

Protective Effect of Polyunsaturated Fatty Acids against Experimental Lung Injury Induced by Acute Ethanol Inhalation

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<http://dx.doi.org/10.13005/bpj/1672>

(Received: 15 April 2019; accepted: 16 May 2019)

Ethanol-induced diseases include oxidative mechanisms by which prolonged endoplasmic reticulum (ER) stress results in genesis and accumulation of cytotoxic total fatty acid ethyl esters (FAEEs, non-oxidative metabolites of ethanol). FAEEs participate in the pathogenesis of alcoholic lung disease. Polyunsaturated fatty acids (PUFA) offer a possible protective effect against damage induced by ethanol inhalation. The present study aimed to investigate the protective effect of flaxseed and fish oils administration against toxicity induced by ethanol inhalation. Forty healthy female albino rats were divided into four groups (control, ethanol, flaxseed and fish oils). Lung superoxide dismutase (SOD) and malondialdehyde (MDA) were measured. Plasma advanced oxidation end product (AOPP) and phosphatidylinositol 3-kinase (PI₃K) were determined. Erythrocyte membrane fatty acids were extracted and fractionated by HPLC. Ethanol inhalation results in significant increase in lung MDA, plasma AOPP and erythrocyte membrane arachidonic acid (AA), linolenic acid (LA), and oleic acid (OA) along with a significant decrease in erythrocyte membrane alpha-linolenic acid (ALA), lung SOD, and plasma PI₃K while pretreatment with flaxseed and fish oils daily (1.2 ml/kg) significantly attenuated these parameters. Supplementation of marine PUFAs reduced the oxidative stress that induced by ethanol inhalation in experimental animals.

Keywords: Arachidonic acid, HPLC, Phosphatidylinositol 3-kinase, Advanced oxidation protein product, Cell membrane.

Chronic administration of alcohol is one of the most prevalent and expensive type of drug abuse with significant multi-systemic pathophysiological consequences¹. Hasin *et al.* reported that about 7 percent of the U.S. adult population attains the diagnostic standard for alcohol abuse and/or alcoholism².

Alcohol is a major component of many products such as solvents, antifreeze, varnish, shellac, canned fuel, and industrial and commercial cleaners. Alcohol toxicity occurs from

various routes of administration including dermal application, inhalation, or methanol ingestion. Obviously, exposure to alcohol via any course leads to significant metabolic acidosis, ocular, central nervous system and respiratory alterations³.

Vapor inhalation is considered as a modality of alcohol administration that stimulates alcohol reliance in rodents⁴. Romero *et al.* (2014) found increase in oxidative stress and inflammatory markers in rats' lung were fed chronically fed Lieber-De Carli ethanol liquid diets⁵.

Moderate exposure to alcohol results in metabolic and immunologic impacts. Chronic alcohol consumption, formation and aggregation of ethanol metabolites in the lungs results in lung and airway disorders, including asthma and pneumonia and consider a potential hazard agent for lung infection ⁶.

Lung parenchyma is believed to be a subject of infectious and environmental factors; this sensitiveness is increased in the state of reduced or abnormalities of innate or adaptive immunity. Ethanol and its metabolites affect redox homeostasis, antioxidants, and immune defense and lead to immunosuppression thus accelerating the lung infection ⁶.

n-3 polyunsaturated fatty acids (PUFAs) are a group of structurally related FAs. ALA is an 18-carbon n-3 PUFA and is a precursor of the other n-3 PUFAs can be produced through a series of metabolic steps. LA and ALA are considered to be an essential nutrient for humans and most animals. DHA is the final end product of ALA elongation and desaturation ⁷.

Hussein *et al.* demonstrated that integration of omega-3 fatty acids modulate inflammatory and immune reactions, it is considered as a potential therapeutic agent for inflammatory and autoimmune diseases. In addition n-3 PUFA inhibits generation of transforming growth factor- β (TGF- β) due to n-3 PUFA-derived lipid mediators ⁸.

