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A Voluntary Oral-feeding Rat Model for Pathological Alcoholic Liver Injury

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1	Introduction.....	12
2	Materials	12
2.1	Animals and Feeding	12
2.2	Assessment of Urine Alcohol	12
2.3	Blood Sample Collection	13
2.4	Tissue Fixation, Processing, and Histological Staining.....	13
2.5	Sirius Red Staining	13
2.6	Immunohistochemical Analysis.....	13
2.7	Biochemical Assay of Serum Alanine Aminotransferase (ALT)	14
2.8	Marker of Oxidative Stress (8-Isoprostane).....	14
2.9	Electrophoretic Mobility Shift Assay	14
2.10	Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).....	15
2.11	Western Blotting	15
2.12	Thiobarbituric Acid Reactive Substances (TBARS).....	17
2.13	Chymotrypsin.....	17
2.14	Endotoxin.....	18
2.15	CYP2E1	18
3	Methods.....	18
3.1	Induction of Fatty Liver, Necrosis, and Inflammation.....	18
3.2	Biochemical assay of ALT.....	20
3.3	Markers of Oxidative Stress.....	21
4	Notes	30
	References.....	30

Summary The variety of animal models used in the study of alcoholic liver disease reflects the formidable task of developing a model that replicates the human disease. We show that oral feeding of fatty acids derived from fish oil and ethanol induces fatty liver, necrosis, inflammation, and fibrosis. Together with the study of oxidative and nitrosative stress markers, cytokines, proteasome function, and protein studies, this model has provided an inexpensive and technically simple method of establishing pathological alcoholic liver injury.

Keywords Alcoholic liver injury; cytokines; rodents; nitric oxide.

1 Introduction

The wide variety of animal models used in the study of alcoholic liver disease (ALD) reflects the formidable task of developing an appropriate model that replicates the human prototype. A major advance in the study of ALD pathogenesis is the development of the intragastric ethanol infusion model, which uses an implanted long-term intragastric catheter for continuous infusion of ethanol and liquid diets. The epidemiologic association between different types of fat and alcoholic liver disease has prompted the study of different types of fatty acids in the development of ALD in the intragastric feeding rat model. Many of the pathological features of ALD in humans are achieved in rats fed ethanol intragastrically. Women develop ALD more rapidly than men, and liver injury progresses in women with alcoholic hepatitis, even after cessation or reduced drinking. Studies using the intragastric feeding rat model confirmed the observation of enhanced susceptibility of females to alcoholic liver injury. Taking advantage of the fact that (1) female rats are more susceptible to alcohol than males and (2) among the different fatty acids, those that are derived from fish oil cause the greatest degree of pathological liver injury, we developed a voluntary oral feeding model for alcoholic liver injury that reproduced many of the pathological and biochemical changes in alcoholic liver injury seen with the intragastric infusion model.

2 Materials

2.1 *Animals and Feeding*

1. Female Wistar rats weighing approx 175-190 g. Animals housed in cages for rats model no. 1291H (Tecniplast, Italy).
2. Preparation of 1 L of diet: 8% ethanol with 40% energy derived from ethanol, 30% from fat, 23% from protein, and 7% from dextrose. The source of fatty acids in the diet is fish oil (*1*).
3. The diet components 1 to 8 (Table 1) are added to a blender with 600 mL of water.
4. The components are mixed thoroughly.
5. The suspending agent, component 9 in Table 1, is added to the mixture and mixed.
6. Water is added up to 1 L and mixed.
7. The mixed diet is served inside a water bottle in the absence of chow.

2.2 *Assessment of Urine Alcohol*

1. Q.E.D. Saliva Alcohol Test (OraSure Technologies, Inc.).
2. 50-mL centrifuge tube (Iwaki Glass Co, Japan).
3. Mineral oil (Sigma-Aldrich Co., St. Louis, MO).

Table 1 Oral Feeding Model Diet Composition

Diet composition	Amount per 100mL diet
1. Ethanol, absolute (Merck, Germany)	7.24 mL
2. Fish oil (Sigma-Aldrich Co., USA)	3.66 mL
3. Mineral mix (Dyets Inc., USA)	0.93 g
4. Vitamin mix (Dyets Inc., USA)	0.26 g
5. Choline bitartrate (Dyets Inc., USA)	0.05 g
6. Dextrose (Dyets Inc., USA)	1.75 g
7. DL-Methionine (Bio-Serv, USA)	0.11 g
8. Lactalbumin hydrolysate (Bio-Serv, USA)	5.75 g
9. Suspending agent K (Bio-Serv, USA)	0.45 g

2.3 Blood Sample Collection

1. 15-mL centrifuge tube (Iwaki Glass Co, Japan).
2. Swing bucket centrifuge (Sigma-Aldrich Co.).

