

FLAXSEED OIL AND PREVENTION OF PULMONARY FIBROSIS

by

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B.S., Ewha Womans University in Seoul, Korea, 2000
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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Human Nutrition
College of Human Ecology

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2012

Abstract

Although omega-3 fatty acids have been a hot issue in nutrition for years, there remains a paucity of research on the topic of omega-3 fatty acid and pulmonary fibrosis and the mechanism is still unclear. The purpose of this research is to investigate the preventive effects of flaxseed oil for bleomycin-induced pulmonary fibrosis in rats and to find the possible underlying mechanisms. There are two experiments demonstrated in this dissertation, one is with various doses of flaxseed oil in the diet (0, 2.5, 5, 7.5, 10, 12.5, and 15 % (w/w)), and the other is with different times of sacrificing animals after oropharyngeal bleomycin treatment (days 7 and 21).

In the first study, three proteins including transforming growth factor- β (TGF- β), interleukin-1 (IL-1), and α -smooth muscle actin (α -SMA), commonly associated with fibrotic inflammation in the lung, were examined by Western blot and fatty acids composition of the diets and tissues were analyzed by gas chromatography (GC). Fifteen percent of flaxseed oil group significantly reduced septal and vascular thickness and fibrosis in the lung, and significant cardiac fibrosis in the heart. The amount of IL-1 and α -SMA decreased significantly as the amount of omega-3 fatty acids increased, whereas TGF- β did not change significantly.

The next study further reported the time-course effect and potential underlying mechanisms. Both interleukin-6 (IL-6), a protein associated with fibrotic inflammation in the lung, and renin, an enzyme related to renin-angiotensin system, were examined by Western blot. The time-dependent increase of IL-6 in response to bleomycin treatment was reversed by flaxseed oil diet. Although renin was not significantly different in the kidney, it suggested that the renin-angiotensin system may be involved locally. In addition, the profiles of fatty acids in both liver and kidney tissues as measured by lipidomics demonstrated a significant increase of omega-3: omega-6 ratio in the flaxseed oil-fed groups.

Overall, these results indicated for the first time that the omega-3 fatty acids rich in flaxseed oil inhibited the formation of pulmonary fibrosis in a dose-dependent manner - however the moderate dose of flaxseed oil was most effective - via anti-inflammatory mechanisms, which appears associated with the modulated fatty acid composition in the tissues.

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Acknowledgements

I would like to express my deepest gratitude to Dr. Richard C. Baybutt and Dr. Weiqun (George) Wang who have served as my academic advisors at Kansas State University. As my advisor and research supervisor, I am deeply appreciative of Dr. Baybutt's guidance, patience and spiritual advice. As my new academic advisor and mentor after Dr. Baybutt joined Wheaton College, Dr. Wang gave me continuous support, advice, and encouragement much like my dad. Without their supervision, I would not have finished my degree in KSU. I am such a lucky person to have advisors like them.

I am also thankful to the rest of my research committee members, Dr. Dingbo Lin for his valuable technique assistance, Dr. Weixing Song from the Department of Statistics for his expertise on statistical analysis, and Dr. John M. Tomich for his critical comments and kind advice as a scientist. Especially, Dr. Lin was the one who always tried to understand and encourage me. I would also like to thank Dr. Agostino Molteni from University of Missouri-Kansas City School of Medicine for his thoughtfulness, insight, and works regarding histological analysis of tissues, and Dr. Mary Roth, Dr. Thilani Samarakoon, and Dr. Ruth Welti from the Kansas Lipidomics Research Center in the Division of Biology for their help and guidance with the fatty acid analysis techniques.

I would also like to thank my colleagues, Dr. Yu Jiang, Dr. Ping Ouyang, Dr. Jennifer Hanson, Dr. Soyoung Lim, Dr. Jaeyong Kim, Tzu-Yu Chen, Joe Standard and Jianteng Xu, for their valuable advice and being my dear friends in such a happy working environment.

I am grateful to my family, especially my parents Dr. Kyungsam Choi and Ms. Haeyun Song, for unlimited love, support and understanding all throughout my life. Also I thank my sister Dr. Seo-eun Choi, who has been my best friend and given me advice as my sister.

Above all, I give praise to my Lord Jesus Christ, who had saved my life and has shown me the greatest love.

Chapter 1 - Introduction

Polyunsaturated fatty acids (PUFA) are represented essentially by two series, the omega-6 and the omega-3 series, whose precursors are linoleic acid (18:2 omega-6, LA) and α -linolenic acid (18:3 omega-3, ALA).¹ (Table 1-1)¹ Their two derivatives arachidonic acid (20:4 omega-6, AA) and eicosapentaenoic acid (20:5 omega-3, EPA), respectively, have been shown to interfere with the synthesis of a variety of inflammatory factors and events. Moreover, omega-3 PUFAs also exert their known anti-inflammatory properties by direct action on the cellular production of major cytokine inflammation mediators, on endothelium dysfunction and on leucocyte chemotaxis.^{3,4} Due to these effects, omega-3 PUFA have been used in the treatment of various chronic inflammatory conditions.⁵

The typical Western diet is rich in omega-6 and relatively low in omega-3 PUFA. Among the fatty acids, the omega-3 PUFA is the one that seems to have the most powerful immunomodulatory actions, and among the omega-3 PUFA those from fish oil (EPA) and docosahexaenoic acid (DHA) are more biologically effective than ALA, which is their precursor. Some of the properties of omega-3 PUFA take place through controlling of the quantities of diverse kinds of eicosanoids made, while other effects are connected with modifications in membrane fluidity, transcription factor activity, gene expression, and intracellular signaling pathways. It is indicated from *in vivo* studies and clinical intervention studies that some omega-3 fatty acids have powerful anti-inflammatory natures and might be helpful in the management of inflammatory and autoimmune disease.⁶

Both LA and ALA are essential fatty acid and as such we have to get them from our diet. Since LA and ALA compete with one another for the enzymes responsible for the synthesis of various eicosanoids, an overload of one kind of fatty acids can interfere with the metabolism of the other, decreasing its incorporation into tissue lipids and modifying their biological effects.⁷ An appropriate balance of essential fatty acids in the diet is important for maintaining good health and optimum performance in our body. Thus, the omega-6:omega-3 fatty acid ratio can impact our body's metabolic and inflammatory state.⁸ In typical Western diet today, the ratio might be as much as 20-30:1.⁹ The optimal ratio recommended by Health Canada is 4:1 to 10:1, the US Food and Drug Administration has not set the official recommendation yet.⁷

Although the benefits of fish oil which is rich in EPA are well known and popular in the market, flaxseed oil which is rich in ALA has not been investigated extensively. Flaxseed oil is a colorless to yellowish oil obtained from the seeds of the flax plant (*Linum usitatissimum*, also known as common flax or linseed). Flax is a blue flowering plant that produces small, flat seeds that range in color from golden yellow to reddish brown. Flaxseed is commonly found as whole seed, ground seed (powder or meal), or flaxseed oil.¹⁰ The oil in flaxseed is unique in that it is comprised of 73 % PUFA, 18 % MUFA, and 9 % saturated fatty acids, making it a low-saturated fat food.¹¹ Flaxseed oil is known as the richest source of the omega-3 fatty acid, ALA, which involves approximately 55 % of the total fatty acids.¹² The percent of fat as ALA in flaxseed is 5.5 times higher than that in walnuts and canola oil, which are the second-richest products.¹³ Flaxseed oil is generally safe when used appropriately, however large doses of 30 g/day or higher can cause loose stools and diarrhea. Some studies reported that ALA could increase the risk of prostate cancer, however it is found to be safe with ALA from plant sources, such as flaxseed oil.^{14, 15, 16}

Previously, our lab was the only lab to demonstrate that short chain omega-3 fatty acid (ALA) prevented bleomycin-induced pulmonary fibrosis. In addition, there have been no studies to determine the mechanism by which short chain omega-3 fatty acid prevent bleomycin-induced pulmonary fibrosis. Furthermore, the role of inflammation in the development of fibrosis has not been clearly defined. The purpose of this dissertation is to establish the preventive effect of omega-3 fatty acids in flaxseed oil on bleomycin-induced pulmonary fibrosis in rats and to find the possible underlying mechanisms.

Table 1-1 Omega-3 and omega-6 series of PUFA²

Notation	Common Name	Formula
C18:2 $\Delta^{9,12}$ (n-6)	Linoleic acid	CH ₃ - (CH ₂) ₄ - CH= CH- CH ₂ -CH= CH-(CH ₂) ₇ -COOH
C18:3 $\Delta^{9,12,15}$ (n-3)	α -Linolenic acid	CH ₃ - (CH ₂ - CH= CH) ₃ -(CH ₂) ₇ -COOH
C20:4 $\Delta^{5,8,11,14}$ (n-6)	Arachidonic acid	CH ₃ - (CH ₂) ₃ -(CH ₂ -CH=CH) ₄ -(CH ₂) ₃ -COOH
C20:5 $\Delta^{5,8,11,14,17}$ (n-3)	Eicosapentaenoic acid	CH ₃ - (CH ₂ - CH= CH) ₅ -(CH ₂) ₃ -COOH
C22:6 $\Delta^{4,7,10,13,16,19}$ (n-3)	Docosahexaenoic acid	CH ₃ - (CH ₂ - CH= CH) ₆ -(CH ₂) ₂ -COOH

*Number of first carbon double bond from methyl end (n system)

+Number of carbon double bond from carboxylic acid end (Δ system)

Reference

1. Nakamura MT and Nara TY. Structure, function, and dietary regulation of delta 6, delta 5, and delta 9 desaturases. *Annu Rev Nutr.* 24:345-76, 2004.
2. Gropper SS, Smith JL, and Groff JL. *Advanced Nutrition and Human Metabolism*, 4th Edition. Belmont, CA: Wadsworth, 2005.
3. James MJ, Gibson RA, and Cleland LG. Dietary polyunsaturated fatty acids and inflammatory mediator production. *Am J Clin Nutr.* 71(Suppl): S343-8, 2000.
4. Calder PC. Polyunsaturated fatty acids, inflammation, and immunity. *Lipids.* 36:1007-24, 2001.
5. Calder PC. N-3 polyunsaturated fatty acids and inflammation : from molecular biology to the clinic. *Lipids.* 38:343-52, 2003.
6. McCowen KC and Bistran BR. Essential fatty acids and their derivatives. *Curr Opin Gastroenterol.* 21:207-215, 2005.
7. Harper CR and Jacobson TA. The fats of life. *Arch Intern Med.* 161:2185-2192, 2001.
8. Tribble EF, Thompson RL, Harrison RA, Summerbell CD, Ness AR, Moore HJ, Worthington HV and Durrington PN. (2006). Risks and benefits of omega 3 fats for mortality, cardiovascular disease, and cancer: systematic review. *BMJ* 332(7544): 752–760, 2006.
9. Connor WE. Importance of omega 3 fatty acids in health and disease. *Am J Clin Nutr.* 71(Suppl):171S-175S, 2000.
10. Bloedon LT and Szapary PO. Flaxseed and cardiovascular risk. *Nutr Reviews.* 62: 18-27, 2004.
11. Morris D. Essential nutrients and other functional compounds in flaxseed. *Nutr Today.* 36: 159-162, 2001.
12. Carter JF. Potential of flaxseed and flaxseed oil in baked goods and other products in human nutrition. *Cereal Foods World.* 38: 753-759, 1993.
13. Baybutt RC, Rosales C, Brady H, and Molteni A. Dietary fish oil protects against lung and liver inflammation and fibrosis in monocrotaline treated rats. *Toxicology.* 175:1-13, 2002.

14. Brouwer IA, Katan MB and Zock PL. Dietary alpha-linolenic acid is associated with reduced risk of fatal coronary heart disease, but increased prostate cancer risk: a meta-analysis. *J Nutr.* 134:919-22, 2004.
15. Chavarro JE, Stampfer MJ and Li H. A prospective study of polyunsaturated fatty acid levels in blood and prostate cancer risk. *Cancer Epidemiol Biomarkers Prev.* 16:1364-70, 2007.
16. Leitzmann MF, Stampfer MJ and Michaud DS. Dietary intake of n-3 and n-6 fatty acids and the risk of prostate cancer. *Am J Clin Nutr.* 80:204-16, 2004.

Chapter 2 - Literature Review

Characteristics of Pulmonary Fibrosis

Definitions and Classification

In 1868, Flint addressed a condition called “chronic pneumonitis” and observed that the fingertips of one patient presumed a bulbous form.^{1,2} This was probably the first recorded case of the condition we now recognize as idiopathic pulmonary fibrosis (IPF), formerly known as cryptogenic fibrosing alveolitis. It is an important and destructive disease with a median mortality of three years. It is worse than many cancers,³ and its incidence continues to rise, doubling in just over a decade (1990-2003).⁴

The interstitium is the microscopic space between the basement membranes of the alveolar epithelium and capillary endothelium and forms part of the blood-gas barrier. Idiopathic interstitial pneumonias (IIPs) are characterized by expansion of the interstitial compartment by inflammatory cells with associated fibrosis in many cases.⁵ The most recent international classification, published in 2002, divides IIPs into seven distinct groups – IPF, nonspecific interstitial pneumonia (NSIP), respiratory bronchiolitis interstitial lung disease (RBILD), desquamative interstitial pneumonia (DIP), acute interstitial pneumonia (AIP), cryptogenic organising pneumonia (COP) and lymphoid interstitial pneumonia (LIP).⁶ IPF is the most common form, explaining approximately 60 % of cases. IPF affects not only the interstitium but also the alveolar spaces, and is associated with a classic pathologic pattern called usual interstitial pneumonia (UIP).⁶ In UIP, “pneumonia” is applied to explain inflammation more willingly than infection, while “usual” indicates that the histological example is that most frequently detected.⁷ IPF has been characterized in the American Thoracic Society, European Respiratory Society, Japanese Respiratory Society, Latin America Thoracic Association (ATS/ERS/JRS/ALAT) consensus statement as “a specific form of chronic, progressive, fibrosing interstitial pneumonia of unknown cause, occurring primarily in older adults, limited to the lungs, and associated with the histopathologic and/or radiologic pattern of usual interstitial pneumonia (UIP)”.^{8,9}

Epidemiology and Pathology

IPF is slightly more general in males and typically presents in older adults with a mean age of onset of 67-69 years.¹⁰ In one of the best population-based studies from Bernalillo County in New Mexico, Coultas et al.¹⁰ recorded all new cases of interstitial lung disease registered over a 2-year period in a population of nearly half a million inhabitants. They reported an incidence/prevalence (male/female) per 105 population of 11/7 and 20/13, respectively. A Norwegian study reported an incidence/prevalence for hospitalized IPF of 4.3 and 20 per 1,000,000.¹¹ The incidence of IPF has been reported as growing by 11 % annually between 1991 and 2003, signifying the number of recorded diagnoses of IPF is doubling every 8 years.⁴

Typical symptoms of IPF contain chronic progressive exertional breathlessness, commonly along with a non-productive cough. As IPF progresses, breathlessness effects on activities of daily living, resulting in many patients becoming housebound, socially isolated and dejected.¹² Clinical examination usually demonstrates end inspiratory fine crackles, which are often ‘high pitched’ or ‘Velcro-like’ in character. Up to 50 % of patients have finger clubbing, and those with more advanced disease may develop cor pulmonale.¹³ Histological biopsies in UIP reveal areas of normal lung with exchanging areas of interstitial inflammation and fibrotic zones formed predominantly of intense collagen with spread foci of proliferating fibroblasts (“fibroblastic foci”), a consistent finding of UIP.¹⁴ Untreated IPF follows a persistently progressive course in most patients. Early studies usually overvalued survival rates, since patients with less destructive interstitial pathology such as NSIP were included in long-term follow-up. More recent studies have demonstrated that median survival is approximately three years.¹⁵ Respiratory failure (39 %) is the most frequent reason of death, while heart failure (14 %), pulmonary cancer (10 %), ischaemic heart disease (10 %), infection (6 %) and pulmonary embolism (3 %) are other causes of mortality.¹⁶

Pathogenesis

The cause of IPF is unknown. The original ‘inflammation/alveolitis’ hypothesis proposed that IPF was a chronic inflammatory disease, taking place in response to an unknown stimulus, initiated progressive lung injury if left untreated, and ultimately fibrosis.¹⁷ The key hypothesis of the etiology of IPF includes irregular wound healing in response to numerous,

microscopic locations of continuing alveolar epithelial injury and activation cooperated with the development of patchy fibroblast-myofibroblast foci, which consequently progress to fibrosis.¹⁸ The role of epithelial cell injury and fibrogenesis is critical to this hypothesis. Following epithelial cell injury from as yet unidentified triggers, there is accumulation of vascular exudates and inflammatory cells within the injured alveolar space. This epithelial cell injury results in the release of several proinflammatory and profibrotic mediators, including tumor necrosis factor alpha (TNF α), transforming growth factor- β (TGF- β), endothelin 1, metalloproteinases, and the coagulation mediator tissue factor, which may have synergistic effects on the induction of fibrosis.^{19, 20}

Various environmental stimuli have been suggested as risk factors for developing IPF, including cigarette smoking, antidepressants, chronic aspiration, metal and wood dusts, and infectious agents, including Epstein Barr virus.⁷ Familial IPF is rare, with a United Kingdom study reporting an incidence of 1.34 cases per million, explaining only 0.5-2.2 % of all IPF cases.²¹ A Finnish study reported similar findings, with familial IPF accounting for 3.3-3.7 % of cases.²² Patients with familial IPF are likely to occur at a younger age and some cases are related with surfactant protein C genes, with two mutations causing protein misfolding, triggering type 2 epithelial cell injury.^{23, 24} Beyond lung transplantation, there are no confirmed effective therapies for the treatment of IPF so far.²⁵

Use of Bleomycin in an Animal Pulmonary Fibrosis Model

There are numerous different methods for modeling pulmonary fibrosis. They involve: contact with bleomycin, silica or fluorescein isothiocyanate (FITC); irradiation; or expression of certain genes through delivery of a viral vector or operation of a transgenic procedure. Among them, bleomycin remains the most frequently used agent and can be given directly into the airway by intratracheal or intranasal routes or systemically via subcutaneous, intraperitoneal or intravenous injection. With bleomycin there is ease of delivery, low cost, and quick development of fibrosis. It is widely used and accepted in literature, but it also has several weaknesses such as lack of many UIP features (temporal heterogeneity, fibroblastic foci, and prominent AEC hyperplasia) and inconsistent fibrosis development. However, in spite of its drawbacks, the animal model using bleomycin remains the best available experimental means. The model is

used for disease pathogenesis research and new pharmaceutical compound tests.²⁶ Among these approaches, the method using a single dose of bleomycin delivered intratracheally is the most commonly used and referenced model. Bleomycin is a chemotherapeutic agent used in the treatment of a variety of tumors including lymphoma, squamous cell carcinoma and testicular carcinoma, due to its minimal immunosuppressive activity and low hematopoietic toxicity. However, this therapy is often complicated by a dose-dependent induction of interstitial pneumonitis that progresses into interstitial pulmonary fibrosis.²⁷ Bleomycin is thought to cause lung damage through direct DNA strand breakage and generation of free radicals, thus inducing oxidative stress.²⁸ When animals are treated with bleomycin, pulmonary fibrosis is induced and this response has been used as a model for studying mechanisms in the development of fibrotic lung diseases. Introduced via the respiratory system, bleomycin induces pathological disturbances similar to that of idiopathic pulmonary fibrosis commonly found in humans.²⁹

Omega-3 Fatty Acids and Pulmonary Fibrosis

Lipids, Inflammation and the Immune System

Inflammatory response is mainly a protective response that, when perpetuated, constitutes the mechanism for many diseases including pulmonary fibrosis. Activated immune system cells such as neutrophils, eosinophils, basophils, monocytes and lymphocytes are capable of modifying the fatty acid profile of their membranes depending on the dietary lipid contribution. During inflammation, the processes of capture, rolling, adhesion, and subsequent transmigration of leukocytes through the endothelium are led by different chemotactic stimulation mechanisms and are determined by the fixation of complementary adhesion molecules between leukocyte and endothelial cell surfaces. In addition, there are several chemical mediators as chemotactic factors and cytokines that have an influence on these processes and modulate surface molecule gene expression as well as fixation intensity. The lipid profile of immune cell membranes will influence the production of chemical mediators, thus determining response intensity.³⁰

Eicosanoids

It has been found that bleomycin-induced pulmonary fibrosis produces a variety of eicosanoids associated with inflammation and collagen deposition. Bleomycin has been shown to generate reactive oxygen species such as superoxide and hydroxyl radicals and to cause an increase in lipid peroxidation in microsomal fractions from the liver and the lung. In addition, an increase in the availability of free radicals has been found to increase the synthesis of certain prostaglandins. Both prostaglandins and leukotrienes have been implicated in the pathogenesis of a variety of inflammatory and immune processes.²⁹ These eicosanoids stimulate lymphocyte and fibroblast proliferation commonly associated with collagen deposition in the lungs and cause pulmonary fibrosis, and it is known that the metabolic activity of eicosanoids are derived from different sources of fatty acids.²⁷ They are products derived from the 20-carbon PUFAs, mainly AA and EPA. Their formation will depend on the composition of the leukocyte membrane phospholipids, in turn determined by the fatty acid profile of the diet.³¹ Eicosanoids have a very short life span, have a local autocrine or paracrine action and act on different biological processes like inflammation and hemostasis, helping to make the inflammatory process chronic.³²

Dietary fish oil has been found to protect against several inflammatory diseases, including rheumatoid arthritis, psoriasis, colitis, and asthma. In addition, fish oil inhibits the inflammatory response to certain endotoxins.^{33, 34, 35} Dietary fish oil enriches phospholipids with omega-3 fatty acids, particularly EPA, while decreasing the content of AA.³⁶ The commonly accepted mechanism for this reaction includes the change in eicosanoids from more biologically active prostaglandins/leukotrienes derived from AA to less biologically active ones derived from EPA or DHA. The different activity of eicosanoids derived from AA versus those derived from EPA is one of the most important mechanisms to explain the anti-inflammatory properties of omega-3 PUFA in many inflammatory diseases. (Figure 2-1³⁷) Since eicosanoids are formed by enzymatic phospholipase cleavage of phospholipids found in cell membranes, dietary induced changes in the fatty acid composition of tissue could conceivably result in alterations of eicosanoid composition and thus, the level of activity in an inflammatory reaction.³⁶ Eicosanoids associated with inflammation may therefore be attenuated by a change in dietary fatty acid. The omega-3 PUFAs in the diet can modify the composition of the micro-domains of T cell membranes, which participate actively in signal transduction mechanisms, modulating *in vivo*

production of pro-inflammatory cytokines coming from the omega-6 fatty acids. These fatty acids also diminish the accumulation of these cells in inflammation sites, either because of proliferation inhibition or increased apoptosis.^{38,39}

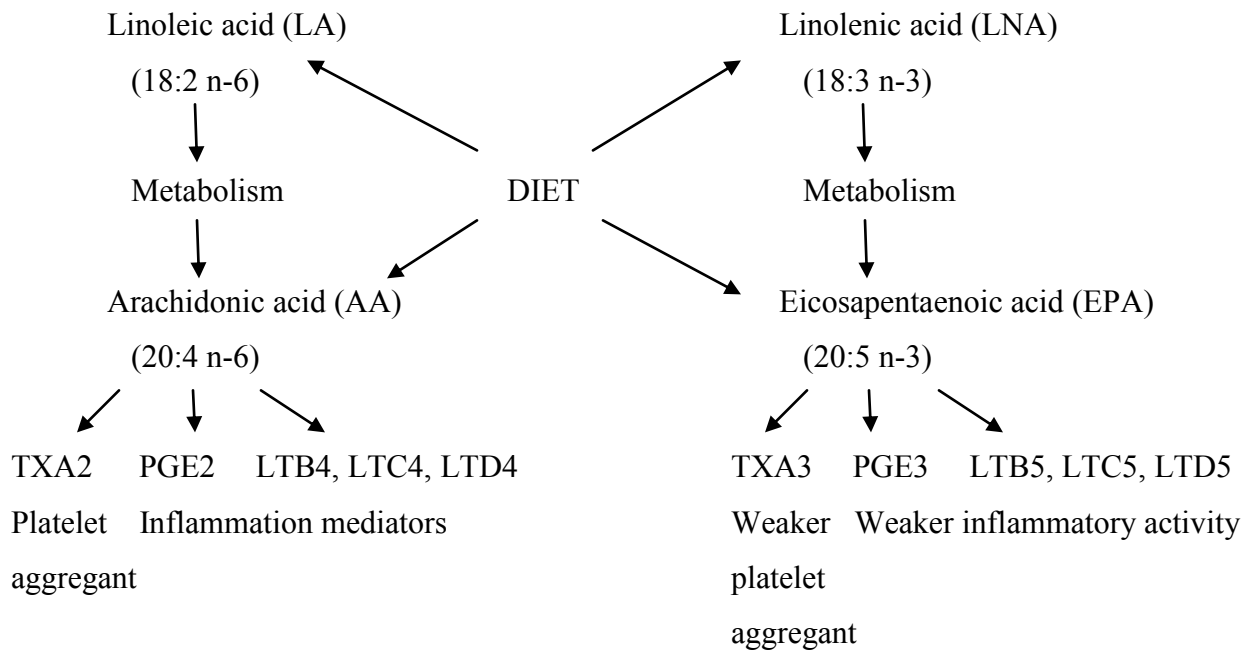


Figure 2-1 Interactions of n-3 PUFA with AA in the synthesis of eicosanoids with pro-inflammatory activity³⁷

Cytokines

Since inflammatory cytokine production is regulated by AA-derived eicosanoids and since dietary omega-3 PUFA affect production of these eicosanoids, it is expected that omega-3 PUFA will affect cytokine production.³⁸

Transforming growth factor β (TGF- β) is an important modulator in the acute repair phase of a wound. It is a profibrotic factor that affects many cellular functions, including fibroblast proliferation and chemotaxis, stimulating the synthesis and deposition of connective tissue, and the inhibition of connective tissue breakdown. When levels of TGF- β are chronically elevated, excessive fibrosis results which is characterized by increased collagen deposition. In general, excess fibrosis contains increased concentrations of collagen, a rich blood supply and myofibroblasts, identified by α -smooth muscle actin (α -SMA) in cytoplasmic stress fibers. TGF- β the major profibrotic growth factor during fibrogenesis is produced by fibroblasts, myofibroblasts, recruited smooth muscle cells, and macrophages.⁴⁰

It is known that production of Interleukin-1 α (IL-1 α), Interleukin-6 (IL-6) and TGF- β is altered in animal models of acute and chronic pulmonary diseases including those exposed to bleomycin.^{41, 42} Studies on IL-1 α and IL-6 have established the primacy of these mediators in upregulation of adhesion molecules that are able to recruit leukocytes.⁴³ Concerning lung inflammation, high concentrations of IL-6 have anti-inflammatory effects, apparently due to their ability to suppress IL-1 α production, thereby inhibiting the upregulation of endothelial adhesion molecules.⁴⁴ *In vivo* expression of IL-6 also significantly enhances lung TGF- β expression.⁴⁵ Since TGF- β is an important regulator of cell proliferation, differentiation, and formation of extracellular matrix and promoting tissue repair after a persistent inflammatory response⁴⁶, IL-6 also has an indirect pro-fibrotic effect.