According to these mechanisms, we aimed to determine the possible protective effect of PUFAs against lung toxicity induced by ethanol inhalation in experimental animal.

MATERIALS AND METHODS

Materials

Chemicals

Ethanol and standards of fatty acids, chloroform, methanol and ethyl ether are all HPLC grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA). 100% natural cold pressed flaxseed oil was purchased from Imtenan Company (El Obour City 1st Industrial Area, Egypt). Fish oil was purchased from SEDICO Pharmaceutical Company (6 October City, Egypt).

Ethanol consumption

Rats were kept in cages and exposed to

5000 ml/m³ ethanol for duration of 15 minutes daily for 14 days, the dose was modified from Mullin and Krivanek ⁹.

Flaxseed oil and fish oil administration

Flaxseed and fish oils were administered (1.2 ml/kg body weight) daily for two months by gavage according to Hussein *et al.* ⁸.

Animals

Forty Wistar female albino rats (180 \pm 20 g) purchased from Animal House, National Research Centre (NRC), Giza, Egypt. Animals were kept in clean cages of polypropylene and maintained in controlled room temperature (37° C) with light and dark cycle (12h:12h), given a standard diet and water *ad libitum* along the experimental period. Animal procedures were performed in accordance with the Ethics Committee of the National Research Centre and followed the recommendations of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

Methods

Experimental design

Rats were assigned into four groups (10 rats each) as follows:

Group (I): Control group: Healthy rats received saline.

Group (II): Ethanol group: Healthy Rats exposed to ethanol for 14 days.

Group (III): Flaxseed group: Healthy Rats received flaxseed oil (1.2 ml/kg body weight) daily for two months by oral gavage then exposed to 5000 ml/m³ ethanol for duration of 15 minutes daily for 14 days, the dose was modified from Mullin and Krivanek (1982) ⁹.

Group (IV): Fish oil group: Healthy Rats received fish oil (1.2 ml/kg body weight) daily for two months by oral gavage then exposed to 5000 ml/m³ ethanol for duration of 15 minutes daily for 14 days, the dose was modified from Mullin and Krivanek ⁹.

After finalizing the experiment; rats were kept fasting overnight (12 hour). Under anesthetic blood withdrawn in heparinized tubes; centrifuged (at 3000 rpm) using cooling centrifuge (Laborzentrifugen, 2K15, and Sigma, Germany); plasma was separated and stored at -20 °C for biochemical analysis. Packed red blood cells were used for estimation of erythrocyte membrane fatty acids fractions. Lung was removed from

each rat quickly; washed with ice-cold saline, homogenized in phosphate buffer (pH 7.4), and centrifuged (at 4000 rpm) for 15 minutes at 4°C and the supernatant was separated for biochemical analysis ⁸.

Determination of lung superoxide dismutase (SOD) and malondialdehyde (MDA)

Lung SOD and MDA levels were determined colorimetrically according to the methods described by Nishikimi *et al.* ¹⁰ Uchiyama and Yamaguchi ¹¹ respectively.

Determination of plasma advanced oxidation protein product (AOPP) and phosphatidylinositol 3-kinase (PI₃K)

Plasma AOPP and PI₃K were estimated by Enzyme-linked immunosorbent assay kits according to Deschamps -Latscha *et al.* ¹² and the manufacture kit.

Determination of erythrocyte membrane fatty acids by High Performance Liquid Chromatography (HPLC)

Sample treatment

Cell membrane was homogenized in 2 %

acetic acid- ethyl ether mixture (2:1 volume ratio). The solution was then filtered and centrifuged at 500 xg, the organic phase was evaporated to dryness. The extract was dissolved in 200 μl acetonitrile ¹³⁻¹⁴.

