lung lipid peroxidation, measured as MDA, following ALI in mice. Mice were subjected to acid aspiration (AA) or intratracheal instillation of LPS (0.5 μg/mouse), and lungs were harvested 24 h after challenge or were exposed to hyperoxia (80% oxygen, 5 d) and lungs were harvested after 5 d. Values are means ± SEM, $n = 4$–11. By 2-way ANOVA there was a main effect of FS supplementation, $P = 0.02$. The effect of diet, evaluated by Student's *t* test within each challenge group, was significant in mice subjected to acid aspiration, $^*P < 0.01$.

Analytical evaluation of FS-derived lignans in mouse plasma following dietary FS supplementation. Plasma concentrations of the SDG metabolite secoisolariciresinol (SECO) were undetectable at all time points evaluated, whereas ED and EL were easily measurable using LC/MS/MS (Fig. 1). With the exception of an initial high plasma concentration of ED after 1 wk of 10% FS supplementation, the plasma concentrations for each lignan were similar after 2, 3, 4, and 10 wk on the diet ($P > 0.1$).

In our initial experiments, which were conducted before obtaining the analytical data using LC/MS/MS (Fig. 1), mice were fed 10% FS for 8 wk before the ALI challenge (Expt. series 1). When data indicated that 2 wk of supplementation was adequate to obtain bioactive FS metabolites in target tissues, all subsequent ALI experiments were performed after 2–3 wk of FS supplementation (Expt. series 2, 3, and 4). The results from the individual experiments did not differ and the results from all ALI models evaluated are shown for each series of experiments (Table 2).

FS supplementation ameliorates hyperoxic- and acid aspiration–induced lung inflammation and oxidation. Hyperoxic exposure and acid aspiration lung injury are clinically relevant, well-standardized, and well-characterized models of ALI. We reasoned that dietary FS supplementation might ameliorate the adverse clinical outcomes associated with these pathological conditions. Indeed, the inflammatory cell infiltrate in the lungs was significantly ameliorated by 10% dietary FS in both the hyperoxic and acid aspiration models of ALI. Specifically, FS treatment decreased the hyperoxia- and acid aspiration–induced neutrophil influx into the alveolar space, and WBC infiltration tended to be lower ($P < 0.11$, Table 2). The alveolar protein level, a sign for increased lung permeability, was not affected by FS treatment.

Oxidative modification of lipids in tissues is associated with ALI and can be quantitated with sensitive in vitro assays. Lung MDA concentrations (Fig. 2) in mice with acid aspiration did not differ from unchallenged mice, whereas they were lower in mice after hyperoxia ($P = 0.0001$) than in unchallenged mice.

FS supplementation is not protective in LPS-induced ALI and does not prevent lipid peroxidation of lung tissue in vivo. FS treatment did not affect any of the ALI outcomes when lung injury was induced with intratracheal instillation of LPS (Table 2). The fact that dietary FS treatment was not able to confer protection to the tissue in LPS-induced lung injury may be an indication that the mechanism of disease may differ in this ALI model as compared with the other models evaluated in this study. Similarly, lung homogenate MDA concentration did not differ between LPS-treated mice and unchallenged control mice, nor was there a difference with FS treatment (Fig. 2).

Plasma FS-derived lignan concentrations and the extent of lung protection. Plasma ED and EL concentrations were similar in unchallenged mice and they both were lower in mice challenged with acid aspiration ($P < 0.05$). The plasma ED concentration was lower in those challenged with hyperoxia ($P < 0.05$); however, EL levels were not significantly different ($P = 0.28$) than in unchallenged mice (Fig. 3). In contrast, plasma lignan concentration was similar in LPS-challenged and unchallenged mice. Most importantly, the lower lignan concentrations occurred only in those ALI models where FS supplementation appeared to be protective, i.e., in hyperoxic and acid aspiration–challenged mice, but not in LPS-challenged mice (**Fig. 3**).