2.4 Tissue Fixation, Processing, and Histological Staining

1. Chloroform (Merck, Germany).
2. 70%, 80%, 90% 95% and 100% ethanol (Merck, Germany)
3. Neutral buffered formalin saline: 40% Formalin (100 mL), $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (4 g), Na_2HPO_4 (6.5 g), NaCl (9 g), distilled H_2O (900 mL).
4. Harris's Hematoxylin (Sigma-Aldrich Co.).
5. Eosin (Sigma-Aldrich Co.).
6. Toluene (Merck, Germany).
7. Permout medium (Fisher Scientific).
8. Poly-L-lysine (Sigma Bioscience).

2.5 Sirius Red Staining

1. Sirius Red F3B (Polysciences Inc.).
2. Ethanol (Merck).
3. Picric acid (BDH Laboratory Supplies, UK).
4. Hydrochloric acid (Merck).

2.6 Immunohistochemical Analysis

1. DAKO EnVision™ + System, Peroxidase (DAB) kit (DakoCytomation Denmark A/S, Denmark).
2. Ethanol (Merck).

Table 2 Antibodies Used in Immunohistochemistry

Target protein	Source	Dilution	Manufacturer
Inducible nitric oxide synthase	Rabbit polyclonal	1:100	Transduction Laboratories, USA
Endothelial nitric oxide synthase	Rabbit polyclonal	1:100	Santa Cruz Biotechnology, CA, USA
Cyclooxygenase-2	Rabbit polyclonal	1:200	Cayman Chemical, USA
Nitrotyrosine	Mouse polyclonal	1:200	Zymed Laboratories Inc., USA

3. TBS Buffer: 0.05 M Tris-HCl pH 7.6; 0.15 M NaCl.
4. Trypsin (Sigma Bioscience).
5. Calcium Chloride (Merck).
6. Normal serum (Vector Laboratories).
7. BSA solution: 1% bovine serum albumin (BSA), 0.05 M TBS.
8. Primary antibodies against (Table 2).

2.7 Biochemical Assay of Serum Alanine Aminotransferase (ALT)

1. ALT Substrate: L-alanine (80 mM), nicotinamide adenine dinucleotide (NADH; 0.2 mM) and lactate dehydrogenase (LDH; 2 units) in potassium phosphate buffer (0.1 M, pH 7.4)
2. α -ketoglutaric acid (10 mM; Sigma-Aldrich Co).

2.8 Marker of Oxidative Stress (8-Isoprostane)

1. 8-isoprostane Competitive Enzyme Immunoassay kit (Cayman Chemical).

2.9 Electrophoretic Mobility Shift Assay

1. Gel Shift Assay Systems (Promega Corporation).
2. Consensus oligonucleotide (Promega Corporation).

NF- κ B: 5'-AGTTGAGGGGACTTCCCCAGGC-3'
3'-TCAACTCCCCCTGAAAGGGTCCG-5'

AP-1: 5'-CGCTTGATGAGTCAGCCGGA-3'
3'-GCGAACTACTCAGTCGGCCTT-5'

3. [γ - 32 P]ATP (Amersham Bioscience Ltd, UK).
4. T4 Polynucleotide Kinase (Promega Corporation).
5. TE buffer (10mM Tris-HCl, pH 8.0; 1mM ethylene diamine tetraacetic acid [EDTA]).
6. 10 \times Reaction buffer: 40% glycerol, 200mM Tris-HCl pH 7.8, 1M NaCl, 50mM MgCl₂, 10mM EDTA, 50mM dithiothreitol.
7. Loading dye: 250mM Tris-HCl, pH 7.5, 0.2% bromophenol blue, 40% glycerol.
8. 4% nondenaturing acrylamide gel prepared in TBE buffer (10 \times TBE buffer (890mM Trisbase, 890mM boric acid, 2mM disodium EDTA).
9. Aquasol (Packard Instrument Company, Inc.).
10. Antibodies for supershift assay (Table 5).
11. TEMED (Sigma-Aldrich Co.).

2.10 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

1. NucleoSpin RNA II extraction kit (Clontech Laboratories, Inc.).
2. β -mercaptoethanol (Sigma-Aldrich Co.).
3. SuperScriptTM First-Strand Synthesis System (Life Technologies).
4. AmpliTaq Gold PCR kit (Roche Applied Science).
5. Primers (Invitrogen Inc.; Tables 3 and 4).
6. 0.05 μ g/mL ethidium bromide.
7. Bromophenol Blue and Xylene-Scyanol (Sigma-Aldrich Co.).