In recent years various *in vivo* studies have shown that certain PUFA contained in fish oil reduce inflammation, diminishing the response to inflammatory and profibrotic cytokines such as IL-1 α and TGF- β .⁴⁷ Fish oil has been employed for the treatment of several pathologies, such as glomerulonephritis, risk of cardiovascular diseases, rheumatoid arthritis, murine lupus nephritis, and even as an adjuvant in cancer therapy. The biological effects of fish oil are attributed to omega-3 PUFA, mainly EPA and DHA.^{47, 48}

Studies on Omega-3 Fatty Acids and Pulmonary Fibrosis

There is a paucity of information concerning the potential benefits of omega-3 fatty acids in protecting against pulmonary fibrosis. More studies and investigation seem to be needed in this area of study.

Kennedy et al.²⁹, reported that a diet rich in EPA could significantly improve bleomycin-induced pulmonary fibrosis, possibly by means of modifications in eicosanoid metabolism. They examined the effect with 27 male Fischer 344 weanling rats, receiving one of two diets (standard lab diet or menhaden oil diet rich in EPA) for eight weeks. In this experiment, they found that the increase in DNA content observed with bleomycin treatment was less in animals fed menhaden oil than in animals fed the laboratory diet. In addition, menhaden oil diet significantly reduced the percentage of morphologically abnormal lung and lung hydroxyproline content following bleomycin treatment. There was also a slight increase in the percent of neutrophils, eosinophils, and lymphocytes in bleomycin-treated animals receiving menhaden oil, although these increases did not reach statistical significance.

In the study of Baybutt et al.³⁶, they found that dietary fish oil, containing long chain omega-3 fatty acids, protected against monocrotaline (MCT, a pulmonary toxin) -induced pulmonary fibrosis.³⁸ They did the experiment with 24 male Sprague-Dawley weanling rats, receiving either corn oil (15 %) or fish oil (13 % menhaden oil with the antioxidant tertiary butyl hydroquinone (30 mg/kg diet)) as the source of fat for 4 weeks. Septal fibrosis and occurrence of pneumonia were improved by dietary fish oil significantly. Vascular thickness was mildly reduced, and parenchymal inflammation and fibrosis were significantly reduced in the fish oil group treated with MCT, suggesting that inflammation of pulmonary parenchyma can be improved in the MCT model.

Recently, Lee et al.⁴⁹, demonstrated that diets with flaxseed prevented pulmonary fibrosis induced by thoracic X-ray radiation therapy (XRT), as they gave flaxseed supplementation to mice pre and post-XRT. Flaxseed is rich in high amount of omega-3 fatty acids and lignans with antioxidant properties. Mice were fed either a control diet or 10 % flaxseed diet, and 13.5 Gy thoracic XRT was treated after three weeks of diet. Lungs were assessed at 24 hours, three weeks, and at four months respectively to see markers for lung damages. As a result, flaxseed-fed mice had reduced expression of lung injury biomarkers such as Bax, p21 and TGF- β 1 at 24

hours, and decreased oxidative lung damage at 3 weeks after XRT. In addition, flaxseed-fed mice showed decreased lung fibrosis and reduced inflammatory cell influx into lungs at 4 months following XRT. Moreover, *in vitro* experiment from Karmiol et al.⁵⁰ demonstrated that α -LNA directly inhibits bleomycin-induced IL-6 production by rat pulmonary endothelial cells.

However, there is one study by Silva et al.²⁷, suggested that fish oil does not prevent bleomycin-induced pulmonary fibrosis. The authors carried out the research with 24 adult female Swiss mice receiving either control diet or fish oil diet. However, there was a point of difference in this study. They administered the fish oil 15 days after the bleomycin treatment, which means they actually tested whether fish oil was effective in treating fibrosis rather than preventing it. It is well known in the omega-3 fatty acid field that it takes about 2-4 weeks of feeding fish oil before the omega-3 fatty acids are incorporated into the phospholipids.

Given published laboratory findings, Iwai et al.⁵¹ performed epidemiological studies in Japan to detect risk factors for IPF, on the basis of 1,311 Japanese IPF autopsy cases. And they also did IPF autopsy cases and live case control study was carried out of 86 subjects with IPF, and found an inverse association between fish intake and IPF development. With another case study in Japan, Miyake et al.⁵¹, found that there was a direct association between dietary omega-6 PUFAs and IPF risk among 104 cases, who were over 40 years old. They revealed intake of saturated fatty acids, mono-unsaturated fatty acids, omega-6 PUFA and meat was independently associated with an increased risk of IPF.^{51, 52}

As shown above, omega-3 fatty acids are beneficial for inflammatory diseases, and they have the potential to prevent pulmonary fibrosis. However, as mentioned earlier, there is paucity of information concerning the potential benefits of omega-3 fatty acids in protecting against pulmonary fibrosis. In addition, many things regarding the underlying mechanisms are still not clear. More well conducted, randomized controlled trials appear warranted. In addition, further studies to determine the optimal dose of omega-3 fatty acids for the prevention of pulmonary fibrosis in human are needed, as well as studies on how they are affected by time.

References

1. Flint A. A treatise on the principles and practice of medicine. Philadelphia: Henry C. Lea; p,193, 1868.
2. Sharma OP and Chan K. Idiopathic interstitial pneumonitis/fibrosis: a historical note. *Curr Opin Pulm Med.* 5:275-7, 1999.
3. Flaherty KR, Travis WD and Colby TV. Histopathologic variability in usual and nonspecific interstitial pneumonias. *Am J Respir Crit Care Med.* 164:1722-7, 2001.
4. Gribbin J, Hubbard RB, Le Jeune I, Smith CJP, West J and Tata LJ. The incidence and mortality of idiopathic pulmonary fibrosis and sarcoidosis in the UK. *Thorax.* 61:980-5, 2006.
5. Visscher DW and Myers JL. Histologic spectrum of idiopathic interstitial pneumonias. *Proc Am Thorac Soc.* 3:322-9, 2006.
6. American Thoracic Society (ATS) / European Respiratory Society (ERS) classification of the idiopathic interstitial pneumonias. *Am J Respir Crit Care Med.* 165:277-304, 2002.
7. American Thoracic Society. Idiopathic pulmonary fibrosis: diagnosis and treatment. International Consensus Statement. American Thoracic Society (ATS) and the European Respiratory Society (ERS). *Am J Respir Crit Care Med.* 161:646-64, 2000.
8. Raghu G, Collard HR and Egan JJ. An official ATS/ERS/JRS/ALAT statement: idiopathic pulmonary fibrosis: evidence-based guidelines for diagnosis and management. *Am J Respir Crit Care Med.* 183:788-824, 2011.
9. American Thoracic Society (ATS) / European Respiratory Society (ERS) classification of the idiopathic interstitial pneumonias. *Am J Respir Crit Care Med.* 165:277-304, 2002.
10. Coultas DB, Zumwalt RE, Black WC and Sobonya RE. The epidemiology of interstitial lung diseases. *Am J Respir Crit Care Med.* 150:967-72, 1994.
11. Von Plessen C, Grinde O and Gulsvik A. Incidence and prevalence of cryptogenic fibrosing alveolitis in a Norwegian community. *Respir Med.* 97:428-35, 2003.
12. Swigris JJ, Kuschner WG, Jacobs SS, Wilson SR and Gould MK. Health-related quality of life in patients with idiopathic pulmonary fibrosis: a systematic review. *Thorax.* 60:588-94, 2005.
13. Dempsey OJ, Kerr KM, Gomersall L, Remmen H and Currie GP. Idiopathic pulmonary fibrosis: an update. *Q J Med.* 99:643-654, 2006.

14. O'Connell O, Kennedy MP and Henry MT. Idiopathic pulmonary fibrosis: Treatment update. *Adv Ther.* 1-14, 2011.
15. Daniil ZD, Gilchrist FC, Nicholson AG, Hansell DM, Harris J, Colby TV and du Bois RM. A histologic pattern of nonspecific interstitial pneumonia is associated with a better prognosis than usual interstitial pneumonia in patients with cryptogenic fibrosing alveolitis. *Am J Respir Crit Care Med.* 160:899-905, 1999.
16. Panos RJ, Mortenson RL, Niccoli SA and King TE Jr. Clinical deterioration in patients with idiopathic pulmonary fibrosis: causes and assessment. *Am J Med.* 88:396-404, 1990.
17. Noble PW and Homer RJ. Back to the future: historical perspective on the pathogenesis of idiopathic pulmonary fibrosis. *Am J Respir Cell Mol Biol.* 33:113-20, 2005.
18. Katzenstein AL and Myers JL. Idiopathic pulmonary fibrosis: clinical relevance of pathologic classification. *Am J Respir Crit Care Med.* 157: 1301-1315, 1998.
19. Chambers RC. Procoagulant signaling mechanism in lung inflammation and fibrosis: novel opportunities for pharmacological intervention? *Br J Pharmacol.* 153:S367-S378, 2008.
20. Selman M and Pardo A. Role of epithelial cells in idiopathic pulmonary fibrosis: from innocent targets to serial killer. *Proc Am Thorac Soc.* 3:364-372, 2006.
21. Marshall RP, Puddicombe A, Cookson WO and Laurent GJ. Adult familial cryptogenic fibrosing alveolitis in the United Kingdom. *Thorax.* 55:143-6, 2000.
22. Hodgson U, Laitinen T and Tukiainen P. Nationwide prevalence of sporadic and familial idiopathic pulmonary fibrosis: evidence of founder effect among multiplex families in Finland. *Thorax.* 57:338-42, 2002.
23. Lawson WE, Grant SW, Ambrosini V, Womble KE, Dawson EP, Lane KB, Markin C, Renzoni E, Lympny P, Thomas AQ, Roldan J, Scott TA, Blackwell TS, Phillips JA 3rd, Loyd JE and du Bois RM. Genetic mutations in surfactant protein C are a rare cause of sporadic cases of IPF. *Thorax.* 59:977-80, 2004.
24. Grutters JC and du Bois RM. Genetics of fibrosing lung diseases. *Eur Respir J.* 25: 915-27, 2005.
25. Frankel SK and Schwarz MI. Update in idiopathic pulmonary fibrosis. *Curr Opin in Pulm Med.* 15: 463-469, 2009.

26. Mouratis MA and Aidinis V. Modeling pulmonary fibrosis with bleomycin. *Curr Opin Pulm Med.* 17(5): 355-61, 2011.
27. Silva LP, Catunda Lemos AP, Curi R and Azevedo RB. Effects of fish oil treatment on bleomycin-induced pulmonary fibrosis in mice. *Cell Biochem Funct.* 24: 387–396, 2006.
28. Moeller A, Ask K and Warburton D. The bleomycin animal model: a useful tool to investigate treatment options for idiopathic pulmonary fibrosis? *Int J Biochem Cell Biol.* 40: 362-82, 2008.
29. Kennedy JI Jr, Chandler DB, Fulmer JD, Wert MB and Grizzle WE. Dietary fish oil inhibits bleomycin-induced pulmonary fibrosis in the rat. *Experimental Lung Research.* 15: 315-329, 1989.
30. Campos FG, Waitzberg DL, Teixeira MG, Mucerino DR, Kiss DR and Habr-Gama A. Pharmacological nutrition in inflammatory bowel diseases. *Nutr Hop.* 18: 57-64, 2003.
31. Serhan CN. Novel eicosanoid and docosanoid mediator: resolvins, docosatrienes, and neuroprotectins. *Curr Opin Clin Nutr Metab Care.* 8: 115-121, 2005.
32. Chlopicki S, Lomnicka M and Gryglewski RJ. Obligatory role of lipid mediators in platelet-neutrophil adhesion. *Thromb Res.* 15 287-292, 2003.
33. Escobar SO, Achenbach R, Iannantuono R and Torem V. Topical fish oil in psoriasis—a controlled and blind study. *Clin Exp Dermatol.* 17: 159–162, 1992.
34. Dry J and Vincent D. Effect of a fish oil diet on asthma: results of a 1-year double-blind study. *Int Arch Allergy Appl Immunol.* 95: 156–157, 1991.
35. Sane S, Baba M, Kusano C, Shirao K, Andoh T, Kamada T and Aikou T. Eicosapentaenoic acid reduces pulmonary edema in endotoxemic rats. *J Surg Res.* 93:21–27, 2000.
36. Baybutt RC, Rosales C, Brady H and Molteni A. Dietary fish oil protects against lung and liver inflammation and fibrosis in monocrotaline treated rats. *Toxicology.* 175:1-13, 2002.
37. Gil A. Polyunsaturated fatty acids and inflammatory diseases. *Biomed Pharmacother.* 56: 388-396, 2002.
38. Calder PC. Dietary modification of inflammation with lipids. *Proc Nutr Soc.* 61: 345-58, 2002.

39. Switzer KC, McMurray DN, Morris JS and Chapkin RS. (n-3) Polyunsaturated fatty acids promote activation-induced cell death in murine T lymphocytes. *J Nutr.* 133: 496-503, 2003.
40. Cutroneo KR, White SL, Phan SH and Ehrlich HP. Therapies for bleomycin induced lung fibrosis through regulation of TGF- β 1 induced collagen gene expression. *J Cell Physiol.* 211:585-589, 2007.
41. Crouch EC. Pathobiology of pulmonary fibrosis. *Am J Physiol Lung.* 259: L159–184, 1990.
42. Costabel U and King TE. International consensus statement on idiopathic pulmonary fibrosis. *Eur Respir J.* 17: 163–167, 2001.
43. Yaqoob P and Calder PC. Effects of dietary lipid manipulation upon inflammatory mediator production by murine macrophages. *Cell Immunol.* 163: 120–128, 1995.
44. Nixon LS, Yung B, Bell SC, Elborn JS and Shale DJ. Circulating immunoreactive interleukin-6 in cystic fibrosis. *Am J Respir Crit Care Med.* 157: 1764–1769, 1998.
45. Kolb M, Margetts PJ, Anthony DC, Pitossi F and Gauldie J. Transient expression of IL-1 beta induces acute lung injury and chronic repair leading to pulmonary fibrosis. *J Clin Invest.* 107: 1529–1536, 2001.
46. Coutts A, Chen G and Stephens N. Release of biologically active TGF-beta from airway smooth muscle cells induces autocrine synthesis of collagen. *Am J Physiol-Lung.* 280: L999–L1008, 2001.
47. Miyasaka CK, Azevedo RB and Curi R. Administration of fish oil by gavage increases the activities of hexokinase, glucose-6-phosphate dehydrogenase, and citrate synthase in rat lymphoid organs. *Gen Pharmacol.* 27: 991–994, 1996.
48. Shahar E, Lori L, Aaron R, Tockman MS, McGovern PG and Eckfeldt JH. Docosahexaenoic acid and smoking-related chronic obstructive pulmonary disease. *Am J Respir Crit Care Med.* 159: 1780–1785, 1999.
49. Lee JC, Krochak R, Blouin A, Kanterakis S, Chatterjee S, Arguiri E, Vachani A, Solomides CC, Cengel KA and Cristofidou-Solomidou M. Dietary Flaxseed prevents radiation-induced oxidative lung damage, inflammation and fibrosis in a mouse model of thoracic radiation injury. *Cancer Biol Ther.* 8: 47-53, 2009.

50. Karmioli S, Remick DG, Kunkel SL and Phan SH. Regulation of rat pulmonary endothelial cell interleukin-6 production by bleomycin: effects of cellular fatty acid composition. *Am J Respir Cell Mol Biol.* 9: 628-636, 1993.
51. Miyake Y, Sasaki S, Yokoyama T, Chida K, Azuma A, Suda T, Kudoh S, Sakamoto N, Okamoto K, Kobashi G, Washio M, Inaba Y and Tanaka H. Japan idiopathic pulmonary fibrosis study group. Dietary fat and meat intake and idiopathic pulmonary fibrosis: a case-control study in Japan. *Int J Tuberc Lung Dis.* 10: 333-339, 2006.
52. Iwai K, Mori T, Yamada N, Yamaguchi M and Hosoda Y. Idiopathic pulmonary fibrosis. Epidemiologic approaches to occupational exposure. *Am J Respir Crit Care Med.* 150: 670-675, 1994.

Chapter 3 - Enriched Short Chain Omega-3 Fatty Acids Protects Against Pulmonary Fibrosis in a Dose-dependent Manner

Abstract

Dietary fish oil, containing long chain omega-3 fatty acids, has been previously found to protect against monocrotaline-induced pulmonary fibrosis. This study investigated the effects of flaxseed oil rich in short chain omega-3 fatty acids on bleomycin-induced pulmonary fibrosis. Forty-two Sprague-Dawley rats were assigned into seven groups with various doses of flaxseed oil at 0, 2.5, 5, 7.5, 10, 12.5, and 15 % (w/w) in the diet for one month prior to treating with bleomycin at 8 U/ kg oropharyngeally. Two weeks after bleomycin treatment, all of the rats were sacrificed and histological analyses of the lung and heart were performed. Three proteins including TGF- β , IL-1, and α -SMA, commonly associated with fibrotic inflammation in the lung, were examined by Western blot analysis. In addition, fatty acids composition of the diets and tissues were analyzed by GC. The fifteen percent flaxseed oil group significantly reduced septal and vascular thickness and fibrosis in the lung, and significant cardiac fibrosis in the heart. The amount of IL-1 and α -SMA decreased significantly as the amount of omega-3 fatty acids increased, whereas TGF- β did not change significantly. These results indicate that the omega-3 fatty acid rich flaxseed oil inhibits development of pulmonary fibrosis in a dose-dependent manner which may be in part related to via anti-inflammatory mechanisms that appear to modulate fatty acid composition in the tissues.

Introduction

Dietary fish oil which has long chain omega-3 fatty acids has been previously found to have protective effects against monocrotaline-induced pulmonary fibrosis.¹ However, the effectiveness of short chain omega-3 fatty acid against fibrosis is not known. Therefore, this study investigated the effects of flaxseed oil, which has short chain omega-3 fatty acids, on bleomycin-induced pulmonary fibrosis. Bleomycin is a chemotherapeutic agent used in the

treatment of a variety of tumors.² However, it has an adverse effect of inducing interstitial pulmonary fibrosis. The animal model using bleomycin remains the best available experimental methods for fibrosis. Bleomycin-induced pulmonary fibrosis is associated with the release of many kinds of eicosanoids also associated with inflammation and collagen deposition, and may be modulated by omega-3 fatty acids since the metabolic activities of eicosanoids derived from these fatty acids are typically less biologically active.

Flax (*Linum usitatissimum*) is a blue flowering plant that produces small, flat seeds that range in color from golden yellow to reddish brown. Flaxseed is commonly found as whole seed, ground seed (powder or meal), or flaxseed oil.³ The oil in flaxseed is unique in that it is comprised of 73 % PUFA, 18 % MUFA, and 9 % saturated fatty acids, characterizing it as a low-saturated fat food.⁴ Flaxseed oil is known as the richest source of the omega-3 fatty acid, ALA, which consists of approximately 55 % of the total fatty acids.⁵ The percent of fat as ALA in flaxseed is 5.5 times higher than that in walnuts and canola oil, which are the second-richest sources.¹

In the fibrotic lungs, pulmonary hypertension is often observed. In 1963, cor pulmonale was identified by the World Health Organization as “hypertrophy of the right ventricle (RV) resulting from diseases affecting the function and/or structure of the lungs, except when these pulmonary alterations are the result of diseases that primarily affect the left side of the heart, as in congenital heart disease.”⁶ The RV is a thin-walled and low-pressure chamber that pumps the same stroke volume as the left ventricle (LV) with around 25 % of the stroke work since the pulmonary vasculature has normally low resistance.⁷ The right coronary artery provides the blood supply to the RV free wall in both systole and diastole. When the pressure is overloaded constantly, the RV hypertrophies and enlarges, which results in both systolic and diastolic malfunction.⁸ Pulmonary fibrosis has an effect on right ventricular function by inflicting an afterload on the right ventricle and, therefore, eventually cardiac output is reduced as pulmonary fibrosis worsens.

In this research three proteins commonly associated with fibrotic inflammation were examined: Interleukin-1 (IL-1), α -Smooth Muscle Actin (α -SMA), and Transforming Growth Factor- β (TGF- β). Decreases in any one of these proteins means an interruption of the inflammatory process. The fatty acid profiles were examined to determine whether flaxseed oil can modulate the fatty acid composition of the rats in pulmonary fibrosis. In addition,

RV+septa/LV ratio of the heart was measured to determine whether pulmonary hypertension was produced by fibrotic lungs and if there was any preventive effects by flaxseed oil. A histological analysis of the heart was also performed to determine if flaxseed oil can modulate any of the above symptoms or pathologies.

Methods and Materials

Animals and Diets

Forty-two male Sprague-Dawley weanling rats (Charles River Laboratories, Wilmington, MA) weighing about 40 - 50 g were housed individually in stainless steel cages at room temperature under a 12 h light : dark cycle (light 06:00 – 18:00 h) and a relative humidity of 50 %. Animal care and use were approved by the Institutional Animal Care and Use Committee of Kansas State University. The animals were randomly assigned into seven different groups containing a control group with 0 % flaxseed oil. They were fed either an AIN 93G basal diet (Reeves et al. 1993, Table 3-1⁹) or a treatment diet that differs only in the type of dietary fat: 0 % flaxseed oil (15 % corn oil), 2.5 % flaxseed oil (12.5 % corn oil), 5 % flaxseed oil (10 % corn oil), 7.5 % flaxseed oil (7.5 % corn oil), 10 % flaxseed oil (5 % corn oil), 12.5 % flaxseed oil (2.5 % corn oil), and 15 % flaxseed oil (0 % corn oil) as the source of dietary fat. The rats were fed ad libitum and the purified diets were purchased from Dyets Inc. (Bethlehem, PA). Food intake was recorded daily and body weights were measured weekly. The experimental design is shown in Figure 3-1.

After four weeks on the diet, half of each diet group were anesthetized and administered 8 U/kg body weight of bleomycin in 0.4 mL sterile saline by oropharyngeal delivery to minimize the stress of the animals.¹⁰ The other half of these groups were administered saline for the negative control groups.

Two weeks after bleomycin treatment the rats were anesthetized with pentobarbitol. Liver, lungs, hearts, and kidneys were collected, weighed and used for chemical/histological studies. Surgical collection of organs was done in the lab separated from the animal facility to minimize stress to the animals. The histopathological analyses were carried by Dr. Molteni and resident pathologists in University of Missouri-Kansas City School of Medicine.