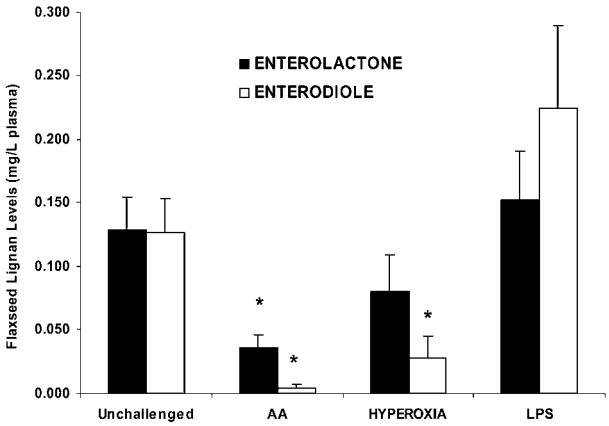

FIGURE 3 Plasma FS-derived lignan concentrations in mice that consumed a 10% FS diet for 10 wk and were then subjected to ALI. Plasma ED and EL were measured in unchallenged mice and in those subjected to acid aspiration (AA) or intratracheal instillation of LIS (0.5 μg/mouse). Values are means ± SEM, $n = 4$. *Different from the unchallenged group, $P < 0.05$.

DISCUSSION

This study investigated the therapeutic role of a nontoxic dietary agent, FS, in the context of acute lung disease. A common concern in dietary research is that the compound under study might reduce food consumption (presumably because of reduced palatability of the food) and therefore energy intake, making the evaluation of potential beneficial effects of the active ingredients complicated. Our study showed that a diet supplemented with 5 and 10% FS (w:w) was well tolerated by mice over a period of 2 mo as judged by weight gain, overall lung tissue oxidative analysis, and most importantly, unremarkable liver pathology.

Secoisolariciresinol diglucoside (SDG) is an antioxidant, isolated from FS, that is metabolized to SECO, ED, and EL in the body (17). These lignans are produced in the colon by the action of bacteria on the plant precursor SDG, a component of FS. These lignans are found in high concentrations in human and animal body fluids after ingestion of FS and some whole grain products (27). To our knowledge, our study provides the first analytical evaluation of ED and EL in mouse plasma using LC/MS/MS, which showed a quick accumulation of both ED and EL in the bloodstream (1 wk) that reached a plateau at about 10 mg/L within 2–3 wk on the FS diet. Our study provides evidence that dietary FS can significantly decrease acute lung injury, as well as inflammation-related variables in mouse models of both hyperoxia- and acid aspiration–induced ALI, whereas FS was not protective, at the dose tested, in LPS-induced ALI. In addition, oxidative stress by lung lipid peroxidation, and measured as MDA, was reduced by FS in mice challenged with acid aspiration and hyperoxia. In addition, circulating plasma lignan concentrations were significantly lower in both acid aspiration and hyperoxia models, but not after LPS challenge. The findings of this study provide novel insights into the usefulness of such dietary agents as potential adjuvant therapeutic tools in the treatment of ALI. Despite extensive studies on the usefulness of FS and FS lignans in the treatment of cancer (28,29), and of FS oils in atherosclerosis (11), nothing is known about the use of these agents in acute lung disease associated with oxidative stress and inflammation. The current study provides a validation of dietary FS as a protective strategy in selected types of oxidative lung injury and, to our knowledge, it is the first comprehensive evaluation of FS in several clinically relevant murine models of ALI.

Murine models of ALI are routinely used for the evaluation of potential therapies. To evaluate antioxidant and protective effects of FS in vivo, we selected 3 models of ALI with high relevance to clinical human ARDS, namely, LPS, acid aspiration injury, as well as prolonged hyperoxic exposure in mice; each of these models is closely linked to reactive oxygen species generation and pulmonary oxidant stress. The intrapulmonary deposition of endotoxin (LPS) from Gram-negative bacteria simulates bacterial pneumonia, a mode of injury characterized by an intense local inflammatory response associated with diffuse neutrophilic infiltrate and cell damage that evolves over several days (30). Intratracheal instillation of LPS, simulating the effects of bacterial pneumonia, results in ALI within just 24 h (25). LPS activates alveolar macrophages and causes early infiltration of neutrophils, further exacerbating the injury (31). Acid aspiration results in a dramatic increase of lung permeability, which is associated with the WBC influx of predominantly PMN in the BAL. Aspiration of gastric contents is one of the most common causes of acute lung injury or acute respiratory distress syndrome, and is reported to be associated with a 25–35% incidence of ARDS (32,33).The aspiration of gastric contents has been modeled in mice by the intratracheal instillation of hydrochloric acid (25). Aspirated HCl evokes direct damage to the alveolar-capillary membrane and promotes PMN adhesion, activation, and sequestration. In addition, HCl aspiration is associated with thromboxane synthesis and the generation of oxygen radicals associated with PMN activation.