HPLC Conditions

HPLC system (Agilent technologies 1100 series) supplied by a quaternary pump (Quat Pump, G131A model) and C18 column (260 X 4.6, particle size 5 μm) was used. Mixture of acetonitrile / water (70/30) v/v used as mobile phase and delivered by isocratic elution with flow rate 1 ml / min and 200 nm wave length. Standards serial dilutions were injected and their peak areas were determined. A linear standard curve was constructed by plotting peak areas versus the corresponding concentrations. Samples concentration was obtained from the standard curve.

Statistical analysis

All data was expressed as mean ± SE. Distribution of the data was verified to be normal using Tests of Normality (SPSS package, version

Table 1. Lung SOD and MDA in different studied groups

Parameters Groups	SOD (U/g tissue)	MDA (nmol/g tissue)
Control	345.5±13	109.7± 4.9
Ethanol	182.7 ^a ± 16.8	220.2 ^a ±10.8
Flaxseed oil	271 ^{a,b} ± 8.5	151.5 ^{a,b} ±4.9
Fish oil	323.5 ^{a,b} ± 11.6	136.7 ^{a,b} ±3.7

P^a value significant difference compared to control group.
P^b value significant difference compared to ethanol group.

Table 2. Plasma AOPP and PI₃K in different studied groups

Parameters Groups	AOPP (ng/ml)	PI ₃ K (ng/ml)
Control	18.7±1.1	21.2±0.75
Ethanol	42.2 ^a ±1.5	10.3 ^a ±1.14
Flaxseed oil	21.5 ^b ± 0.86	19.2 ^b ±0.94
Fish oil	20.5 ^b ± 2.0	17.2 ^{a,b} ±1.25

P^a value significant difference compared to control group.P
P^b value significant difference compared to ethanol group.

Table 3. Erythrocyte membrane fatty acids in different studied groups

Parameters Groups	ALA (mg/ml RBCs)	AA (mg/ml RBCs)	LA (mg/ml RBCs)	OA (mg/ml RBCs)
Control	0.31±.08	0.29±.03	0.55±.24	0.25±.05
Ethanol	0.13 ^a ±.04	0.72 ^a ±.14	0.27 ^a ±.02	0.61 ^a ±.13
Flaxseed oil	0.40 ^b ±.07	0.31 ^b ±.09	0.23 ^a ±.14	0.39 ±.28
Fish oil	0.28 ^b ±.13	0.26 ^b ±.11	0.43 ±.25	0.13 ^b ±.03

P^a value significant difference compared to control group.
P^b value significant difference compared to ethanol group.

16). Statistical significance was tested by one way analysis of variance (ANOVA). For all tests a probability ($P < 0.05$) was considered significant.

RESULTS AND DISCUSSION

Severe ethanol exposure is a leading factor in the progression of liver, cardiovascular, and pulmonary diseases¹⁵. A lot of alcohol administration methods have been used in experimental animals to investigate the potential mechanisms of alcohol to induce alcoholic liver, lung, and cardiovascular diseases in addition to offering diverse merits to researchers, including the invalidation of rat's natural aversion to alcohol, and the reliable achievement of consistently high blood alcohol levels with relative ease¹⁶.

In this study, rats exposed to ethanol showed a significant decline in lung SOD with significant elevation in lung MDA (table 1).

Chronic ethanol consumption boosts the levels of activated transforming growth factor- β_1 (TGF- β_1) that activates NADPH oxidase (Nox). Nox₄ upregulates Nox₁ and Nox₂, causing generation of reactive oxygen species (ROS) and reduction in antioxidants thus impair the function of alveolar macrophages¹⁷.

In agreement with our results, D'Onofrio *et al.* reported that alcohol consumption depletes levels of antioxidant in the lungs which results in chronic oxidative stress, and then impairs alveolar macrophage functions. Thus, decreasing both cellular-based microbial lung clearance and alveolar macrophage cell viability and resulting in increase in oxidative stress¹⁸.