Histopathological analysis

Histological analysis of lung tissue was performed in a semi quantitative manner.^{11, 12, 13} A degree scale of damage in the lung was carried out by blinded scoring of the degrees of severity by skilled pathologists. They scored by subjective comparisons with the normal tissue. The score ranged from 0 to 40, with the score 40 meaning extensive amounts of inflammation, fibrosis, or vasculitis (Appendix A). The right lung from each rat were inflated with 10 % buffered formalin and then placed in the same solution and fixed for one week. Sagittal sections embedded in paraffin, and sections 4 micro meters thick were stained by hematoxylin eosin for light microscopy. Masson's trichrome staining for collagen, the marker for pulmonary fibrosis, was performed on representative samples from rats from each group. A lobe of liver from each animal also was collected at necropsy, immediately placed in 10 % buffered formalin, and fixed for 1 week. Hematoxylin eosin and Masson's trichrome staining were performed on 4 micro meter-thick sections of this tissue. Hydroxyproline levels were determined (via a kit) as a measure of collagen synthesis. In addition, RV/LV ratio of the heart was evaluated. Blinded to the experimental group, hearts were dissected and weighed on an analytical balance, and RV+septa/LV ratio calculated.

Fatty Acids Analysis

For fatty acids analysis, lipids were extracted from the lung and the liver and analyzed by Gas Chromatography using Folch method¹⁴ arranged by Kansas Lipidomics Research Center. To 0.8 parts tissue (homogenized) in aqueous solution, 1 part chloroform and 2 parts methanol were added. After being shaken well, 1 part chloroform and 1 part water were added. After shaking again, it was centrifuged at low speed for 5 - 10 min. After removing the lower layer, 1 part chloroform was added. Then it was shaken, centrifuged, and the lower layer was again removed. After repeating this one more time, the combined lower layers were washed once with a small volume 1.0 M KCl and once with a small volume of water. After drying down, it was dissolved in 1 mL of chloroform. The extracted lipid was then derivatized. The fatty acid C15:0 internal standard was added to the lipid extract and fatty acids were methylated after hydrolysis by methanoic-HCl. 1 mL of 1.5 M methanoic HCl was added, bubbled with nitrogen gas for 10 s, then it was heated at 78 °C on heat block for 30 min. After cooling down, 2 mL of water and 2

mL of pentane were added and shaken for 15 s. Then it was centrifuges at low speed (700-800 rpm) for 20 min. Upper pentane layers were collected to tubes containing sodium sulfate, then 2 mL of pentane was added, shaken, and centrifuged again. The upper pentane layer was collected and combined with the previous layer extraction, and then all pentane layers were transfer to clean tubes. Pentane layers were dried down after transferred, and redissolved in small volume of hexane, then the sample would be ready to be injected in the Gas Chromatograph.

Fatty acid composition of the lung tissue total lipids was determined by measuring the fatty acid methyl esters using a capillary gas chromatography with a flame ionization detector. The Gas chromatography-FID (Flame Ionization Detector) analysis was performed at the Kansas Lipidomics Research Center with a 6890N GC (Agilent Technologies) coupled to a FID. The GC was fitted with a HP-88 capillary column with a bis (Cyanopropyl) Polysiloxanes stationary phase (column length - 100 cm, internal diameter - 250 μ m, film thickness - 0.25 μ m). Helium was used as the carrier gas at a flow rate of 1.2 mL/min. The back inlet was operating at a pressure and temperature of 32.36 psi and 275 $^{\circ}$ C respectively. Agilent 7683 autosampler was used to inject 1 μ L of the sample in the split mode with a split ratio of 10:1. The GC temperature ramp was operated as follows, initial temperature of 70 $^{\circ}$ C, ramp 1 at 15 $^{\circ}$ C/min to 175 $^{\circ}$ C, ramp 2 at 1 $^{\circ}$ C/min to a final temperature of 235 $^{\circ}$ C. The FID detector was operated at 260 $^{\circ}$ C. The hydrogen flow to the detector was 30 mL/min and air flow was 400 mL/min. The sampling rate of the FID was 20 Hz. The data were processed using Chemstation software.

Western Blot Analysis

Protein analysis of IL-1, α -SMA, and TGF- β was conducted for stored lung tissues (-80 $^{\circ}$ C) via Western Blot analysis after protein extraction. Rat lung tissue was smashed into powder in liquid nitrogen, mixed with cell lysis buffer (5 mM Tris-HCl, pH 7.4, 2 mM EDTA, 10 % Triton X-100, protease inhibitor cocktail and phosphatase inhibitor cocktail) and then homogenized. The lysate was then centrifuged in 20,000 rpm for 15 min at 4 $^{\circ}$ C. The supernatant was collected and sonicated for 10 s. After sonication, it was centrifuged with the speed of 10,000 rpm for 15 min at 4 $^{\circ}$ C. The supernatant was collected and the protein concentration was measured by the Bio-Rad protein assay (Bio-Rad, Hercules, CA) with Bovine Serum Albumin (BSA) standards. Laemmli sample buffer (Bio-Rad, Hercules, CA) was used to mix with and

denature the proteins. The proteins were separated by 10 % SDS-PAGE gel and transferred to nitrocellulose membrane using Trans-Blot SD SEMI-DRY Transfer Cell (Bio-Rad, Hercules, CA) with 15 V for 20 min. The membrane was blocked with 5 % blocking buffer containing 5 % non-fat milk at room temperature for 1 hr, and incubated with first antibody (IL-1, α -SMA, TGF- β , and β -actin (as the loading control)) (Santa Cruz Biotechnology Inc, Santa Cruz, CA) overnight. After the first antibody incubation, the membrane was washed with TDN-T (Tris-EDTA-NaCl with Tween 20) twice for 5 min each, then incubated with anti-mouse HRP-conjugation secondary antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA) for 1 hr. Finally the bands were visualized and protein density was determined using the Fluorchem densitometry program.

Statistical Analysis

All data were expressed as means \pm SEM. Some of the histological data will be semi-quantitative, that is a subjective evaluation by a trained pathologist. The quantitative data was analyzed by one-way and two-way ANOVA using SAS software (SAS Institute, Cary, NC). Values of p less than 0.05 represented a significant difference. Post hoc t-tests (or Tukey) were performed with the differences between means when the overall main or interaction effect was significant at $p < 0.05$.

Results

Food Intake and Body Weight Gain

The average amount of daily food intake was described in Figure 3-2 and 3. Overall, the food intake increased as the rats grew. There was not a significant difference between the saline groups and the bleomycin groups before the treatment with bleomycin. However, the daily food intake decreased after the treatment, and returned to pretreatment intake after one week.

The body weight gain from the rats is shown in Figure 3-4 and 5. There was a similar tendency as shown in food intake. There was not a significance difference between saline groups and bleomycin groups, and the bodyweights increased as the rats grew. For bleomycin groups,

they showed a decreased body weights after the treatment with bleomycin, however they recovered after one week.

Organ Weights and Gross Evaluation

There was no significant difference between control and treatment groups for the liver and the kidney. However, in the lung and the heart, the weights from the bleomycin treatment groups were significantly heavier than control groups ($p < 0.01$). Also, there were significant differences among flaxseed oil groups treated with bleomycin ($p < 0.05$) in the liver. The results are shown in Table 3-2. At gross evaluation, there were hemorrhaging and cobblestone appearances observed in the lungs of bleomycin treated rats. Among the flaxseed oil group, there was a less of tendency to observe discoloration or hemorrhaging, and cobblestone appearances compared to those without flaxseed oil from the groups with higher doses of flaxseed oil.

Histopathological Analysis of the Lung and the Heart

Trichrome and H&E staining figures are shown in Figure 3-6 and 7. Saline, with low or high concentration of flaxseed oil, showed minimal inflammation. Staining for collagen with Trichrome also showed minimal presence of fibrosis. Severe inflammation occurred without flaxseed oil with septal and vascular thickness and marked collagen deposition. Fifteen percent flaxseed oil diet significantly reduced septal and vascular thickness and fibrosis when the damage was semi-quantified by blinded pathologists. Also, extensive lung damage was produced by bleomycin as seen in Figure 3-8. Comparing to saline control groups, all of the bleomycin treated groups showed lung damages. All were significant, therefore it could be predicted from these data that flaxseed oil was effective in preventing fibrosis, inflammation and vasculitis in the lung.

For the histopathological analysis from the heart (Figure 3-9), significant cardiac fibrosis was seen, especially around vessels, in animals receiving bleomycin and 0 % flaxseed oil while it was not seen in the saline control group. Pretreatment of 15 % flaxseed oil protected the heart from the bleomycin effect.

Figure 3-10 showed RV/LV ratios of the heart. Although there was a tendency of decreasing with the ratio as the dose of flaxseed oil in the diet increased, there was no significant

difference among groups. 15 % flaxseed oil group seemed exceeding saline control group, however, it was with a much larger standard deviation.

Fatty Acids Analysis from the Diet

Fatty acids composition of the diet analyzed by GC is shown at Figure 3-11. Comparing with the control group (0 % flaxseed oil diet), it showed a significant increase of α -linolenic acids and a significant decrease of linoleic acids as the dose of flaxseed oil increased, proving that the flaxseed oil was rich in α -linolenic acids. ($p < 0.01$)

Fatty Acids Analysis from the Tissues

The results from GC analysis with the tissues of the rats are shown in Figure 3-12. There was an increase of omega-3 fatty acid and a decrease of omega-6 fatty acid significantly among the rats as the dose of flaxseed oil increased in the diets ($p < 0.001$), demonstrating that the α -linolenic acid within the dietary flaxseed oil is displacing the linoleic acid within the tissue. Especially, a significant increase of omega-3 fatty acid was observed from the group of 7.5 % when compared to the group of 5 % flaxseed oil diet ($p < 0.05$). Overall, the amount of omega-6 fatty acid was elevated with the bleomycin treatment.

Western Blot Analysis from the Lung

Interleukin-1 (IL-1)

Figure 3-13 shows the result from the Western Blot analysis for IL-1 from the lung. The amount of IL-1 expression was significantly higher in the bleomycin groups than that of the saline groups ($p < 0.0001$). It gradually decreased as the dose of flaxseed oil increased in both of saline and bleomycin groups, showing that it did in a dose-dependent manner overall. However, IL-1 in 12.5 % saline group increased a little bit as compared with others.

Alpha-Smooth Muscle Actin (α -SMA)

Alpha-SMA Western Blot analysis result from the lung is shown in Figure 3-14. The amount of α -SMA expression was higher in the bleomycin groups than that of the saline groups.

It significantly decreased with any dose of flaxseed oil, showing a dose-dependent manner. Especially with α -SMA, flaxseed oil showed the preventive effect with any doses from 2.5 %.

Transforming Growth Factor β (TGF- β)

The result from Western Blot analysis of TGF- β is shown in Figure 3-15. Like other two proteins, the amount of TGF- β expression was also higher in the bleomycin groups than that of the saline groups. It significantly decreased at first comparing with the control group, however it showed an inconclusive result as the dose of flaxseed oil gets higher.

Discussion

The main purpose of this study was to investigate the effectiveness of flaxseed oil, rich in short chain omega-3 fatty acids, on preventing bleomycin-induced pulmonary fibrosis in rat model, especially if there was a dietary dose-dependent effect of ALA in preventing bleomycin-induced pulmonary fibrosis. Other studies regarding omega-3 fatty acids and preventing pulmonary fibrosis only demonstrated with a single dose of omega-3 fatty acids, either control diet or experimental diet^{1, 15, 16}. This study was the first one to show if there was dose-dependent effect of ALA and to find appropriate dose of flaxseed oil to prevent pulmonary fibrosis. The overall results showed that preventive effect of flaxseed oil was dose-dependent at low doses, however it was most effective at the moderate dose. Especially fatty acid data suggested that 7.5 % seemed to be an optimal dose to enrich the tissues with omega-3 fatty acids. Pretreatment of flaxseed oil with diet for 4 weeks successfully prevented from pulmonary fibrosis and cardiac fibrosis as found in histological analyses of the lung and the heart. The severe inflammation and fibrosis observed in 0 % flaxseed oil diet induced by bleomycin treatment was significantly reduced with flaxseed oil diets.

Another purpose of this study was to determine if flaxseed oil prevented pulmonary hypertension, which was often associated with pulmonary fibrosis. Although flaxseed oil diets successfully protected the heart from cardiac fibrosis, according to the results from RV/LV weights on the rats, they did not show significant improvement on the pulmonary hypertension induced by bleomycin-induced fibrosis. It was interesting since cardiac fibrosis seemed to be prevented in the heart by flaxseed oil, the pulmonary hypertension which was secondary to a fibrotic lung was not prevented by flaxseed oil in this study.

One of possible mechanisms underlying these preventive effects of flaxseed oil is through anti-inflammatory effects by omega-3 fatty acids. Omega-3 fatty acids are known to have anti-inflammatory properties by direct action on the cellular production of major cytokine inflammation mediators, on endothelium dysfunction and on leukocyte chemotaxis. Due to these effects, omega-3 fatty acids have been used in the treatment of various chronic inflammatory conditions. It has been found that bleomycin-induced pulmonary fibrosis produces a variety of eicosanoids associated with inflammation and collagen deposition. Since eicosanoids are formed by enzymatic phospholipase cleavage of phospholipids found in cell membranes, dietary induced changes in the fatty acid composition of tissue could conceivably result in alterations of eicosanoid composition and thus, the level of activity in an inflammatory reaction. Therefore, eicosanoids associated with inflammation may be attenuated by a change in dietary fatty acid. One of the most important mechanisms to explain the anti-inflammatory properties of omega-3 PUFA in many inflammatory diseases is the different activities of eicosanoids derived from AA versus those derived from EPA, since eicosanoids from EPA or DHA are less biologically active than those from AA². From the results of fatty acids profile in diets and tissues analyzed by GC, it was demonstrated that the α -linolenic acid which was omega-3 fatty acid in flaxseed oil diet displaced linoleic acid in the tissues, which was omega-6 fatty acid. Especially, 7.5 % flaxseed oil diet group showed significant elevation of amount of omega-3 fatty acids in the tissues. Omega-3 fatty acids in flaxseed oil were effectively incorporated into the lung tissue, therefore they could exert the biologically protective effects. However, neither of EPA nor DHA was evaluated in this study, therefore further study should be performed with analysis with EPA and DHA considering of the low conversion rates from α -linolenic acid to EPA.

In addition, since inflammatory cytokine production is regulated by AA-derived eicosanoids and since dietary omega-3 PUFA affects production of these eicosanoids, it was expected that omega-3 PUFA will affect cytokine production. In this study, biomarkers associated with fibrotic inflammation were used, IL-1, α -SMA, and TGF- β , through Western Blot analysis. The major advantages of the bleomycin-induced fibrosis models are the facts that it is easy to deliver the drug and takes a short time to demonstrate fibrosis.¹⁷ After bleomycin delivery, intense inflammation and edema with an elevation in cytokines such as tumor necrosis factor (TNF) α and IL-1 β occur during the first week.^{18, 19} By approximately 14 days after the treatment, TGF- β peaks as well.^{20, 21} By the second and third week after the treatment of

bleomycin, patchy isolated collagen deposits resembling fibrosis builds up, with remarkable deposition of extracellular matrix components including fibronectin and collagen-1.¹⁷ Through the Western blot analysis, it was found that the inflammation in tissues decreased with flaxseed oil in dose-dependent manner, and moderate doses of flaxseed oil were more effective than the 15 % of flaxseed oil. However TGF- β expression was inconclusive though it is the mostly well-known profibrotic factor. It might be due to the short working period of TGF- β . Since the animals were sacrificed after two weeks of bleomycin treatment and TGF- β peaks at that time, the right time for TGF- β may have been missed.

In addition, we used oropharyngeal delivery of bleomycin to rats instead of intratracheal delivery to minimize stress of the animals and improve the delivery of bleomycin. The rats were fed flaxseed oil incorporated into their food for one month prior to treating with bleomycin to determine whether these fatty acids will block or prevent the development of the pulmonary fibrosis. There was a concern about the delivery efficiency since it was the first time to apply this method in our lab, however the result of pulmonary fibrosis induced in organs showed that ample bleomycin was delivered.

The purpose of this study was to determine if omega-3 fatty acids in flaxseed oil prevented bleomycin-induced pulmonary fibrosis, and how it differs along different doses of flaxseed oil. As shown in the results above, the omega-3 fatty acids in flaxseed oil diet successfully prevented pulmonary fibrosis and cardiac fibrosis in rats via modulation of fatty acids composition of the tissues and its associated anti-inflammatory effects.

Table 3-1 AIN-93G control diet⁹

Ingredient	g/kg diet
Cornstarch	397.486
Casein (≥85 % protein)	200.000
Dextrinized cornstarch (90-94 % tetrasaccharides) ¹	132.000
Sucrose	100.000
Soybean oil (no additives)	70.000
Fiber ²	50.000
Mineral mix (AIN-93G-MX)	35.000
Vitamin mix (AIN-93-VX)	10.000
L-Cystine	3.000
Choline bitartrate (41.1 % choline) ³	2.500
Tert-butylhydroquinone	0.014

¹Deytrose (Dyets, Bethlehem, PA) and Lo-Dex 10 (American Maize, Hammond, IN) meet these specifications. An equivalent product may also be used.

²Solka-Floc, 200 FCC (FS&D, St. Louis, MO) or its equivalent is recommended.

³Based on the molecular weight of the free base.

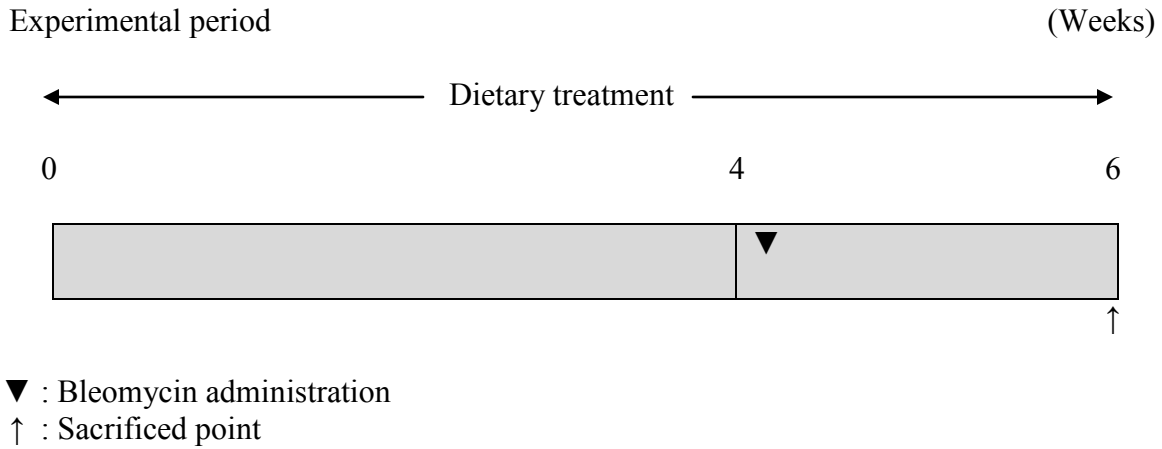


Figure 3-1 Experimental design

The overall study period was for 6 weeks. After 4 weeks on the diet, half of each diet group were administered 8 U/kg body weight of bleomycin by oropharyngeal delivery. The other half of these groups were administered saline for the negative control groups. Two weeks after bleomycin treatment, the rats were sacrificed.

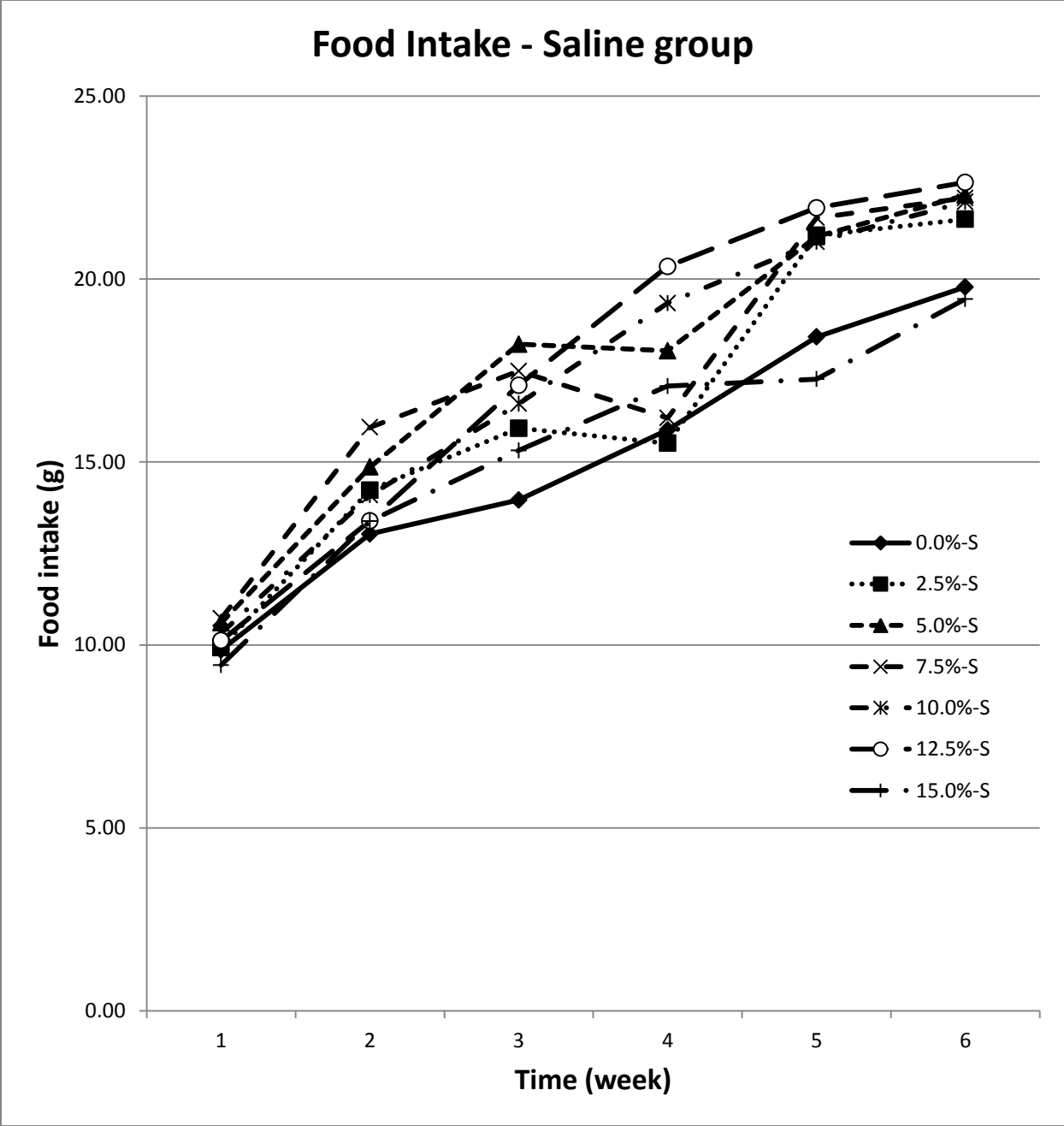


Figure 3-2 Food intake of the rats fed different doses of flaxseed oil in diet without treatment of bleomycin

The average amount of daily food intake increased as the rats grew. There was no significant difference among saline groups, implying the taste of flaxseed oil did not affect the amount of food intake.