Finally, the pathogenesis of pulmonary oxygen toxicity is thought to be initiated by the generation of highly reactive oxygen and nitrogen species (34,35). These radicals can react with and damage many important biomolecules, including enzymes, structural proteins, membrane lipids, and DNA, which leads to direct endothelial and epithelial cell damage. This response is simulated in mice after prolonged hyperoxic exposure at 80% oxygen levels. High concentrations of oxygen cause deleterious alterations in the lungs of humans and other animals (36,37). These changes occur in a progressive fashion and include both direct damage and death to endothelial and epithelial cells, as well as damage induced by the bioactive product release of neutrophils, macrophages, and platelets that are recruited to the lung. Oxygen injury is associated with pulmonary edema, hemorrhage, and increased alveolar-capillary leakage. Alveolar-capillary leakage increases the abundance of plasma protein in the alveolar space.

Our studies indicate a protective effect of dietary FS in lung tissue oxidation and inflammation in at least acid aspiration- and hyperoxia-, but not LPS-induced ALI. Because we did not have defatted or lignan-free FS available to test, we were unable to clearly attribute the antioxidant effects of the diet to either of the bioactive components alone. However, we can provide a possible explanation as to why FS was protective in acid aspiration and hyperoxic injury. Acid aspiration–induced ALI is associated with tissue oxidative stress, as evidenced by lipid peroxidation products such as lipid peroxides (38). Hyperoxia is also associated with detectable levels of MDA formation (39). Lipid peroxides are labile and can undergo further enzymatic and nonenzymatic decomposition to produce products such as malondialdehydes, which we were able to quantify and, indeed, to show a decrease of with the use of FS supplementation. In contrast, at least under the testing conditions of our model, LPS instillation was not associated with an increase of lipid oxidation in the lung above baseline values. This may explain why FS did not have an effect.

The flaxseed lignan SDG and its metabolites (ED and EL) have known antioxidant activities, shown in both in vitro (18) and in vivo systems (19), that are exerted mainly through the inhibition of lipid peroxidation. Pattanaik and Prasad (19) investigated cardiac dysfunction and tissue injury, conditions related to increased levels of oxygen free radicals, during endotoxemia in dogs fed FS-containing diets. They measured antioxidant enzyme activity, as well as cardiac MDA concentration, and concluded that pretreatment with FS for up to 6 d attenuated cardiac dysfunction and cellular damage. This result correlates well with our findings that, after dietary FS supplementation, plasma concentrations of ED and EL were detectable as early as 1 wk and then reached steady levels that persisted for several weeks on the FS diet. Future analysis of whole-lung homogenates will determine tissue lignan levels following dietary FS administration.

Circulating levels of cell adhesion molecules (CAM) as well as endothelial CAM levels are known to change in ARDS (40). Thies et al. (41) showed that FS oil, which is rich in α-linolenic acid, reduced the plasma concentrations of soluble vascular cell adhesion molecule-1, VCAM-1, and soluble E-selectin, but not soluble ICAM-1, under unchallenged baseline conditions. However, in response to E. coli, FS oil did not affect the number of inflammatory cells in the circulation, nor neutrophil and monocyte phagocytosis, nor respiratory burst. Most importantly, treatment did not decrease the production of tumor necrosis factor-α, interleukin-1-β, and interleukin-6 in response to LPS. This could potentially explain why FS did not protect against inflammation and lung injury in our models of LPS-induced lung injury.

The fact that the circulating lignans, ED and EL, were dramatically decreased only in those models of injury (hyperoxia and acid aspiration) where FS supplementation was protective, but not in the model where there was no conferred tissue protection (LPS model), is puzzling. This inverse correlation of plasma lignan concentrations and lung protection could possibly be explained due to differential *1*) clearance by the kidney; *2*) uptake (efflux) into tissues such as the lung, whereby these agents can exert their antioxidant protection; or *3*) conversion to oxidized metabolites with mass spectra different from the parent compounds (42). The evaluation of tissue concentrations of lignans was beyond the scope of this study, but we anticipate that future studies evaluating lung tissue levels of lignans during ALI challenge will conclusively differentiate among these possibilities.

In conclusion, the lung is an organ particularly susceptible to oxidative stress. To our knowledge, our study shows here the first supporting evidence to indicate that dietary supplementation with FS can ameliorate oxidative tissue damage and inflammation in certain forms of experimental acute lung injury.

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