The current study showed the elevation of AOPP level in alcohol group compared to the control group. AOPP is a novel described protein marker formed under oxidative damage status and it is not only a marker for oxidative stress but also represent a modern class of pathogenic mediator via redox-dependent pathway in addition it is implicated in oxidation-associated diseases. AOPP is strongly associated with overexpression of Nox which is known as the major cytosolic source of ROS thus results in cell damage and involved in diseases progression¹⁹.

As were observed in this study. Indeed, PI₃K was significantly decreased in ethanol

group compared to the control group (table 2). Cederbaum, (2012) suggested that PI3K/Akt signaling has been described to activate NADPH oxidase (Nox), therefore producing ROS. Alcohol-associated oxidative stress in the lungs is associated with alcohol-driven changes in Nox enzymes functions and depletion of antioxidants including GSH and SOD that protect the cells against oxidative stress²⁰.

Liver alcohol dehydrogenase (ADH) and microsomal mono-oxygenases (cytochrome P450 2E1 isozyme) are part of ethanol oxidative detoxification. Over consumption of ethanol results in impairment of the detoxification mechanisms and related to production of acetaldehyde that binds to cellular proteins and DNA results in oxidation of proteins that enhancing oxidative stress²¹.

Kaphalia and Calhoun (2013) reported that ethanol oxidation, ER stress, and generation of FAEEs directly lung inflammation through bronchial circulation⁵. Cederbaum, (2012) reported another potential metabolic pathway in which chronic ethanol exposure motivate hepatic cytochrome P450 2E1, forming different profile of metabolites, which may have overlapping or distinct toxicities such as FAEEs²⁰. On the other hand, suppression of hepatic ADH expedite the FAEEs formation *through* non-oxidative metabolism of ethanol catalyzed by FAEE synthase, which occurs in target organs including lungs of mammals exposed to ethanol²¹.

Our data revealed that rats exposed to ethanol showed a significant decrease in erythrocyte membrane ALA and LA along with increase in AA and OA compared to the control group table (3).

Being a major component of cell membrane, decreased amount of PUFAs affects cell surface and intracellular receptors and thus impairs the anti-inflammatory gene expression. PUFAs are critical factor in lung inflammation in COPD patients²².

Our results suggested that prophylactic treatment with flaxseed and fish oils significantly ameliorate oxidative stress and inflammatory markers compared to the ethanol group.

Omega-3 and omega-6 fatty acids have competitive interactions that are critical in modifying inflammation²³. Studies showed

an inverse association between dietary intake of PUFAs and systemic inflammation across different populations including people with chronic obstructive pulmonary disease (COPD) ²⁴.

Since the mechanisms of inflammation comprise activation of both nuclear factor κ B (NF- κ B) and peroxisome proliferator agonist receptors (PPAR) which known as a ligand-activated transcription factor. Interestingly, it was found that n-3 PUFAs binding effectively to PPAR. PUFAs-PPAR binds to peroxisome proliferator response elements on DNA to control gene expression, prevent the energizing of NF- κ B, which can prohibit genes encoding for inflammatory factors including interleukin 6 (IL-6) and tumor necrosis factor α (TNF- α). This explains the anti-inflammatory and antioxidant mechanisms of n-3 PUFAs ²⁵.

Titz *et al.* (2016) suggested a complex interplay between smoke exposures, lung disease, and systemic alterations in serum lipid profiles and reported that high dietary intake of anti-inflammatory ω -3 PUFAs was associated with a possible protective effect against smoking-related COPD ²⁶.

Several studies reported that supplementation of PUFAs attenuate cell membrane fatty acids disturbance and inhibit inflammation progression as well as oxidative stress ^{14, 27}.

CONCLUSION

We concluded that PUFAs may protect lung from toxicity induced by ethanol inhalation through incorporation into cell membrane thus increasing arachidonic acid and reducing protein glycation and oxidative stress.

ACKNOWLEDGMENT

Authors are grateful to National Research Centre, Egypt for supporting this work.

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