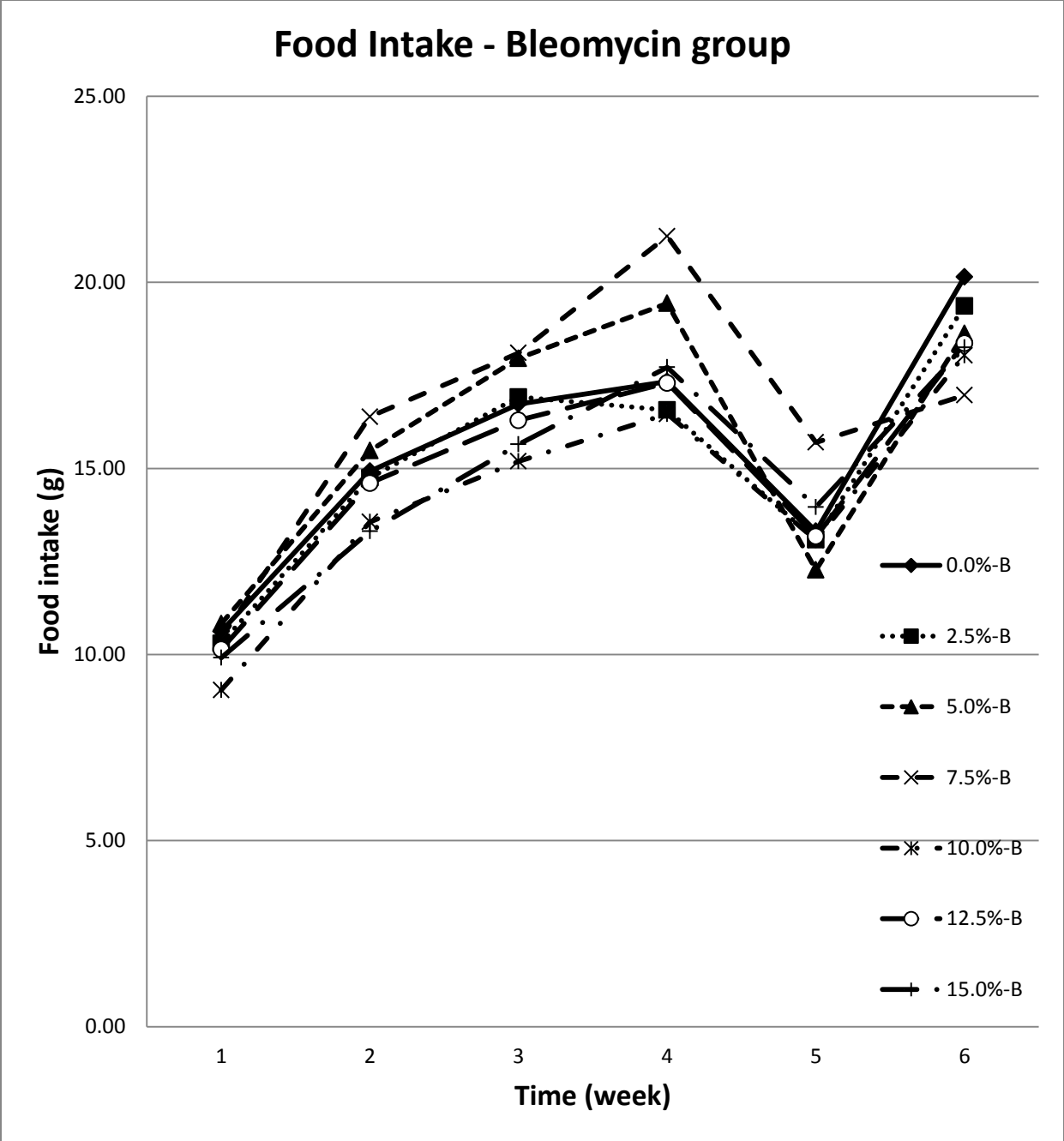


Figure 3-3 Food intake of the rats fed different doses of flaxseed oil in diet with treatment of bleomycin

There was same tendency as with saline groups, the food intake increased as the animal grew. After the bleomycin treatment, the food intake decreased among bleomycin groups significantly, it might be due to the toxicity of bleomycin. However they returned to pretreatment intake after one week

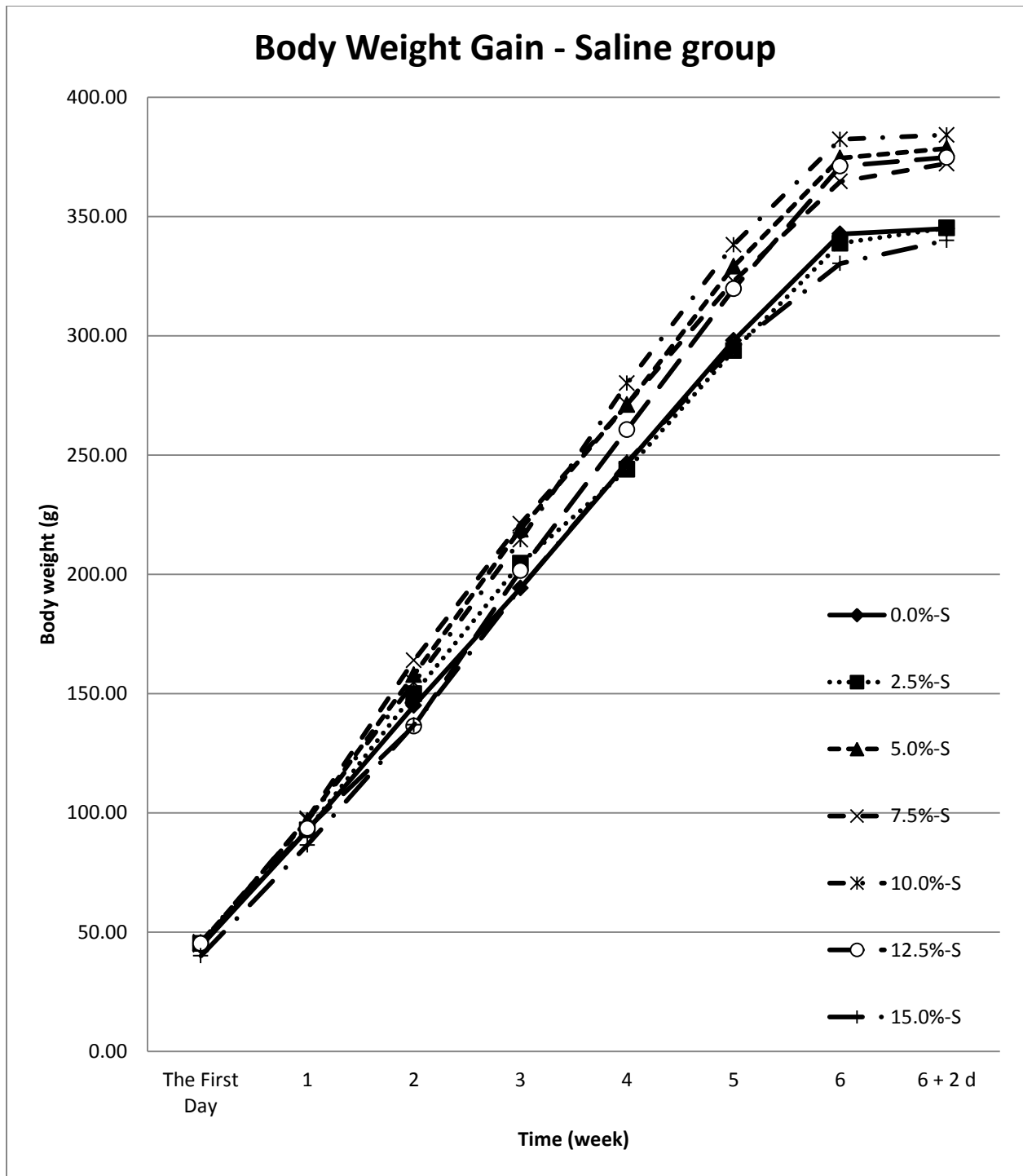


Figure 3-4 Body weight change in rats fed diets containing different doses of flaxseed oil, without bleomycin treatment

The body weights increased as the time passed and the rats grew. There was not a significant difference among the control and test groups.

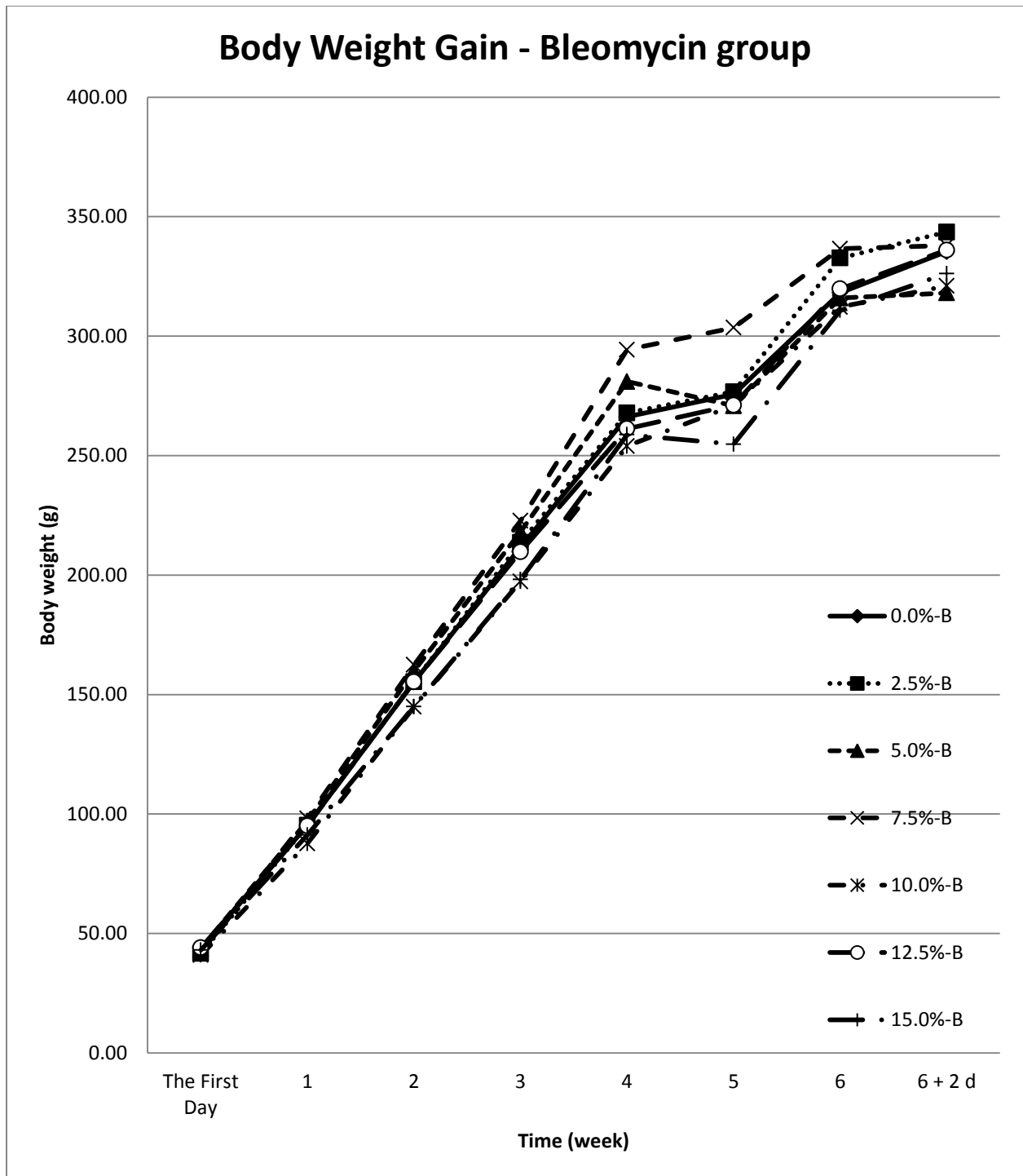


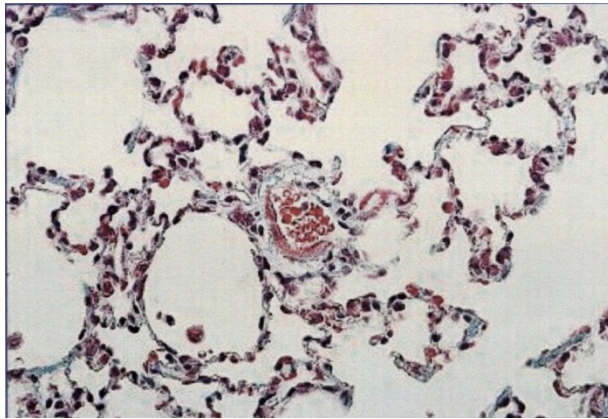
Figure 3-5 Body weight change in rats fed diets containing different doses of flaxseed oil, with bleomycin treatment

There was a similar tendency as shown in food intake. After the bleomycin treatment, body weights decreased as the food intake decreased and possibly from the toxicity of bleomycin. And they recovered after one week.

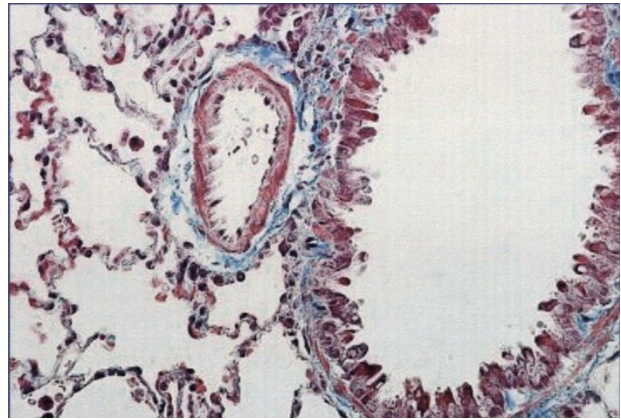
Table 3-2 Organ weights in rats fed diets containing different dose of flaxseed oil with or without bleomycin treatment

Treatment	Flaxseed oil	Lung	Liver	Heart	Whole Kidney
Saline	0.0 %	0.55 ± 0.07 ¹⁾	3.56 ± 0.31	0.37 ± 0.01	0.81 ± 0.01
	2.5 %	0.47 ± 0.04	3.36 ± 0.07	0.37 ± 0.01	0.77 ± 0.01
	5.0 %	0.40 ± 0.09	3.29 ± 0.07	0.38 ± 0.02	0.75 ± 0.09
	7.5 %	0.54 ± 0.16	3.23 ± 0.22	0.36 ± 0.01	0.75 ± 0.04
	10.0 %	0.71 ± 0.29	3.24 ± 0.10	0.35 ± 0.01	0.79 ± 0.03
	12.5 %	0.37 ± 0.09	3.43 ± 0.19	0.37 ± 0.01	0.78 ± 0.06
	15.0 %	0.51 ± 0.06	3.26 ± 0.22	0.33 ± 0.01	0.71 ± 0.08
Bleomycin	0.0 %	0.79 ± 0.39	3.76 ± 0.04 ^{a2)}	0.39 ± 0.02	0.81 ± 0.03
	2.5 %	0.60 ± 0.11	3.65 ± 0.05 ^{ab}	0.40 ± 0.02	0.83 ± 0.03
	5.0 %	1.18 ± 0.07	3.79 ± 0.16 ^a	0.39 ± 0.01	0.86 ± 0.03
	7.5 %	0.80 ± 0.13	3.20 ± 0.05 ^{cd}	0.39 ± 0.01	0.79 ± 0.03
	10.0 %	0.58 ± 0.04	2.92 ± 0.10 ^d	0.40 ± 0.02	0.82 ± 0.04
	12.5 %	0.70 ± 0.12	3.35 ± 0.06 ^{bc}	0.39 ± 0.01	0.74 ± 0.01
	15.0 %	1.18 ± 0.38	3.06 ± 0.06 ^{cd}	0.37 ± 0.03	0.73 ± 0.04
Significant factor(2-way) ³⁾		B	A	B	-

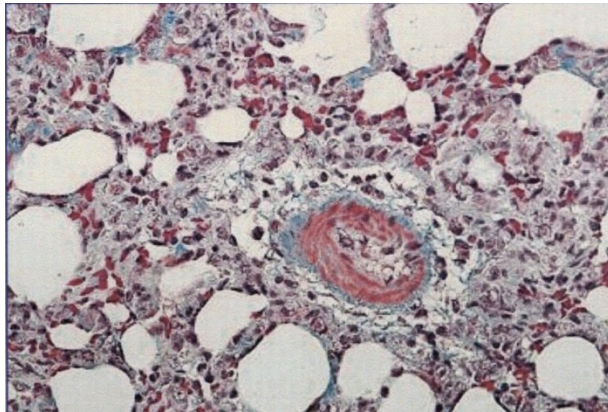
- 1) Data were expressed as mean ± SEM (n=3) in weight of organ/100g bodyweight.
- 2) Values with different letter within the column in the same treatment group are significantly different at p < 0.05 by Tukey test.
- 3) Statistical significance of experimental factors was calculated based on 2-way ANOVA.
 A : Effect of flaxseed oil diet with different doses was significant at p < 0.05.
 B : Effect of bleomycin treatment was significant at p < 0.05.



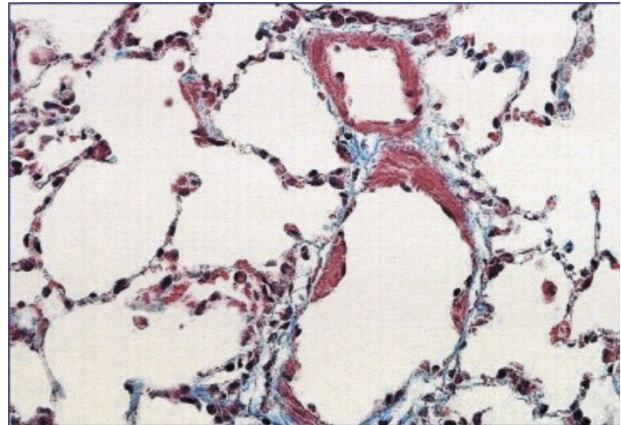
a) Saline + 0 % flaxseed oil



b) Saline + 15 % flaxseed oil



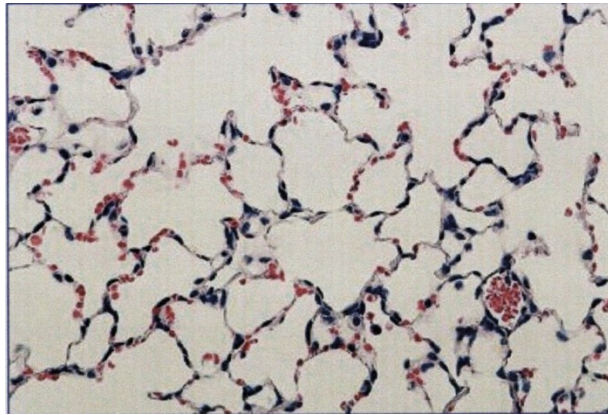
c) Bleomycin + 0 % flaxseed oil



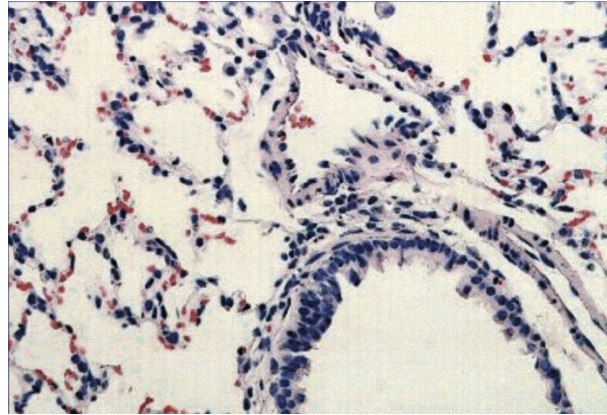
d) Bleomycin + 15 % flaxseed oil

Figure 3-6 Representative histological pictures with Mason's Trichrome staining of lung from the rats fed diets containing different doses of flaxseed oil with or without bleomycin treatment

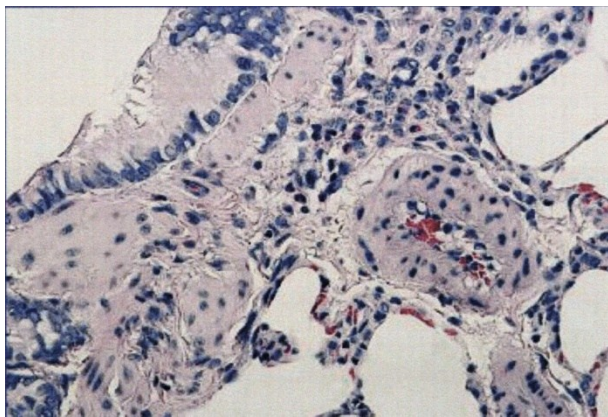
Saline groups (figure a, b) showed minimal fibrosis with low or high doses of flaxseed oil. There are some collagen found in figure b, however it is due to the different part of the tissues. In figure c, severe inflammation and fibrosis were observed without flaxseed oil in bleomycin treatment, however in figure d, significantly reduced septal and vascular thickness and fibrosis were observed with 15 % flaxseed oil in diet with bleomycin treatment.



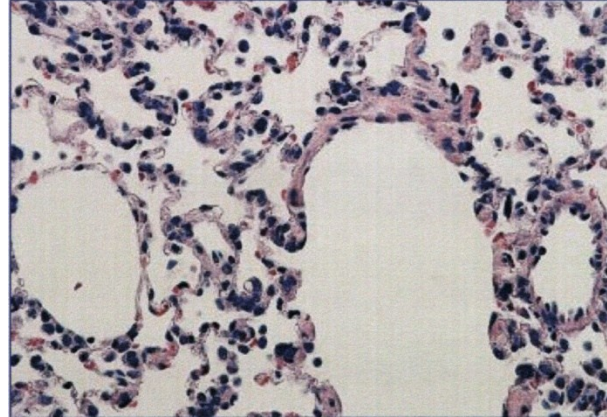
a) Saline + 0 % flaxseed oil



b) Saline + 15 % flaxseed oil



c) Bleomycin + 0 % flaxseed oil



d) Bleomycin + 15 % flaxseed oil

Figure 3-7 Histopathological pictures with H&E staining of lung from the rats fed diets containing different doses of flaxseed oil with or without bleomycin treatment

There was the same tendency as shown with Trichrome staining of lung in figure 3-6. There was minimal inflammation among saline groups either low or high dose of flaxseed oil. In figure c without flaxseed oil in bleomycin treatment, severe inflammation was occurred, and significantly reduced septal and vascular thickness was observed with 15 % flaxseed oil in diet with bleomycin treatment in figure d.

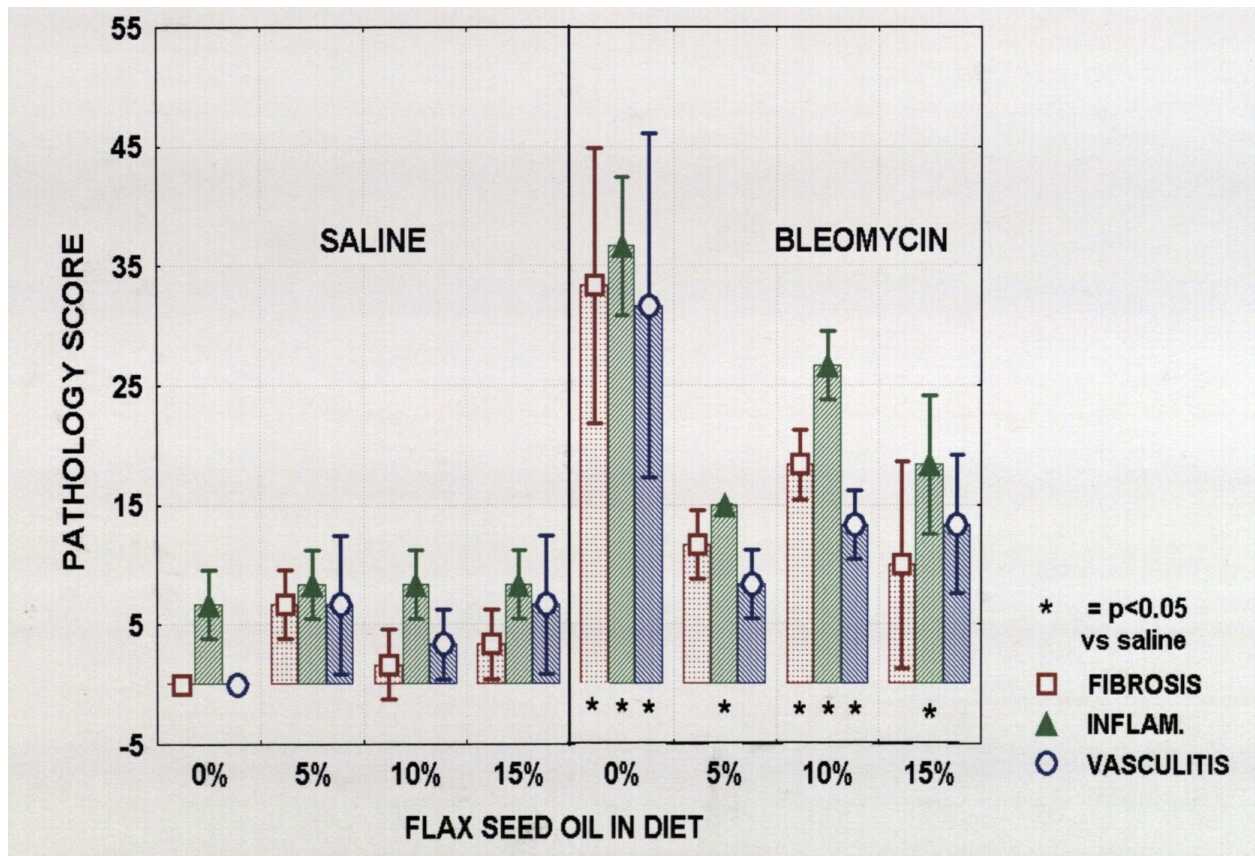
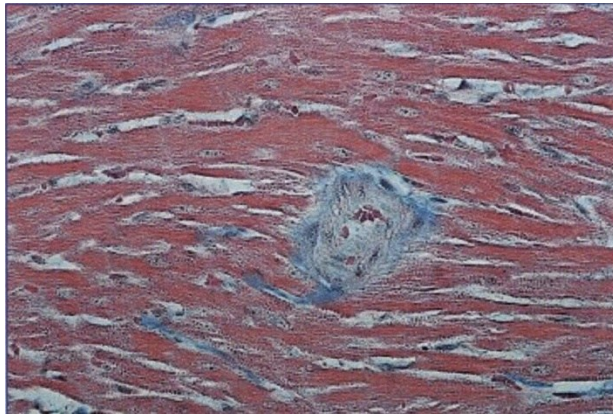
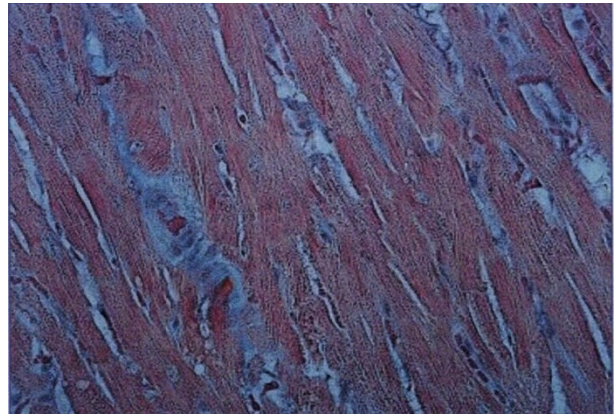


Figure 3-8 Extensive lung damage produced by bleomycin versus saline groups

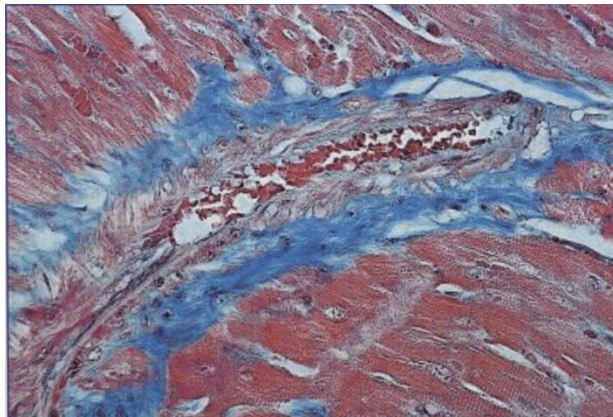
* : Significant difference between the groups of saline and bleomycin treatment at $p < 0.05$
 *** : Significant difference between the groups of saline and bleomycin treatment at $p < 0.001$
 Compared with saline groups, all of the bleomycin treatment groups showed significant lung damages and they were improved by flaxseed oil in diet. Five percent flaxseed oil group was most effective in preventing of fibrosis, inflammation and vasculitis in the lung.



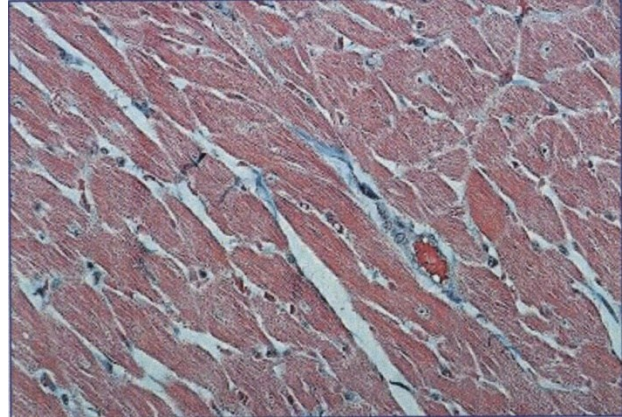
a) Saline + 0 % Flaxseed oil



b) Saline + 15 % Flaxseed oil



c) Bleomycin + 0 % Flaxseed oil



d) Bleomycin + 15 % Flaxseed oil

Figure 3-9 Histopathological pictures with Trichrome staining from the heart of the rats fed diets containing different doses of flaxseed oil with or without bleomycin treatment

Significant cardiac fibrosis was observed in figure c, without flaxseed oil in bleomycin treatment, especially around vessels. In figure a and b saline control groups, there was no fibrosis seen in tissues. In figure d with 15 % flaxseed oil with bleomycin treatment, the tissue seemed as a control group, showing that flaxseed oil protected the heart from the bleomycin effect.

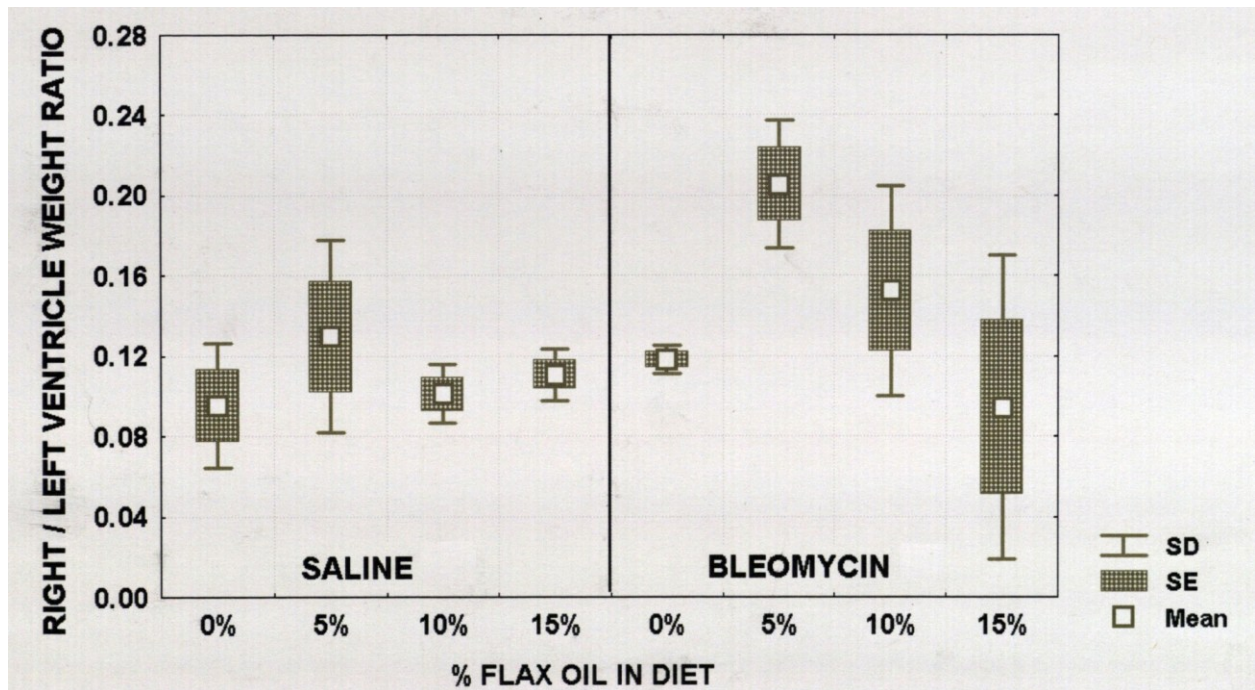


Figure 3-10 Right ventricle+saptal wall / Left ventricle weight ratio from the heart of the rats

Although 15 % flaxseed oil group seemed to have preventive effect, it was with large standard deviation and without significance. There was no significant difference among groups, showing no improvement of pulmonary hypertension with flaxseed oil.

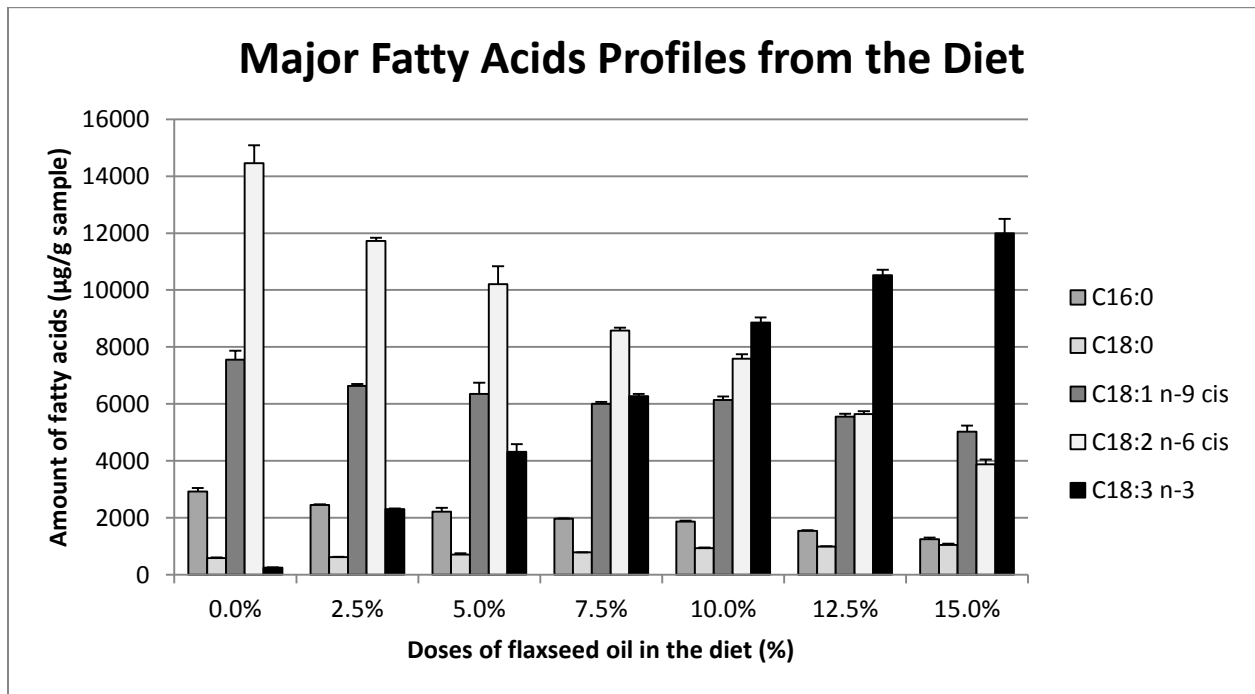


Figure 3-11 Major fatty acids profiles from the diets with different doses of flaxseed oil

Fatty acids profiles in the diet analyzed by GC. As the dose of flaxseed oil increased in diet, the amount of α -linolenic acid increased and the amount of linoleic acid decreased significantly, showing that flaxseed oil was rich in α -linolenic acid.

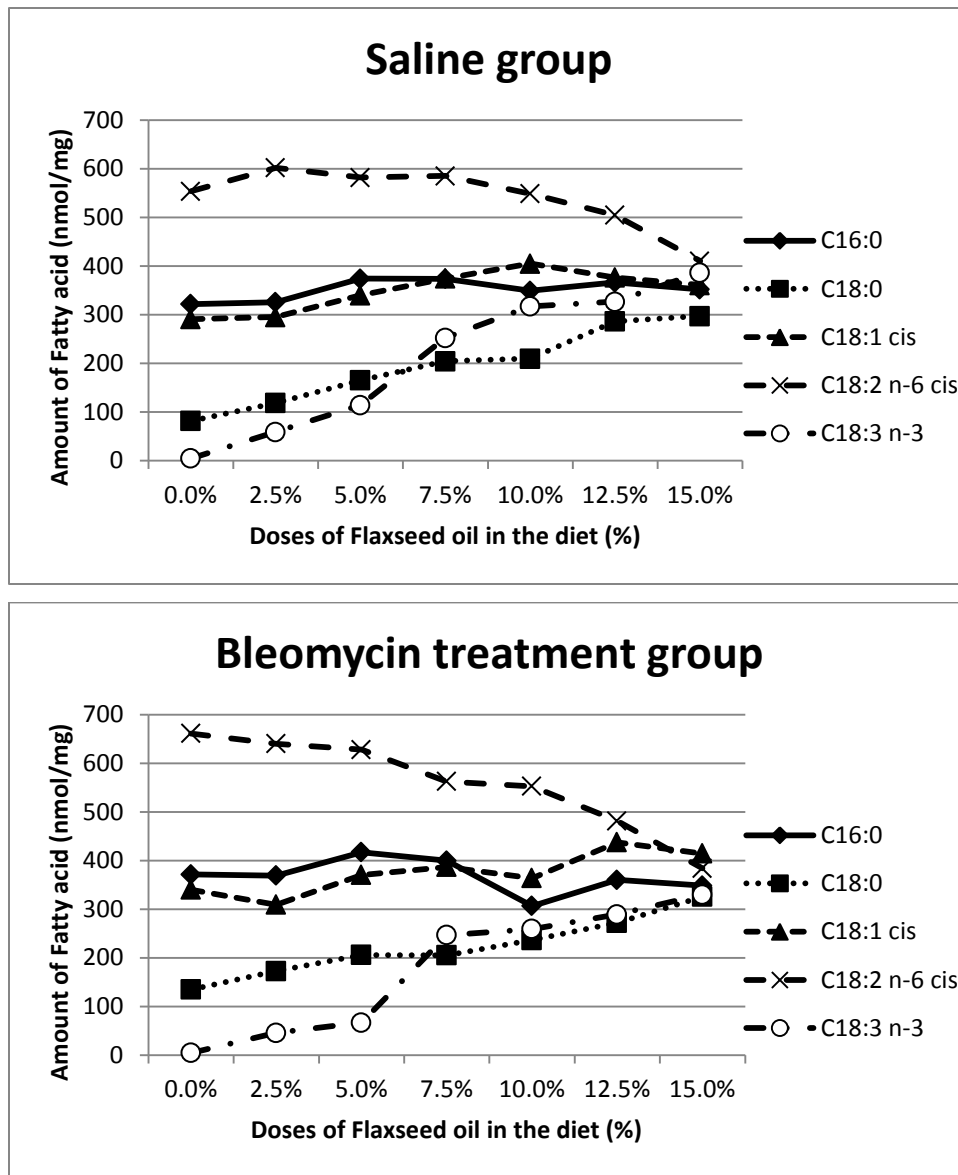


Figure 3-12 Fatty acid composition of the liver from the rats fed different doses of flaxseed oil with or without bleomycin treatment

Fatty acids profile from the liver of the rats performed by GC. The amount of α -linolenic acid increased as the dose of flaxseed oil increased, and the amount of linoleic acid decreased, demonstrating that α -linolenic acid in flaxseed oil replaced linoleic acid in the tissue. Among α -linolenic acids, a significant increase of α -linolenic acid was observed from the group of 7.5 % flaxseed oil in diet when compared to the group of 5 % flaxseed oil diet.

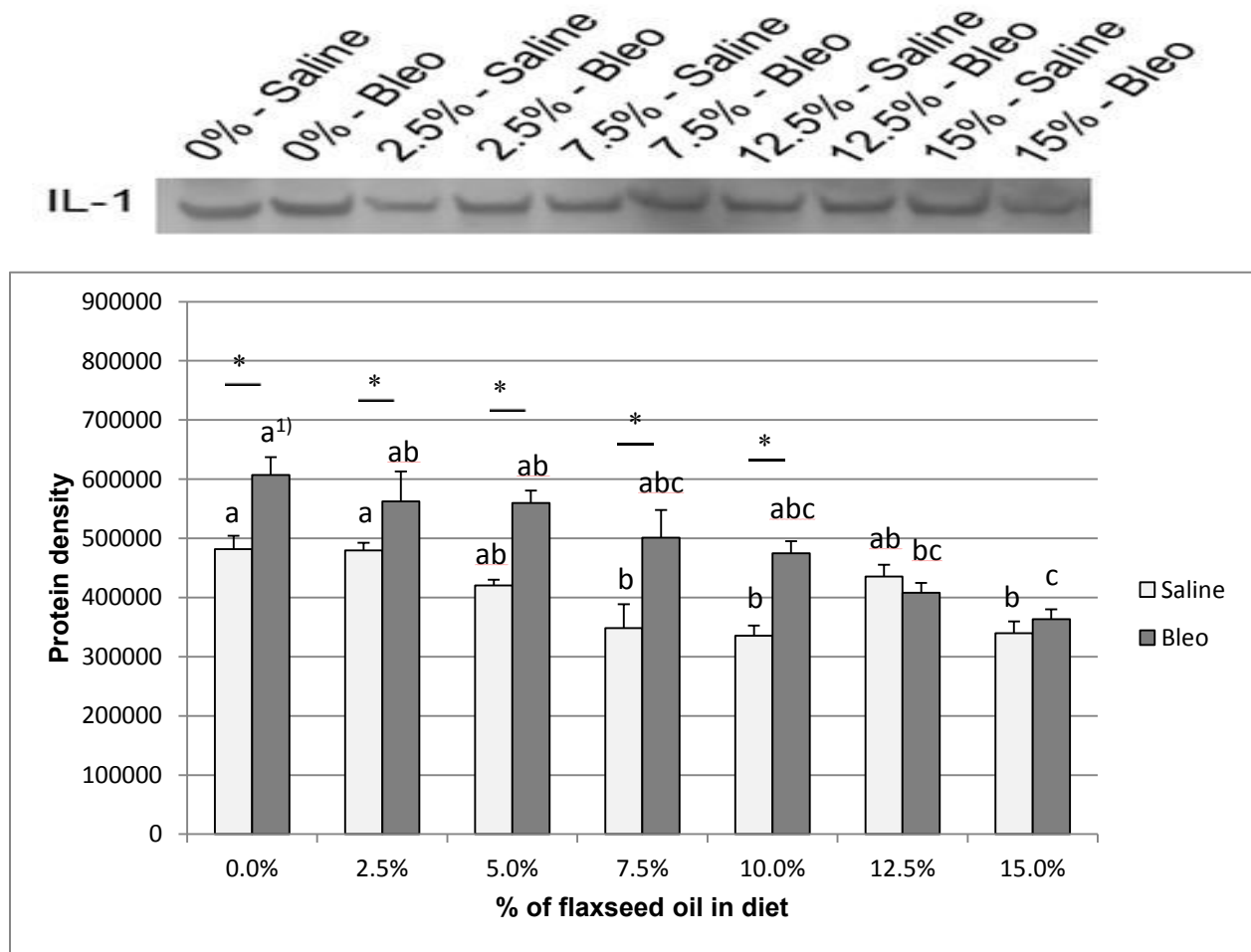


Figure 3-13 Western Blot analysis of IL-1 from the lung of rats fed diets with different doses of flaxseed oil

- 1) Values with different alphabet within the same color bars are significantly different at $p < 0.001$.
- 2) *Significant difference between saline control and bleomycin treatment group at $p < 0.05$.

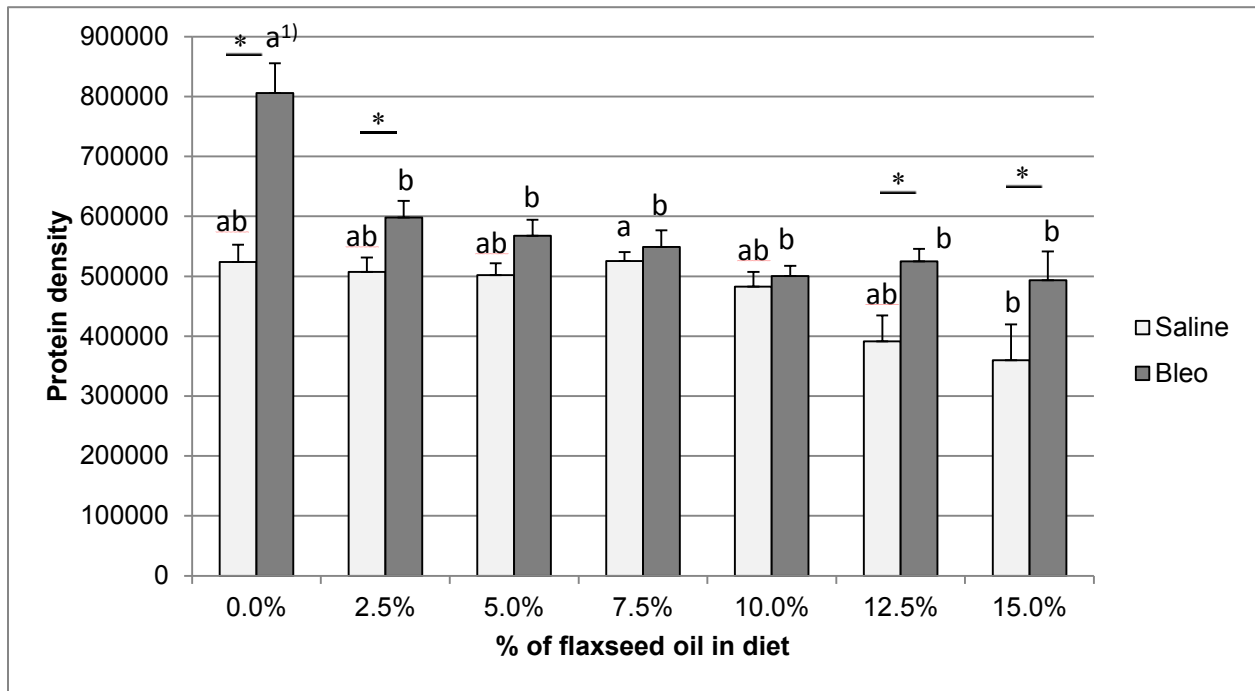


Figure 3-14 Western Blot analysis of α -SMA from the lung of the rats fed diets with different doses of flaxseed oil

- 1) Values with different letter within the same color bars are significantly different at $p < 0.001$.
- 2) * Significant difference between saline control and bleomycin treatment group at $p < 0.05$.

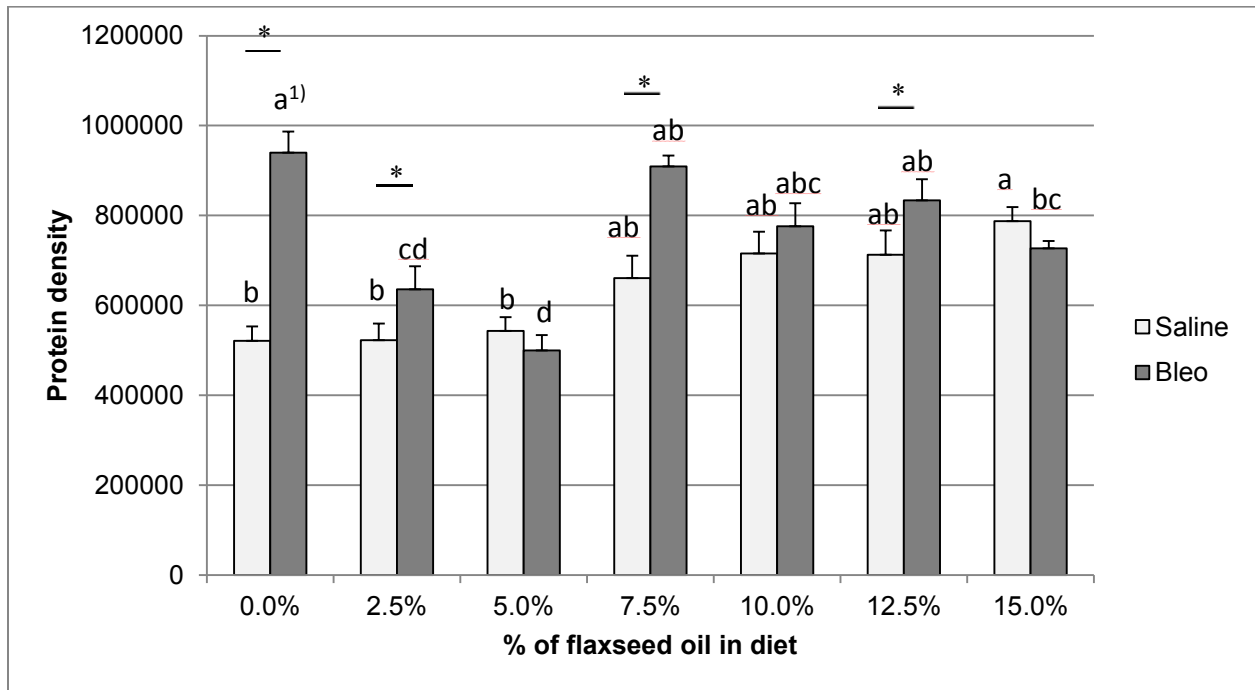
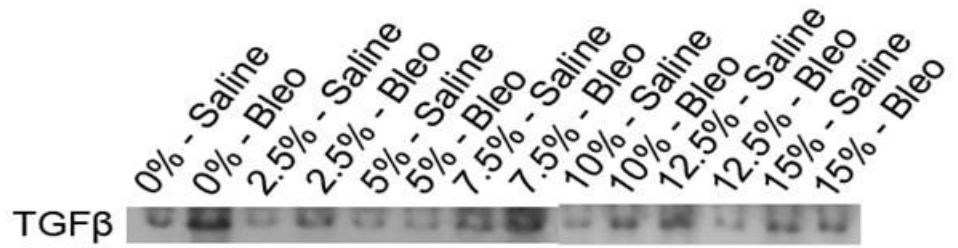


Figure 3-15 Western Blot analysis of TGF- β from the lung of rats fed diets with different doses of flaxseed oil

- 1) Values with different letter within the same color bars are significantly different at $p < 0.001$.
- 2) * Significant difference between saline control and bleomycin treatment group at $p < 0.05$.

Reference

1. Baybutt RC, Rosales C, Brady H and Molteni A. Dietary fish oil protects against lung and liver inflammation and fibrosis in monocrotaline treated rats. *Toxicology*. 175:1-13, 2002.
2. Silva LP, Catunda Lemos AP, Curi R and Azevedo RB. Effects of fish oil treatment on bleomycin-induced pulmonary fibrosis in mice. *Cell Biochem Funct*. 24: 387–396, 2006.
3. Bloedon LT and Szapary PO. Flaxseed and cardiovascular risk. *Nutr Reviews*. 62: 18-27, 2004.
4. Morris D. Essential nutrients and other functional compounds in flaxseed. *Nutr Today*. 36: 159-162, 2001.
5. Carter JF. Potential of flaxseed and flaxseed oil in baked goods and other products in human nutrition. *Cereal Foods World*. 38: 753-759, 1993.
6. World Health Organization. Chronic cor pulmonale: a report of the expert committee. *Circulation*. 27:5 94 –598, 1963.
7. Voelkel NF, Quaife RA, Leinwand LA, Barst RJ, McGoon MD, Meldrum DR, Dupuis J, Long CS, Rubin LJ, Smart FW, Suzuki YJ, Gladwin M, Denholm EM and Gail DB. Right ventricular function and failure: report of a National Heart, Lung, and Blood Institute working group on cellular and molecular mechanisms of right heart failure. *Circulation*. 114: 1883–1891, 2006.
8. Weitzenblum E. Chronic cor pulmonale. *Heart*. 89: 225-230, 2003.
9. Reeves PG, Nelsen FH and Fahey GC Jr. AIN-93 purified diets for laboratory rodents: Final report of the American Institute of Nutrition Ad Hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr*. 123: 1939-1951, 1993.
10. Lakatos HF, Burgess HA, Thatcher TH, Redonnet MR, Hernady E, Williams JP and Sime PJ. Oropharyngeal aspiration of a silica suspension produces a superior model of silicosis in the mouse when compared to intratracheal instillation. *Exp Lung Res*. 32: 181-99, 2006.
11. Baybutt RC, Hu L and Motleni A. Vitamin A deficiency injures lung and liver parenchyma and impairs function of rat type 2 pneumocytes. *J Nutr*. 130: 1159-1165, 2000.

12. Baybutt RC and Molteni A. Dietary beta-carotene protects lung and liver parenchyma of rats treated with monocrotaline. *Toxicology*. 137: 69-80, 1999.
13. Molteni A, Ward WF, Ts'ao C and Soliday NH. Monocrotaline-induced cardiopulmonary damage in rats: amelioration by the angiotensin-converting enzyme inhibitor CL242817. *Proc Soc Exp Biol Med*. 182: 483-493, 1986.
14. Folch J, Lees M and GHS Stanley. A simple method for the isolation and purification of total lipids from animal tissues. *J Biochem*. 497-509, 1956.
15. Lee JC, Krochak R, Blouin A, Kanterakis S, Chatterjee S, Arguiri E, Vachani A, Solomides CC, Cengel KA and Cristofidou-Solomidou M. Dietary Flaxseed prevents radiation-induced oxidative lung damage, inflammation and fibrosis in a mouse model of thoracic radiation injury. *Cancer Biol Ther*. 8: 47-53, 2009.
16. Kennedy JI Jr, Chandler DB, Fulmer JD, Wert MB and Grizzle WE. Dietary fish oil inhibits bleomycin-induced pulmonary fibrosis in the rat. *Experimental Lung Research*. 15: 315-329, 1989.
17. Hoshino T, Okamoto M and Sakazaki Y. Role of proinflammatory cytokines IL-18 and IL-1beta in bleomycin-induced lung injury in humans and mice. *Am J Respir Cell Mol Biol*. 41:661-70, 2009.
18. Piguet PF, Collart Ma and Grau GE. Tumor necrosis factor/cachectin plays a key role in bleomycin-induced pneumopathy and fibrosis. *J Exp Med*. 170:655-63, 1989.
19. Moeller A, Ask K and Warburton D. The bleomycin animal model: a useful tool to investigate treatment options for idiopathic pulmonary fibrosis? *Int J Biochem Cell Biol*. 40: 362-82, 2008.
20. Higashiyama H, Yoshimoto D and Okamoto Y. Receptor-activated Smad localization in bleomycin-induced pulmonary fibrosis. *J Clin Pathol*. 60:283-9, 2007.

Chapter 4 - Short Chain Omega-3 Fatty Acids prevent Pulmonary Fibrosis in Time-course

Abstract

We have previously found that dietary flaxseed oil, containing omega-3 fatty acids, protects against bleomycin-induced pulmonary fibrosis. This study evaluates the development of pulmonary fibrosis over time and potential underlying mechanisms. Forty Sprague-Dawley rats were assigned into four groups with either corn oil (15 % w/w) or flaxseed oil (15 % w/w) in the diet for one month prior to treating with bleomycin at 8 U / kg oropharyngeally. Half of the rats were sacrificed 7 days after bleomycin treatment. Both interleukin-6 (IL-6), a protein associated with fibrotic inflammation in the lung, and renin, an enzyme related to renin-angiotensin system, were examined by Western blot. The time-dependent increase of IL-6 in response to bleomycin treatment was significantly reversed by flaxseed oil diet. Although renin was not significantly different in the kidney, it suggested that the renin-angiotensin system may be involved locally. In addition, the profiles of fatty acids in both liver and kidney tissues as measured by lipidomics demonstrated a significant increase of omega-3 : omega-6 ratio in flaxseed oil-fed groups. These results indicated for the first time that flaxseed oil rich in omega-3 fatty acid might inhibit the formation of pulmonary fibrosis via suppressing inflammation.

Introduction

As reported in Chapter 3, dietary flaxseed oil, which has short chain omega-3 fatty acids, was found to have protective effects against bleomycin-induced pulmonary fibrosis. Therefore, this study was conducted to further investigate the effects of flaxseed oil on bleomycin-induced pulmonary fibrosis and to determine the potential underlying mechanisms. Bleomycin is a chemotherapeutic agent used in the treatment of a variety of tumors.¹ However, it has an adverse effect of inducing interstitial pulmonary fibrosis. Associated with bleomycin-induced pulmonary fibrosis is the release of many kinds of eicosanoids associated with inflammation and collagen

deposition, and they can be modulated by omega-3 fatty acids since the metabolic activities of eicosanoids are derived from different sources of fatty acids.

Chapter 3 focused on the anti-inflammatory effects of flaxseed oil with different doses over time and detailed the effects on organs including the lung and heart. Therefore this chapter will further explore additional organs such as the liver and the kidney, and also possible underlying mechanisms other than inflammation. In addition, this study is a follow-up study of Dr. Baybutt². Therefore the same animals have been used for both studies, they focused just on the lung.

It is known that fibrosis can occur in the liver and kidney along with pulmonary disease. Thus we chose a different cytokine, IL-6 to see the potential anti-inflammatory effects of flaxseed oil on the liver and kidney against bleomycin-induced fibrosis. Another potential mechanism is by the renin-angiotensin pathway. Renin is an enzyme that participates in the body's renin-angiotensin system (RAS), and angiotensin II (ANGII) is related to the inflammatory reaction involving NF- κ B activation.³ Changes in renin expression would suggest a potential role for the renin-angiotensin pathway.

Methods and Materials

Animals and Diets

Forty weanling male Sprague-Dawley rats weighing 40-60 g each were randomly assigned into one of four different groups and were fed an AIN 93G basal diet (Reeves et al. 1993)⁴ containing either corn oil (15 % w/w) or flaxseed oil (15 % w/w) as the source of dietary fat. The animals were housed individually in stainless steel cages at room temperature under a 12 h light : dark cycle (light 06:00 – 18:00 h) and a relative humidity of 50 %. Animal care and use were approved by the Institutional Animal Care and Use Committee of Kansas State University. The rats were fed ad libitum and the purified diets were purchased from Dyets Inc. (Bethlehem, PA). Food intake was recorded daily and body weights were measured weekly.

After four weeks on the diet, half of each of these groups were anesthetized and administered 8 U/kg body weight of bleomycin in 0.4 mL sterile saline by oropharyngeal

delivery to minimize the stress of the animals. The other half of these groups were administered saline for the negative control groups.

Half of the rats were sacrificed 7 days after bleomycin treatment, and the others were sacrificed after 21 days. (Figure 4-1) The rats were anesthetized with pentobarbital and organs including the heart, lung, liver, and kidney were collected for weighing and analysis. Lung, liver and kidneys were collected for chemical and histological studies.

Fatty Acids Analysis

For fatty acids analysis, lipids were extracted from the liver and the kidney and analyzed by Gas Chromatography using Folch method⁵ arranged by Kansas Lipidomics Research Center. To 0.8 parts tissue (homogenized) in aqueous solution, 1 part chloroform and 2 parts methanol were added. After being shaken well, 1 part chloroform and 1 part water added. After shaking again, it was centrifuged at low speed for 5 - 10 min. After removing the lower layer, 1 part chloroform was added. Then it was shaken, centrifuged, and got removed the lower layer again. After repeating this one more time, the combined lower layers were washed once with a small volume 1.0 M KCl and once with a small volume of water. After drying down, it was dissolved in 1 mL of chloroform. Finishing with extraction, it was gone through derivatization. The fatty acid C15:0 internal standard was added to the lipid extract and fatty acids were methylated after hydrolysis by methanoic-HCl. 1 mL of 1.5 M methanoic HCl was added, bubbled with nitrogen gas for 10 s, then it was heated at 78 °C on heat block for 30 min. After cooling down, 2 mL of water and 2 mL of pentane were added and shaken for 15 s. Then it was centrifuges at low speed (700-800 rpm) for 20 min. Upper pentane layers were collected to tubes containing sodium sulfate, then 2 mL of pentane was added, shaken, and centrifuged again. The upper pentane layer was collected and combined with the previous layer extraction, and then all pentane layers were transfer to clean tubes avoiding taking any of sodium sulfate. Pentane layers were dried down after transferred, and redissolved in small volume of hexane, then the sample would be ready to be injected in the Gas Chromatograph.

Fatty acid composition of the liver and the kidney tissue total lipids was determined by measuring the fatty acid methyl esters using a capillary gas chromatography with a flame ionization detector. The Gas chromatography-FID (Flame Ionization Detector) analysis was

performed at the Kansas Lipidomics Research Center with a 6890N GC (Agilent Technologies) coupled to a FID. The GC was fitted with a HP-88 capillary column with a bis (Cyanopropyl) Polysiloxanes stationary phase (column length - 100 cm, internal diameter - 250 μ m, film thickness - 0.25 μ m). Helium was used as the carrier gas at a flow rate of 1.2 mL/min. The back inlet was operating at a pressure and temperature of 32.36 psi and 275 $^{\circ}$ C respectively. Agilent 7683 autosampler was used to inject 1 μ L of the sample in the split mode with a split ratio of 10:1. The GC temperature ramp was operated as follows, initial temperature of 70 $^{\circ}$ C, ramp 1 at 15 $^{\circ}$ C/min to 175 $^{\circ}$ C, ramp 2 at 1 $^{\circ}$ C/min to a final temperature of 235 $^{\circ}$ C. The FID detector was operated at 260 $^{\circ}$ C. The hydrogen flow to the detector was 30 mL/min and air flow was 400 mL/min. The sampling rate of the FID was 20 Hz. The data were processed using Chemstation software.

Western Blot Analysis

Protein analysis of IL-6, and renin was conducted for stored liver and kidney tissues via Western Blot analysis after protein extraction. Rat liver and kidney tissues were smashed into powder in liquid nitrogen, mixed with cell lysis buffer (5 mM Tris-HCl, pH 7.4, 2 mM EDTA, 10 % Triton X-100, protease inhibitor cocktail and phosphatase inhibitor cocktail) and then homogenized. The lysate was then centrifuged in 20,000 rpm for 15 min at 4 $^{\circ}$ C. The supernatant was collected and sonicated for 10 s. After sonication, it was centrifuged with the speed of 10,000 rpm for 15 min at 4 $^{\circ}$ C. The supernatant was collected and the protein concentration was measured by Bio-Rad protein assay (Bio-Rad, Hercules, CA) with Bovine Serum Albumin (BSA) standards. Laemmli sample buffer (Bio-Rad, Hercules, CA) was used to mix with and denature the proteins. The proteins were separated by 10 % SDS-PAGE gel and transferred to nitrocellulose membrane using Trans-Blot SD SEMI-DRY Transfer Cell (Bio-Rad, Hercules, CA) with 15 V for 20 min. The membrane was blocked with 5 % blocking buffer containing 5 % non-fat milk at room temperature for 1 hr, and incubated with first antibody (IL-6, renin, and β -actin (as the loading control)) (R&D Systems, Minneapolis, MN, Santa Cruz Biotechnology Inc, Santa Cruz, CA) overnight. After the first antibody incubation, the membrane was washed with TDN-T (Tris-EDTA-NaCl with Tween 20) twice for 5 min each, then incubated with anti-mouse HRP-conjugation secondary antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA) for 1 hr.

Finally the bands were visualized and protein density was determined using the Fluorchem densitometry program.

Statistical Analysis

All data were expressed as means \pm SEM. One-way, two-way and three-way ANOVA using SAS software (SAS Institute, Cary, NC) were performed to test pre-determined hypotheses. Values of p less than 0.05 represented a significant difference. Post hoc t-tests (or Tukey) were performed with the differences between means when the overall main or interaction effect was significant at $p < 0.05$.

Results

Food Intake and Body Weight Gain

The average amount of daily food intake among bleomycin groups was significantly less than the vehicle group after bleomycin treatment. ($p < 0.01$, Table 4-1) In 21 days groups, the decreased amount of daily food intake recovered as time went on, and after 2 weeks of bleomycin treatment, there was no significant difference between vehicle control and bleomycin treatment groups.

The body weight gain or loss from the rats after bleomycin treatment is described in Table 4-2. At 7 days after the treatment, there was significantly less weight loss in the BF treated groups than the BC treated group. ($p < 0.03$) All the rats treated with bleomycin had weight loss for a while, but they began to gain weight again and after 21 days of treatment, most of the bleomycin treated rats recovered from the weight loss.

Organ Weights and Gross Evaluation

Compared to the organs of the vehicle controls at 7 days group, the weights of all of the organs from bleomycin treated rats were all significantly less than control groups. (Table 4-3) Interestingly, the significant differences between diet groups were observed only in 7 days group. There was no significant difference in organ weights between diet groups in 21 days group. For

all of the organs, there was a significant difference between 7 days and 21 days groups, organ weights from 21 days groups were higher than those from 7 days since the animals were still growing. There were discoloration, hemorrhaging and a cobblestone appearance observed in the lungs of BC groups at 7 days, and more severely at 21 days. Comparing among bleomycin treated group, the lungs from BC group were appeared to have more discoloration and hemorrhaging than BF group.

Fatty Acids Analysis from the Diet

Fatty acids composition of the diet analyzed by GC is shown at Table 4-4. The result was confirmed to be the same as in Chapter 3 since we used the same diets. Comparing with the control group (0 % flaxseed oil diet), it showed a significant increase of α -linolenic acids and a significant decrease of linoleic acids in 15 % flaxseed oil diet group, proving that the flaxseed oil was rich in α -linolenic acids.

Fatty Acids Analysis from the Tissues

The Liver

The results from GC analysis with the liver tissues of the rats are shown in Figure 4-2. There was a huge significant increase in omega-3 : omega-6 ratio in all of the flaxseed oil-fed groups. As the amount of α -linolenic acid increased, the amount of linoleic acid decreased along with it. In the 7 days groups, there were significant differences in palmitic acid and stearic acid between corn oil group and flaxseed oil group. However, there was no significant difference with oleic acid in all groups. In the bleomycin treated groups, generally less amounts of fatty acids were observed in the 21 days group compared to the 7 days group in the rats fed corn oil diet, while there was an increase in flaxseed oil diet group compared with corn oil diet group. For EPA and DHA, a few amounts of them were found in the liver of rats, showing that the low conversion rate from α -linolenic acid. However, both of the amount of EPA and DHA significantly increased with flaxseed oil diet group. In addition, the amount of EPA and DHA in bleomycin treatment group was higher than those in vehicle group, implying higher conversion rate with bleomycin treatment.

The Kidney

Most of the fatty acids appeared to decrease in the flaxseed oil fed group except α -linolenic acid, which significantly increased over all groups with flaxseed oil diet. Especially there were significant decrease in palmitic, stearic and oleic acid with 7 days bleomycin treated group. Comparing with vehicle and bleomycin treated groups, there was a tendency for stearic and linoleic acid to increase with bleomycin treatment, and it was attenuated in flaxseed oil diet groups. For EPA and DHA, it was interesting to note that the amount of EPA were not significantly different between corn oil and flaxseed oil diet in the kidney, implying there might be the basal amount of EPA in the kidney. There was no significant difference with EPA or DHA in bleomycin treatment groups.

Western Blot Analysis from the Tissues

Interleukin-6 (IL-6) from the Liver and Kidney

Figure 4-3 and 4-4 showed the results from the Western Blot analysis of IL-6 from the liver and the kidney. The amount of IL-6 expression was significantly higher in the bleomycin groups than that of the vehicle groups ($p < 0.05$), and increase of IL-6 by bleomycin was significantly reversed by flaxseed oil diet in 7 days after treatment in the liver. Although there was no significant difference in the kidney, the amount of IL-6 was less expressed in 7 days flaxseed oil fed group ($p < 0.05$).

Renin from the Kidney

The result from renin Western Blot analysis in the kidney is shown in Figure 4-5. It increased, but not significantly, in flaxseed oil groups among bleomycin treated groups. There also was no significant difference between 7 days and 21 days groups.

Discussion

This study was performed to further investigate the effects of 15 % flaxseed oil on bleomycin-induced pulmonary fibrosis with time-course. As same as in Chapter 3, 15 % flaxseed oil diet successfully and significantly prevented from bleomycin-induced pulmonary fibrosis in

the lung of rats, which was revealed with histological data from our lab.² The lungs of bleomycin treated flaxseed oil diet groups were obviously attenuated 21 days versus those of rats sacrificed after 7 days.

In addition, the purpose of this study was to determine the potential underlying mechanisms with the liver and the kidney. Besides cardiac fibrosis, liver and kidney fibrosis were often observed in fibrotic lungs. As mentioned earlier in this chapter, this research was focused on the liver and the kidney. Especially for the kidney, renin-angiotensin system might be involved.

Through Western blot analysis, significantly increased amount of IL-6 in control group with bleomycin treatment indicated that there was an ongoing process of inflammation induced by bleomycin in both of the liver and the kidney. Elevated expression of IL-6 due to bleomycin treatment was significantly reversed by flaxseed oil diet. IL-6 is an important cofactor for neutrophil activation and B cell stimulation, and it works at the early stage of inflammation⁶, as the results showed that the expression of IL-6 of 7 days group was much elevated than that of 21days group. However in the kidney, the amount of IL-6 was very small when compared with that of the liver, implying there might not be severe inflammation in the kidney as produced in the liver.

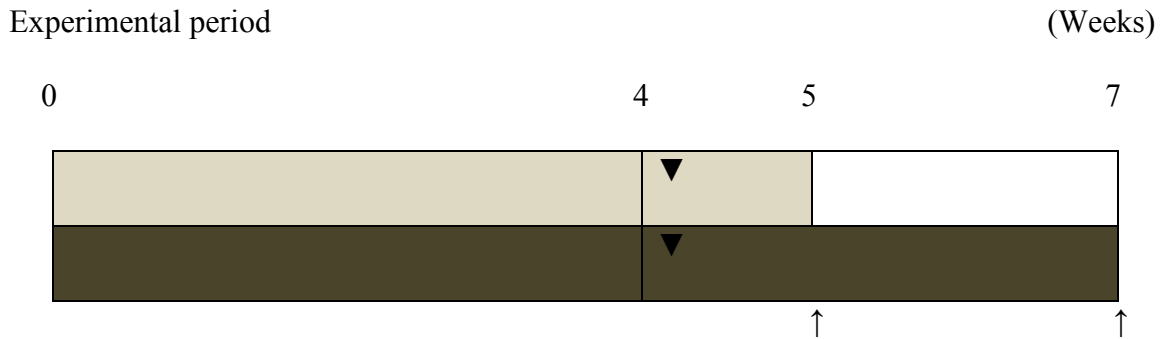
In both of liver and kidney tissues, there was a significant increase in amount of ALA, reducing the ratio of tissue omega-6 : omega-3 fatty acid. Less amount of ALA in bleomycin groups were observed, and flaxseed oil diet significantly changed the fatty acid composition in the liver and the kidney. In addition, the amount of LA significantly decreased in 21 days group. In this study, amount of EPA and DHA in the liver and the kidney was evaluated to determine the relationship with ALA to protect from pulmonary fibrosis and inflammation from bleomycin treatment, since dietary flaxseed oil does not contain both of EPA and DHA.

It should be noted that the amount of EPA and DHA in the tissues was very small when compared to other study⁷ on prevention of pulmonary fibrosis, although they were significantly increased with flaxseed oil diet. The conversion rate of ALA to EPA is about 10 % in rats and 5 % in men^{8,9}. Women have higher conversion efficiency than men, might be due to the importance for demanding of fetus and neonate for DHA¹⁰. Due to these low conversion rates, dietary sources of ALA tend to be underestimated than those of EPA or DHA. However in this study, it clearly showed that ALA alone would be enough to play the important role for preventing


pulmonary fibrosis and inflammation. It is well known that ALA competes with LA for the metabolic enzyme and inhibit the conversion of LA to AA, giving rise to decreased amounts of substrate available for the formation of proinflammatory eicosanoids.¹¹


The angiotensin system is known to be triggered after tissue damage to support tissue repair, while tissue gets fibrosis when exacerbated. There is significant *in vivo* evidence suggesting that the angiotensin system is involved in pulmonary fibrosis.¹² Earlier study revealed that constitutive expression of active TGF- β 1 induced by human lung myofibroblasts was downregulated of collagen synthesis, therefore local upregulation of either angiotensin or TGF- β 1 expression could induce the other. Also, the synergy between two systems should be inhibited in order to block the development of lung fibrotic disease.¹³ Also recently, it was found that angiotensin signaling is related with fibrotic lungs.¹⁴ Although the kidney initiated renin-angiotensin system, maybe involved, it is possible to and a number of studies support, the existence of “local” angiotensin (ANG) systems in diverse organs and tissues.¹⁵ It has to be noted that there is a key difference between the endocrine and local tissue ANG systems. It is that many local ANG systems have been shown to be not related with renin and angiotensin converting enzyme (ACE), but relatively related with other enzymes such as cathepsin D, tonin, cathepsin G, or chymase, for the enzymatic translation of angiotensinogen (AGT) to ANGII.¹⁶ Therefore we need to think about those two possibilities together. Since in this study only renin was measured by Western blot analysis, we cannot convincingly state that renin-angiotensin system is related. Moreover, renin was not significantly different in the kidney. It might be because kidney fibrosis was not strong enough to determine the effect of renin, or it could suggest a potential mechanism with ‘local’ angiotensin systems regarding pulmonary fibrosis. Further study on angiotensin and receptors will be needed.

In conclusion, these results indicated that the omega-3 fatty acids showed preventive effects from fibrosis in the liver and the kidney. Flaxseed oil diet significantly changed the fatty acid composition in the liver and the kidney, especially with low concentration of EPA and DHA. Although there was no significant difference of renin in the kidney, it suggests that the renin-angiotensin system may be involved locally. With overall data from fatty acids analysis and Western blot analysis, ALA in flaxseed oil showed preventive effects for each of 7 days and 21 days group, by attenuating the acute phase of inflammation in the liver and the kidney, and modulating fatty acid composition in the tissues sufficiently as time went on.



▼ : Bleomycin administration

 : Experimental period 1 with sacrificing 7 days after bleomycin treatment

 : Experimental period 2 with sacrificing 21 days after bleomycin treatment

↑ : Sacrificed point

Figure 4-1 Experimental Design

The overall study period was for 7 weeks. After 4 weeks on the diet, half of each diet group were administered 8 U/kg body weight of bleomycin by oropharyngeal delivery. The other half of these groups were administered saline for the negative control groups. Half of the animals were sacrificed 7 days after bleomycin treatment, and the rest of the animals were sacrificed 21 days after bleomycin treatment.

Table 4-1 Food intake averages by experimental group after bleomycin treatment²

Group		Weeks after bleomycin treatment		
Time	Treatment	1	2	3
7 days	VC ¹⁾	22.32 ± 1.73 ²⁾	-	-
	VF	22.15 ± 1.35		
	BC	2.80 ± 1.13 ^{a3)}		
	BF	8.32 ± 1.58 ^b		
Significant factor (2-way)		B	-	-
21 days	VC	21.62 ± 1.27	21.92 ± 1.42	20.63 ± 1.95
	VF	21.51 ± 1.43	18.85 ± 1.77	19.61 ± 1.91
	BC	11.17 ± 2.57	14.64 ± 2.84	16.96 ± 2.83
	BF	8.37 ± 1.30	17.29 ± 2.12	20.22 ± 2.03
Significant factor (2-way) ⁴⁾		B	B	-

- 1) VC : Vehicle Corn oil diet group, VF : Vehicle Flaxseed oil diet group, BC : Bleomycin Corn oil diet group, BF: Bleomycin Flaxseed oil diet group
- 2) Data were expressed as mean (n=13-18).
- 3) Values with different letter within the column in the same treatment group are significantly different at p < 0.01 by Tukey test.
- 4) Statistical significance of experimental factors was calculated based on 2-way ANOVA.
B : Effect of bleomycin treatment was significant at p < 0.05.

Table 4-2 Body weight gain or loss shown by experimental group after bleomycin treatment²

	VC ¹⁾	VF	BC	BF
7 days	23.6 ± 2.2 ²⁾	31.0 ± 1.8	-71.7 ± 2.5	-37.6 ± 15.4 ^{*3)}
21 days	97.3 ± 1.7	88.4 ± 11.9	4.2 ± 62.4	16.9 ± 31.0

- 1) VC : Vehicle Corn oil diet group, VF : Vehicle Flaxseed oil diet group, BC : Bleomycin Corn oil diet group, BF: Bleomycin Flaxseed oil diet group
- 2) Data are expressed as mean ± SEM (n=3-5).
- 3) *Significantly different between BC and BF groups, p < 0.03.

Table 4-3 Weights of organs at 7 and 21 days after bleomycin treatment from the rats fed either corn oil or flaxseed oil diet²

Days	Group	Lung ³⁾	Liver	Heart	Whole Kidney
7	VC ¹⁾	0.44 ± 0.01 ²⁾	3.30 ± 0.06	0.55 ± 0.02 ^a	0.82 ± 0.02
	VF	0.40 ± 0.01	3.27 ± 0.02	0.47 ± 0.02 ^b	0.84 ± 0.02
	BC	1.55 ± 0.08 ^a	3.78 ± 0.19 ^a	0.49 ± 0.01 ^a	1.06 ± 0.03 ^a
	BF	1.00 ± 0.15 ^b	3.23 ± 0.08 ^b	0.43 ± 0.03 ^b	0.94 ± 0.03 ^b
Significant Factor (2-way ANOVA) ³⁾		A, B, A*B	A, A*B	A	B, A*B
21	VC	0.40 ± 0.01	3.02 ± 0.04	0.38 ± 0.01	0.75 ± 0.01
	VF	0.41 ± 0.01	2.94 ± 0.03	0.37 ± 0.02	0.79 ± 0.02
	BC	0.95 ± 0.29	3.14 ± 0.29	0.48 ± 0.06	0.90 ± 0.14
	BF	0.84 ± 0.18	3.20 ± 0.22	0.44 ± 0.05	0.88 ± 0.07
Significant Factor (2-way ANOVA) ⁴⁾		B	-	B	-
Significant Factor (3-way ANOVA)		A, B, C, A*B, B*C	B, C	A, B, C	B, C

- 1) VC : Vehicle Corn oil diet group, VF : Vehicle Flaxseed oil diet group, BC : Bleomycin Corn oil diet group, BF: Bleomycin Flaxseed oil diet group
- 2) Data were expressed as mean ± SEM (n=3-5) in weight of organ/100g bodyweight.
- 3) Lung and heart data were from Dr. Baybutt's work.²
- 4) Statistical significance of experimental factors was calculated based on 2-way and 3-way ANOVA.
 - A : Effect of flaxseed oil diet was significant at p < 0.05.
 - B : Effect of bleomycin treatment was significant at p < 0.05.
 - C : Effect of time with bleomycin treatment was significant at p < 0.05.
 - A*B : Interaction between flaxseed oil diet and bleomycin treatment was significant at p < 0.05.
 - B*C : Interaction between bleomycin treatment and time with bleomycin treatment was significant at p < 0.05.

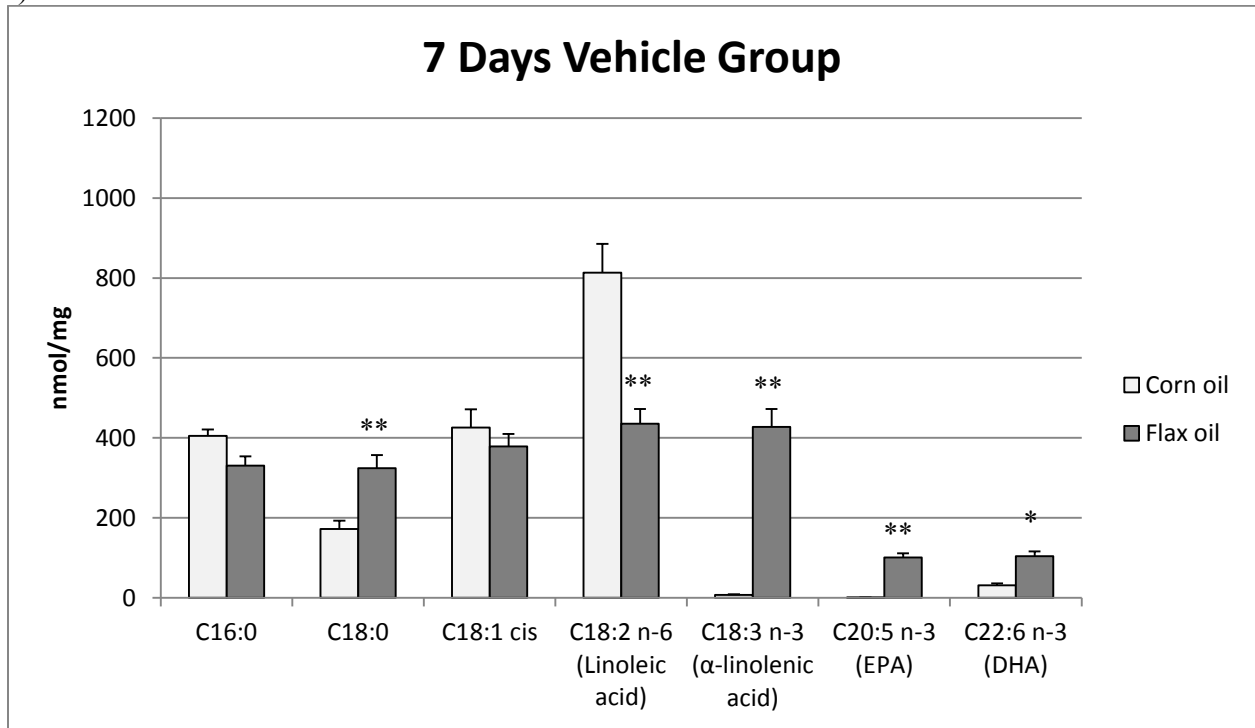
Table 4-4 Fatty acids composition of the diet analyzed by GC¹⁾

Fatty acid	Corn oil diet	Flaxseed oil diet
C16:0	2922.11 ± 124.67 ²⁾	1248.55 ± 52.76
C18:0	583.85 ± 25.16	1052.02 ± 41.99
C18:1 n-9 cis	7551.47 ± 321.2	5027.15 ± 214.48
C18:2 n-6 cis	14459.07 ± 621.28	3882.91 ± 165.84
C18:3 n-3	252.895 ± 10.625	11992.8 ± 511.81

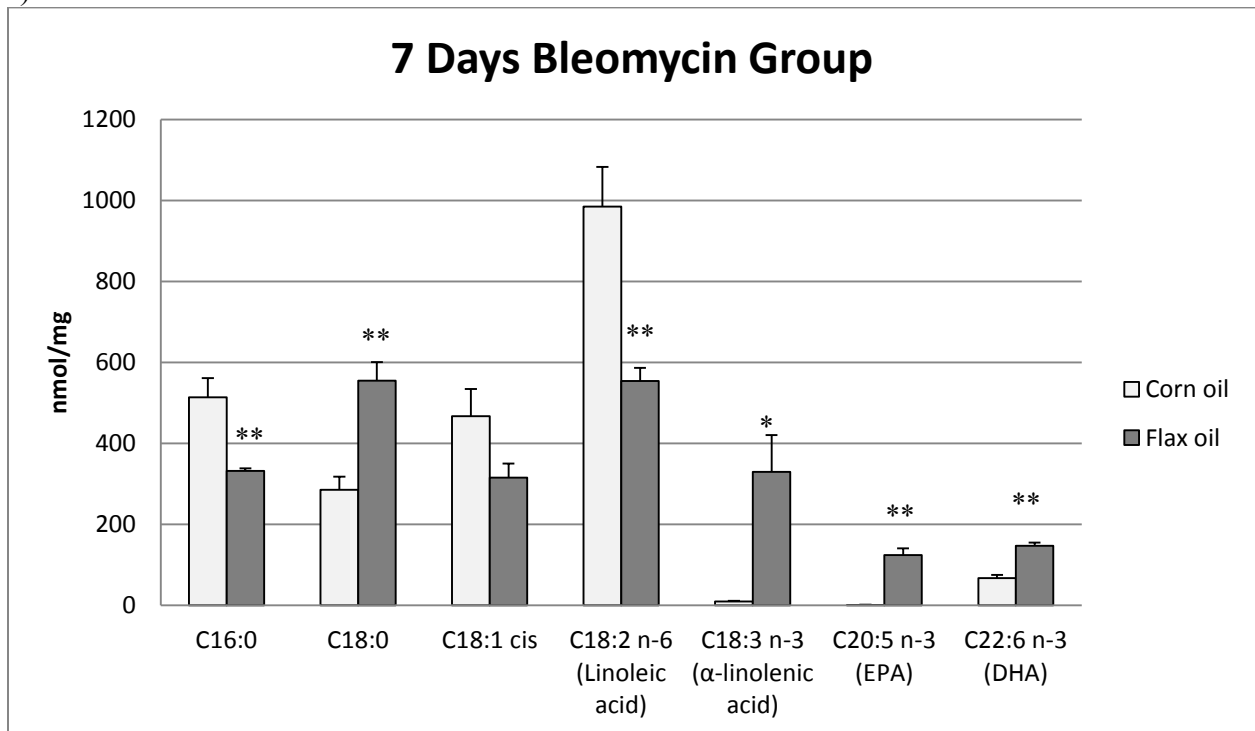
- 1) Data were expressed as µg/g sample.
- 2) Data were expressed as mean ± SEM.

The diets were AIN-93G control diets containing either corn oil (15 % w/w) or flaxseed oil (15 % w/w) as the source of dietary fat.

a)



b)



c), d)

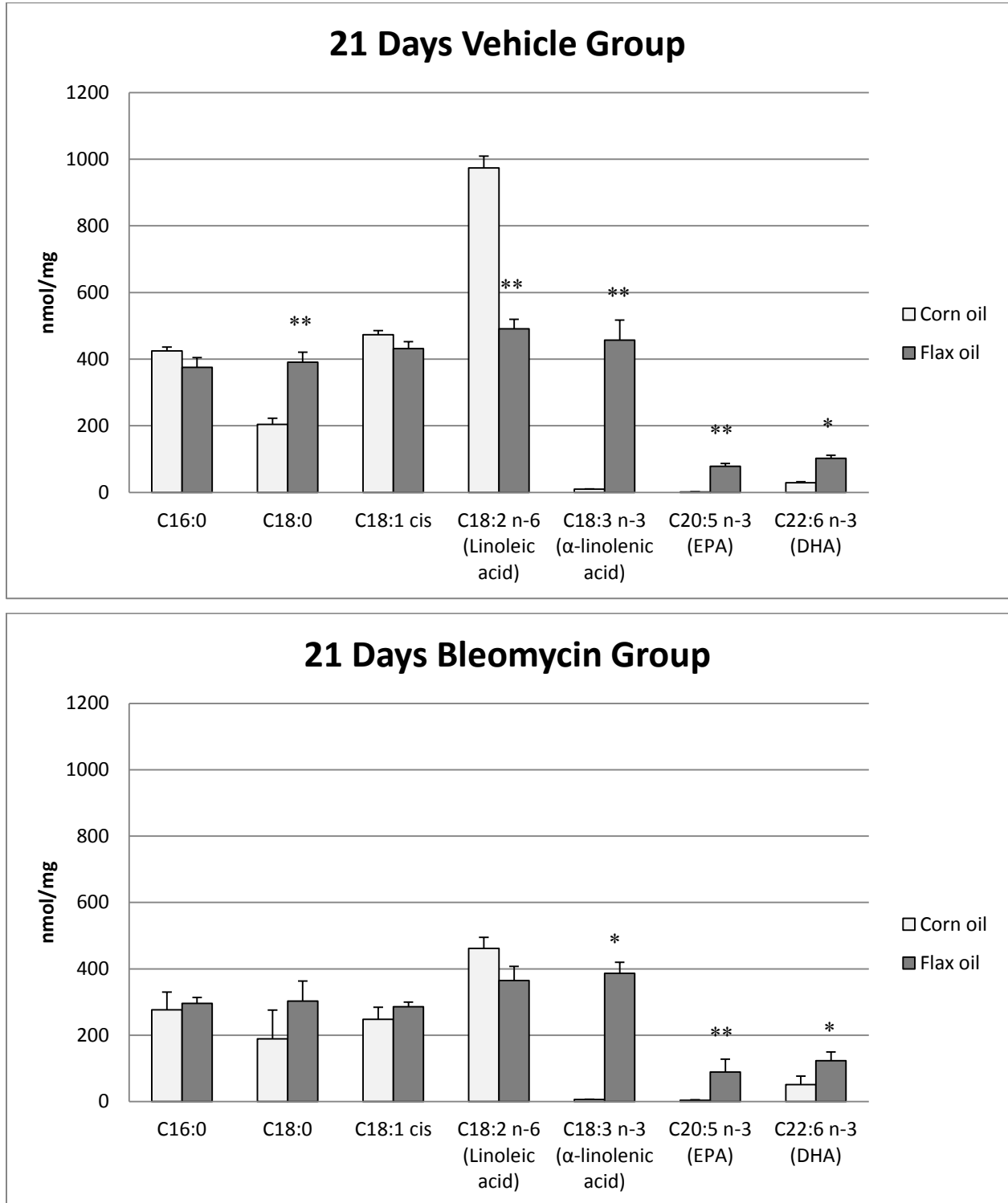
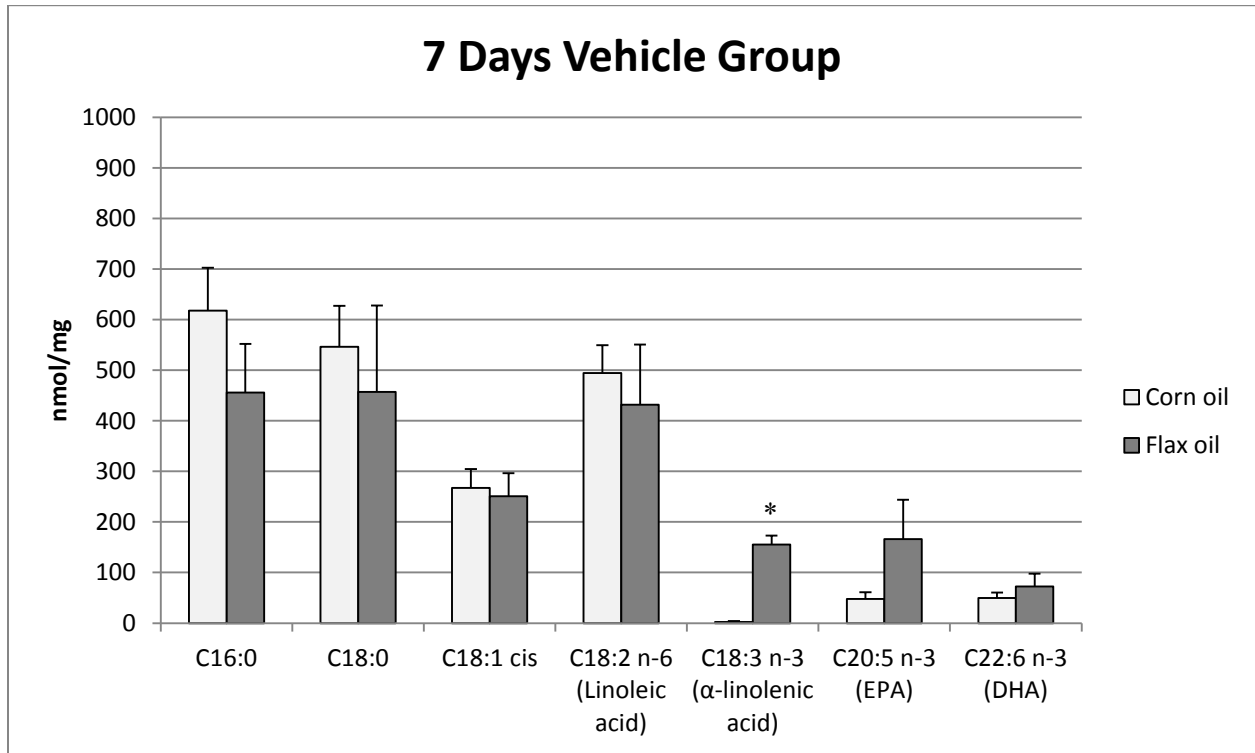


Figure 4-2 Major fatty acids profile of the liver analyzed by GC.

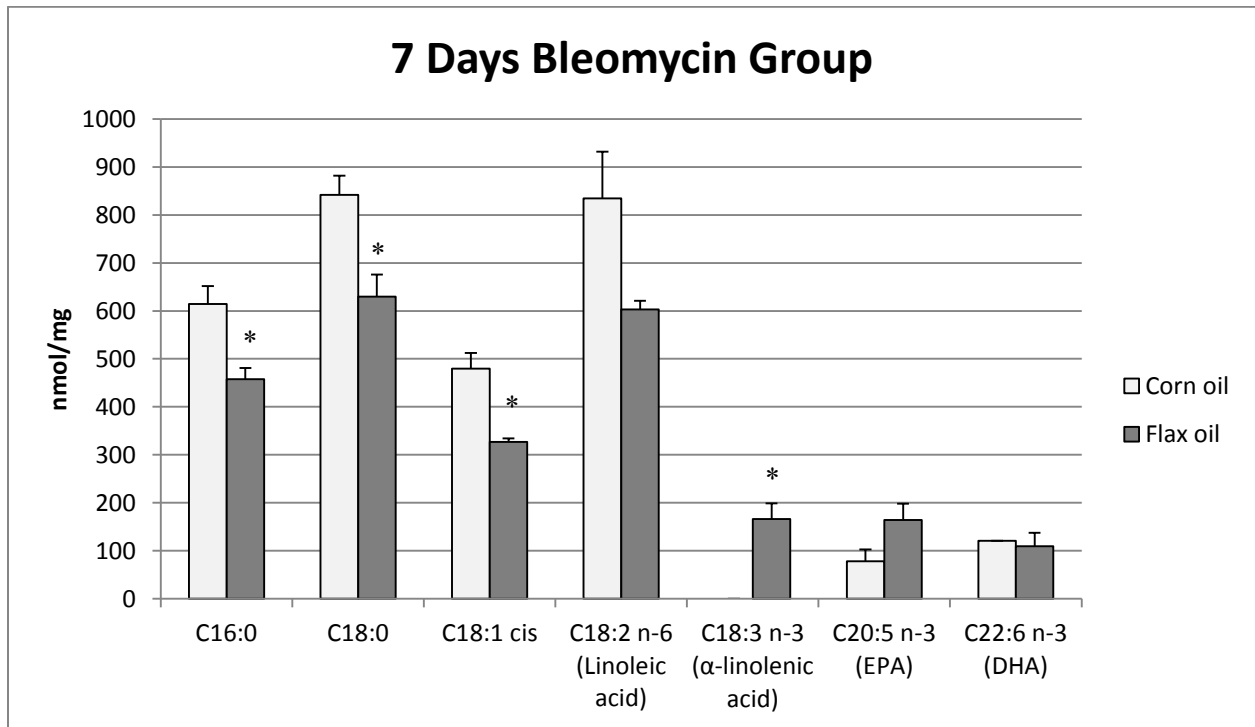
*Significantly different from vehicle group, $p < 0.05$

**Significantly different from vehicle group, $p < 0.01$

a)



b)



c), d)

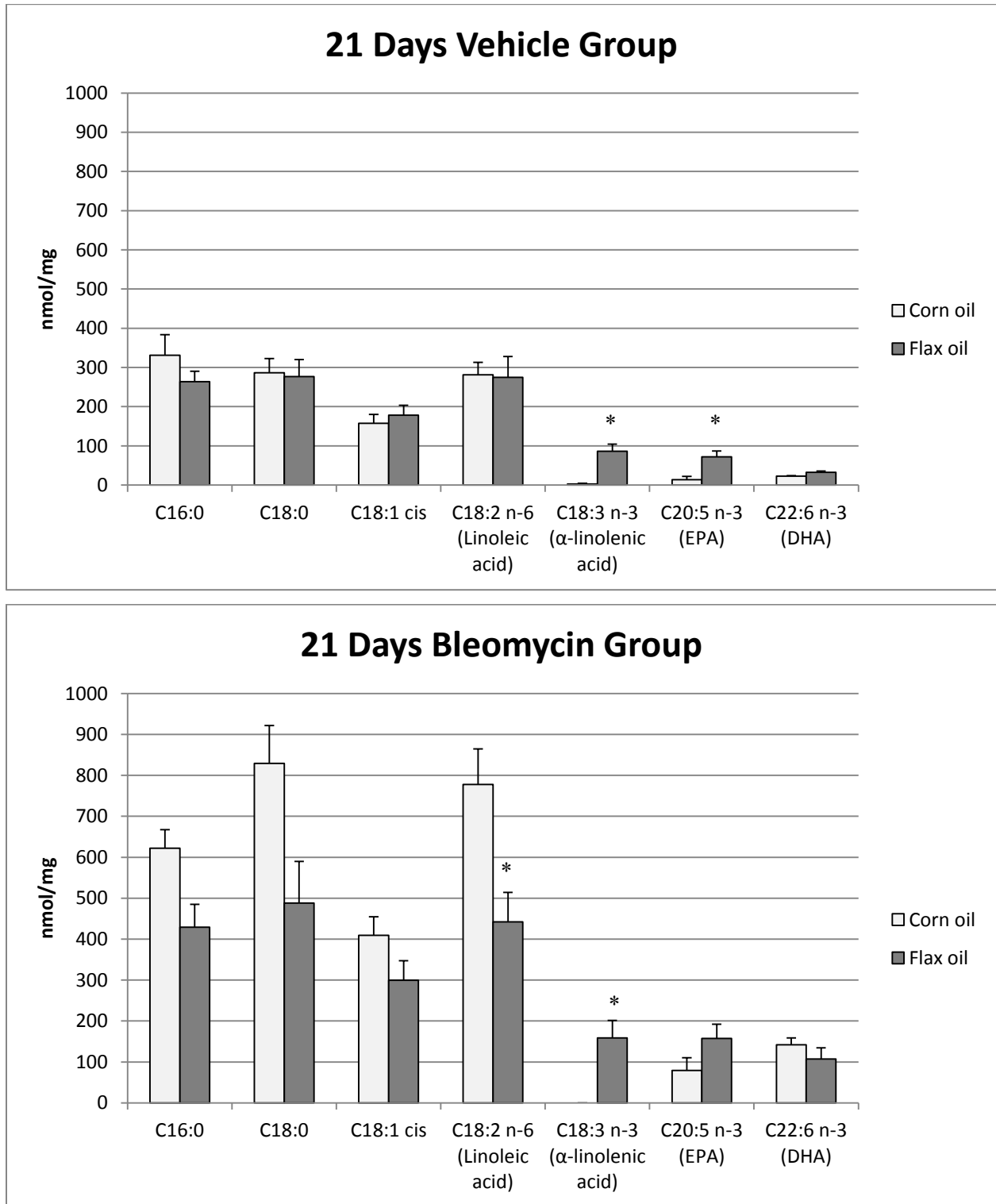


Figure 4-3 Major fatty acids profiles in the kidney analyzed by GC

*Significantly different from vehicle group, $p < 0.05$.

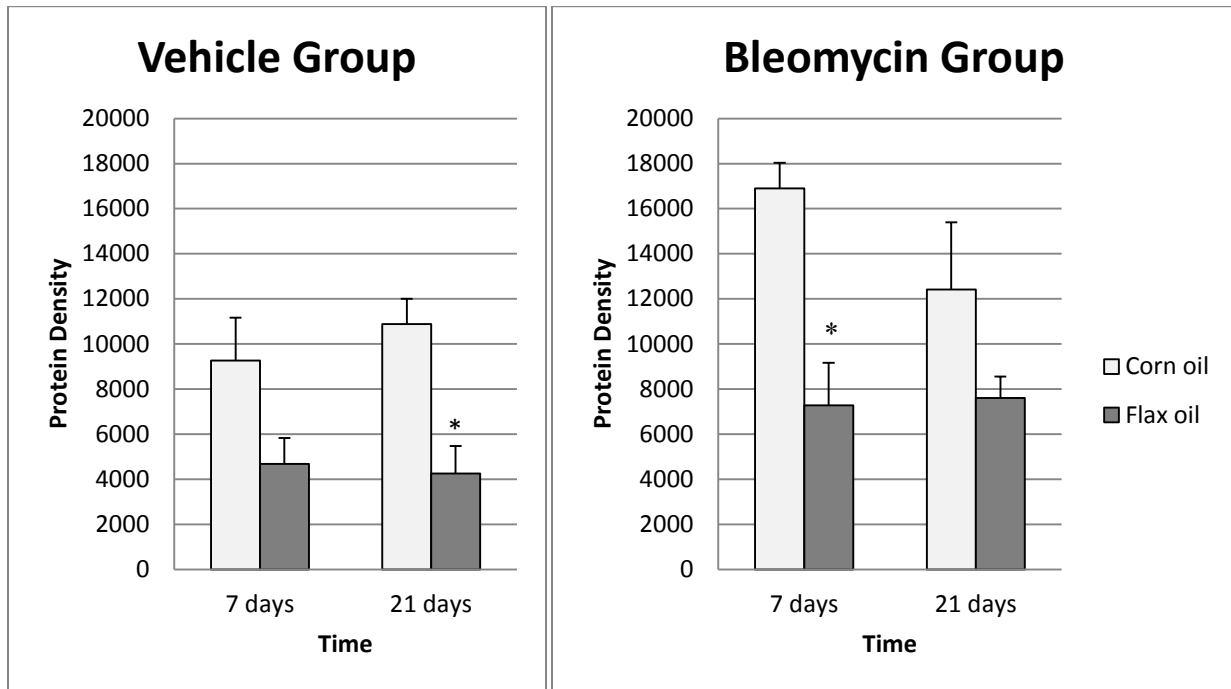
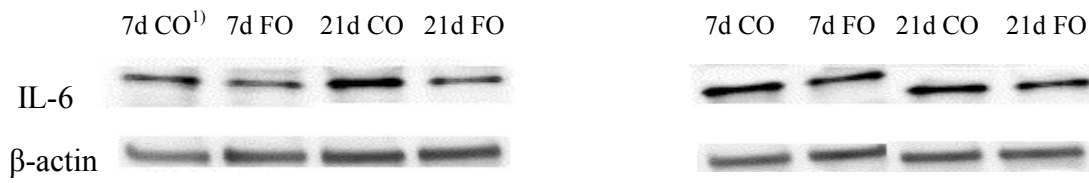


Figure 4-4 Western Blot analysis of IL-6 from the liver in the rats with or without bleomycin treatment

1) CO : Corn Oil diet group, FO : Flaxseed Oil diet group

*Significantly different from vehicle group, $p < 0.05$.

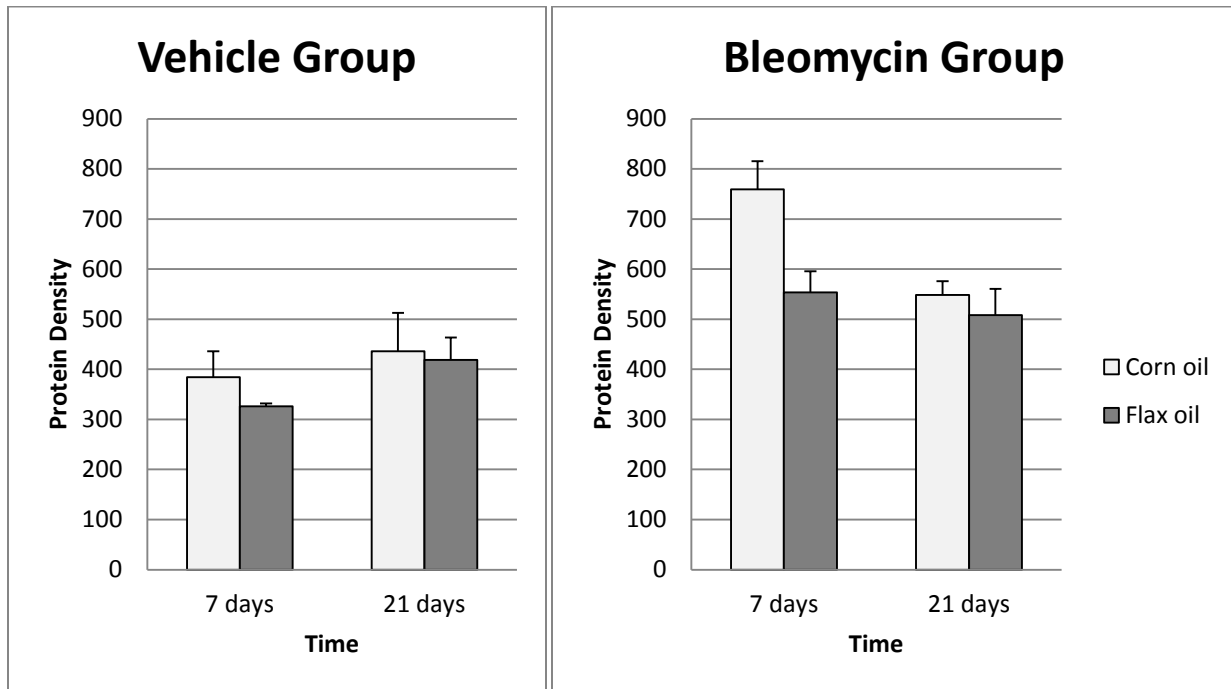
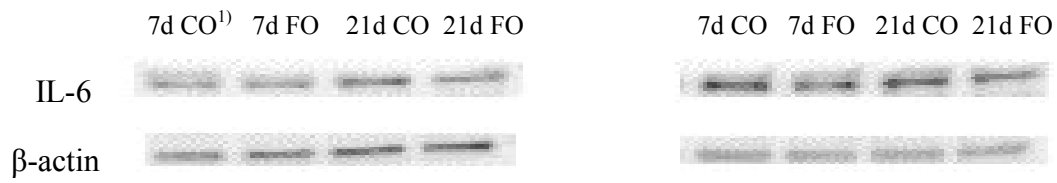


Figure 4-5 Western Blot analysis of IL-6 from the kidney in the rats with or without bleomycin treatment

1) CO : Corn Oil diet group, FO : Flaxseed Oil diet group

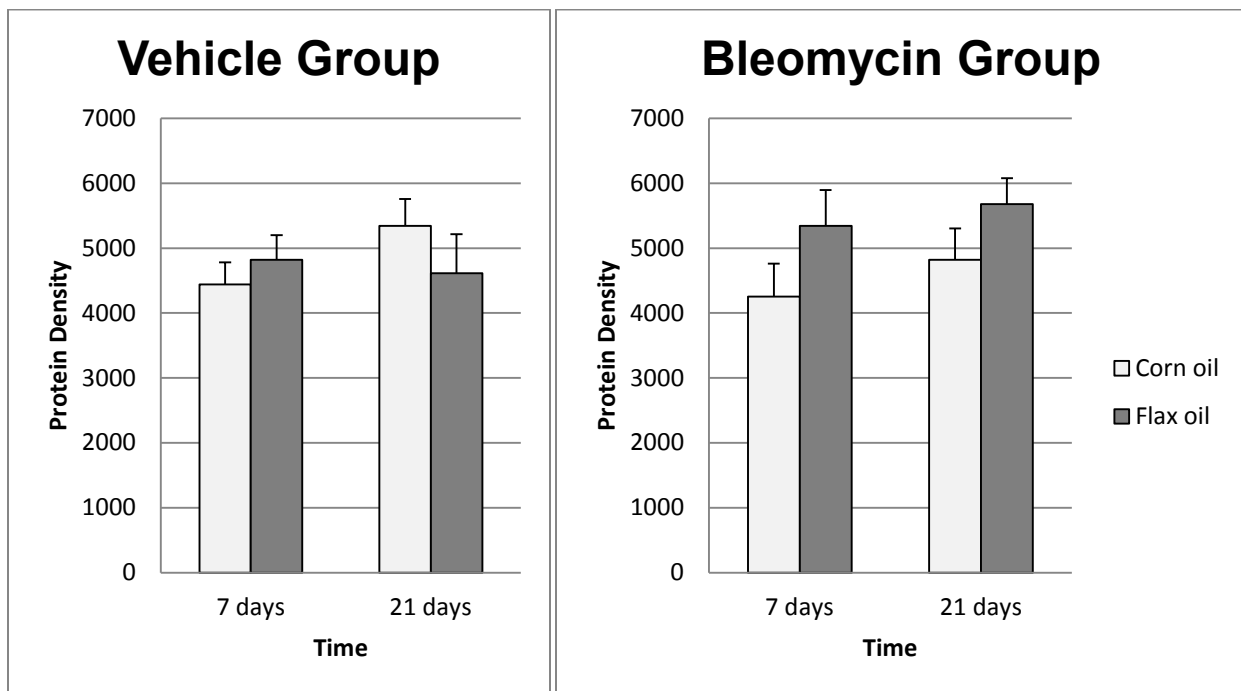
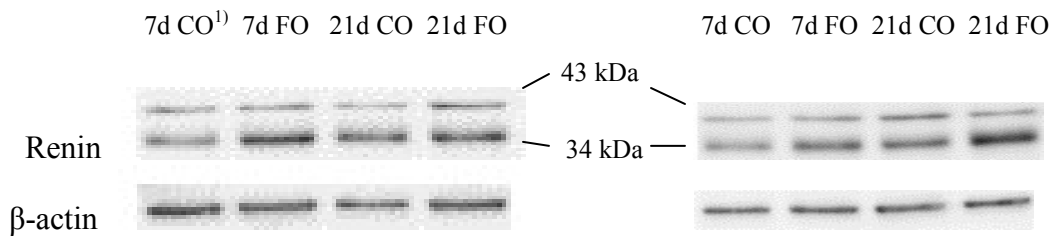


Figure 4-6 Western Blot analysis of renin from the kidney of rats with or without bleomycin treatment

1) CO : Corn Oil diet group, FO : Flaxseed Oil diet group

Reference

1. Silva LP, Catunda Lemos AP, Curi R and Azevedo RB. Effects of fish oil treatment on bleomycin-induced pulmonary fibrosis in mice. *Cell Biochem Funct.* 24: 387–396, 2006.
2. Lawrenz JM, Herndon B, Kamal A, Mehrer A, Dim DC, Baidoo, C, Gasper D, Nitz J, Molteni A and Baybutt RC. Dietary flaxseed oil protects against bleomycin-induced pulmonary fibrosis in rats. *Pulm Med.* 2012:1-11, 2012.
3. Ganong WF. 2005. *Review of medical physiology* : 23rd Edition. San Francisco: McGraw-Hill. pp.454-456,
4. Reeves PG, Nelsen FH and Fahey GC Jr. AIN-93 purified diets for laboratory rodents: Final report of the American Institute of Nutrition Ad Hoc writing committee on the reformulation of the AIN-76A rodent diet. *J Nutr.* 123: 1939-1951, 1993.
5. Folch J, Lees M and GHS Stanley. A simple method for the isolation and purification of total lipids from animal tissues. *J Biochem.* 497-509, 1956.
6. Karmiol S, Remick DG, Kunkel SL and Phan SH. Regulation of rat pulmonary endothelial cell Interleukin-6 production by bleomycin: Effects of cellular fatty acid composition. *Am J Respir Cell Mol Biol.* 9:628-636, 1993.
7. Baybutt RC, Rosales C, Brady H and Molteni A. Dietary fish oil protects against lung and liver inflammation and fibrosis in monocrotaline treated rats. *Toxicology.* 175:1-13, 2002.
8. Gerster H. Can adults adequately convert alpha-linolenic acid (18:3n-3) to eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3)? *Int J Vitam Nutr Res.* 68(3): 159–173, 1998.
9. Brenna JT. Efficiency of conversion of alpha-linolenic acid to long chain n-3 fatty acids in man. *Curr Opin Clin Nutr Metab Care.* 5(2): 127–132, 2002.
10. Burdge GC and Calder PC. Conversion of alpha-linolenic acid to longer-chain polyunsaturated fatty acids in human adults. *Reprod Nutr Dev.* 45(5): 581–597, 2005.
11. Budowski P and Crawford MA. Alpha-linolenic acid as a regulator of the metabolism of arachidonic acid: dietary implications of the ratio, n-6:n-3 fatty acids. *Proc Nutr Soc.* 44:221-229, 1985.
12. Uhal BD, Li X, Piasecki CC and Molina-Molin M. Angiotensin signaling in pulmonary fibrosis. *Int J Biochem Cell Biol.* 44: 465-468, 2012.

13. Uhal BD, Kim JK, Li X and Molina-Molina M. Angiotensin-TGF-beta1 crosstalk in human idiopathic pulmonary fibrosis: autocrine mechanisms in myofibroblasts and macrophages. *Curr Pharm Des.* 13: 1247-56, 2007.
14. Renzoni E, Shi-Wen X, Nicholson AG, Maher T, Dusmet M, Abraham DJ and Wells UW. The increased contractility of idiopathic pulmonary fibrosis lung fibroblasts is dependent on angiotenin signaling, *Eur Respir J.* 36: 4178-84, 2010
15. Uhal BD, Dang MT, Li X and Abdul-Hafez A. Angiotensinogen gene transcription in pulmonary fibrosis. *Int J Pept.* Epub 2012 Feb 20.
16. Paul M, Poyan Mehr A and Kreutz R. Physiology of local renin-angiotensin systems. *Physiol Rev.* 86(3): 747–803, 2006.

Chapter 5 - Summary

The overall purpose of this dissertation was to establish the preventive effect of omega-3 fatty acids in flaxseed oil on bleomycin-induced pulmonary fibrosis in rats and to find the possible underlying mechanisms. Before this dissertation, very little was known about the flaxseed oil and prevention of bleomycin-induced pulmonary fibrosis. There have been no studies to determine the mechanism by which short chain omega-3 fatty acid prevent bleomycin-induced pulmonary fibrosis, and further the role of inflammation in the development of fibrosis has not been clearly defined.

Figure 5-1 summarized the major findings from this research and suggested possible mechanisms which might direct the future studies as following :

- Measuring various kinds of cytokine to determine if ALA affects on each of phases with inflammation on pulmonary fibrosis over a period of time
- Further analysis of local renin-angiotensin system and investigating the relationship with pulmonary fibrosis
- Applying multiple deliveries of bleomycin instead a single treatment to induce pulmonary fibrosis more effectively
- Exploring cardiac fibrosis and ALA : further preventive effects on the heart

Although there were some limitations in this research, the results revealed for the first time that flaxseed oil diet successfully prevented bleomycin-induced pulmonary fibrosis in rats via modulation of fatty acids composition of the tissues and its associated anti-inflammatory effects, along with preventive effects on the heart, the liver, and the kidney. This dissertation is also looked at determining the optimal dose of flaxseed oil and underlying mechanism of mitigating bleomycin-induced pulmonary fibrosis in rats. Additional studies are warranted to investigate further unique mechanisms of ALA on idiopathic pulmonary fibrosis.

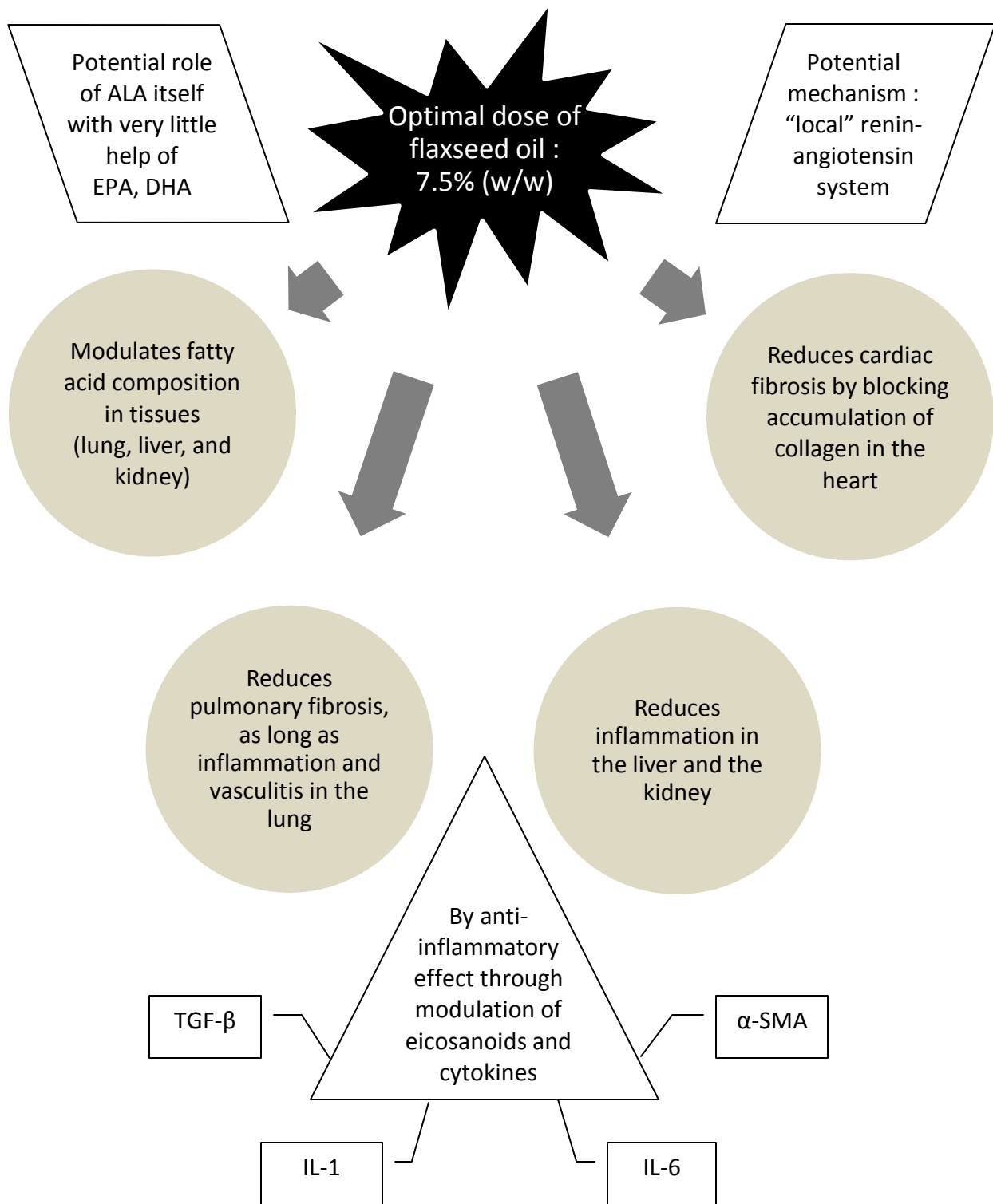


Figure 5-1 Summary of major findings from the dissertation

Appendix A - Pathology Score Sheet

STATISTICA File Server												
File Edit View Analysis Graphs Options Window Help												
-3999. Vars Cases												
Data: flax+bleo full.BAK 14v * 43c												
TEXT VALUES	1	2	3	4	5	6	7	8	9	10	11	12
	DRUG_CON	%FLAXOIL	S_FIBROS	S_INFLAM	VASCULIT	BRONCHIT	HEMORHAG	EMPHYSEM	PERIVLGA	PERISMA	INTR_TUB	GLOMERUL
rat#380	saline	0%	0.000	5.000	0.000	5.000	5.000	5.000	5.000	5.000	0.000	5.000
381	saline	0%	0.000	5.000	0.000	5.000	5.000	5.000	5.000	0.000	0.000	5.000
382	saline	0%	0.000	10.000	0.000	5.000	10.000	5.000	5.000	5.000	5.000	5.000
383	bleomycn	0%	20.000	30.000	15.000	15.000	20.000	20.000	15.000	15.000	10.000	10.000
384	bleomycn	0%	40.000	40.000	40.000	20.000	30.000	10.000	20.000	25.000	10.000	10.000
385	bleomycn	0%	40.000	40.000	40.000	20.000	20.000	10.000	25.000	30.000	10.000	15.000
386	saline	2.5%	10.000	20.000	20.000	20.000	10.000	0.000				
387	saline	2.5%	5.000	15.000	10.000	10.000	10.000	0.000				
388	saline	2.5%	5.000	10.000	5.000	20.000	10.000	0.000				
389	bleomycn	2.5%	20.000	15.000	5.000	20.000	10.000	20.000				
390	bleomycn	2.5%	10.000	15.000	15.000	10.000	10.000	10.000				
391	bleomycn	2.5%	20.000	30.000	20.000	20.000	10.000	15.000				
392	saline	5%	5.000	10.000	10.000	10.000	10.000	10.000				
393	saline	5%	5.000	5.000	0.000	10.000	10.000	10.000				
394	saline	5%	10.000	10.000	10.000	10.000	5.000	5.000				
395	bleomycn	5%	15.000	15.000	5.000	20.000	5.000	10.000				
396	bleomycn	5%	10.000	15.000	10.000	15.000	5.000	5.000				
397	bleomycn	5%	10.000	15.000	10.000	15.000	10.000	10.000				
398	saline	7.5%	0.000	5.000	5.000	5.000	20.000	20.000				
399	saline	7.5%	5.000	10.000	5.000	5.000	20.000	10.000				
400	saline	7.5%	5.000	5.000	0.000	5.000	10.000	10.000				
401	bleomycn	7.5%	20.000	25.000	15.000	20.000	10.000	10.000				
402	bleomycn	7.5%	20.000	25.000	20.000	15.000	10.000	10.000				
403	bleomycn	7.5%	30.000	40.000	25.000	20.000	20.000	30.000				
404	saline	10%	5.000	10.000	5.000	15.000	20.000	15.000				
405	saline	10%	0.000	5.000	5.000	5.000	10.000	10.000				
406	saline	10%	0.000	10.000	0.000	5.000	15.000	20.000				
407	bleomycn	10%	20.000	30.000	15.000	20.000	10.000	15.000				
408	bleomycn	10%	20.000	25.000	15.000	15.000	10.000	10.000				
409	bleomycn	10%	15.000	25.000	10.000	20.000	10.000	10.000				
410	saline	12.5%	5.000	5.000	5.000	5.000	0.000	0.000				
411	saline	12.5%	10.000	10.000	5.000	10.000	10.000	5.000				
412	saline	12.5%	20.000	20.000	25.000	10.000	10.000	10.000				
413	bleomycn	12.5%	10.000	10.000	10.000	20.000	10.000	5.000				
414	bleomycn	12.5%	20.000	25.000	20.000	20.000	10.000	10.000				
415	bleomycn	12.5%	5.000	10.000	5.000	5.000	5.000	10.000				
416	saline	15%	5.000	10.000	10.000	5.000	5.000	5.000	10.000	10.000	5.000	5.000
417	saline	15%	5.000	10.000	10.000	5.000	20.000	5.000	10.000	10.000	5.000	5.000
418	saline	15%	0.000	5.000	0.000	5.000	0.000	5.000	5.000	5.000	5.000	5.000
419	bleomycn	15%	5.000	15.000	10.000	10.000	5.000	10.000	5.000	5.000	5.000	5.000
420	bleomycn	15%	5.000	25.000	20.000	10.000	20.000	10.000	10.000	10.000	5.000	5.000
421	bleomycn	15%	20.000	15.000	10.000	10.000	5.000	10.000	5.000	5.000	5.000	5.000

Appendix A-1 Pathology score sheet

A degree of damage in the lung was carried out by scoring its degree of severity by blinded pathologists who were not aware of the treatment. They scored by subjective comparisons with the normal tissue. The score ranged from 0 to 40, with the score 40 meaning the most extensive degree of damage.