2.11 Western Blotting

1. Liver homogenizing Buffer 1: 250mM sucrose, 15mM NaCl, 5mM EDTA, 1mM EGTA, 0.15mM spermine, 0.5mM spermidine, 1mM dithiothreitol,

Table 3 Primers Used in RT-PCR

Target gene	Primer sequence
Inducible nitric oxide synthase	5'-GTGGTGACAAGCACATTTGG-3' 5'-GGCTGGACTTTTCACTCTGC-3'
Endothelial nitric oxide synthase	5'-GACCCTCACCGCTACAACAT-3' 5'-CACAGAAAGTGGGGGTATGCT-3'
Cyclooxygenase-2	5'-GGAGAGACTATCAAGATAGTGATC-3' 5'-ATGGTCAGTAGACTTTTACAGCTC-3'
Tumor necrosis factor- α	5'-ATGAGCACAGAAAGCATGATC-3' 5'-TACAGGCTTGTCACCTCGAATT-3'
Glyceraldehydes-3-phosphate dehydrogenase	5'-CCTTCATTGACCTCAACTACATGGT-3' 5'-TCATTGTCATACCAGGAAATGAGCT-3'

Table 4 Reaction Mixture and Temperature Profile of PCR

Target genes	Reaction mixture (per sample)		Temperature profile	
iNOS	10× PCR buffer	2.5 µL		
	25 mM MgCl ₂	2.5 µL	95°C	15 min
	10 mM dNTP	1.0 µL	95°C	1 min
	10 µM forward primer	0.5 µL	60°C	1 min
	10 µM reverse primer	0.5 µL	72°C	1 min
	Taq Polymerase	0.25 µL		37 cycles
	Milli-Q H ₂ O	13.75 µL	72°C	10 min
eNOS	CDNA	4.0 µL		
	10× PCR buffer	2.5 µL		
	25 mM MgCl ₂	2.5 µL	95°C	15 min
	10 mM dNTP	0.5 µL	95°C	1 min
	10 µM forward primer	0.5 µL	60°C	1 min
	10 µM reverse primer	0.5 µL	72°C	1.5 min
	Taq Polymerase	0.125 µL		35 cycles
COX-2	Milli-Q H ₂ O	17.375 µL	72°C	10 min
	cDNA	1.0 µL		
	10× PCR Buffer	2.5 µL		
	25 mM MgCl ₂	2.5 µL	95°C	15 min
	10 mM dNTP	0.5 µL	95°C	1 min
	10 µM forward primer	0.5 µL	60°C	1 min
	10 µM reverse primer	0.5 µL	72°C	1.5 min
TNF-α	Taq Polymerase	0.125 µL		37 cycles
	Milli-Q H ₂ O	17.375 µL	72°C	10 min
	cDNA	1.0 µL		
	10× PCR buffer	2.5 µL		
	25 mM MgCl ₂	1.5 µL	95°C	15 min
	10 mM dNTP	0.5 µL	95°C	1 min
	10 µM forward primer	0.5 µL	50°C	1 min
GAPDH	10 µM reverse primer	0.5 µL	72°C	1 min
	Taq Polymerase	0.125 µL		34 cycles
	Milli-Q H ₂ O	18.375 µL	72°C	10 min
	cDNA	1.0 µL		
	10× PCR buffer	2.5 µL		
	25 mM MgCl ₂	1.5 µL	95°C	15 min
	10 mM dNTP	0.5 µL	95°C	1 min
	10 µM forward primer	1.25 µL	55°C	1 min
	10 µM reverse primer	1.25 µL	72°C	1 min
	Taq Polymerase	0.15 µL		25 cycles
	Milli-Q H ₂ O	16.85 µL	72°C	10 min
	cDNA	1.0 µL		

15 mM Tris-HCl, pH 7.9, 60 mM KCl, 0.1% leupeptin [Leu], 0.1% aprotinin [Apr]; 0.5% phenylmethylsulfonyl fluoride [PMSF]).

2. Buffer 2: 10 mM Hepes, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.1% Leu, 0.1% Apr; 0.5% PMSF.
3. Buffer 3: 0.5 mM Hepes, pH 7.9, 0.75 mM MgCl₂, 0.5 mM EDTA, 0.5 mM KCl, 12.5% glycerol, 0.1% NP-40, 0.1% Leu, 0.1% Apr, 0.5% PMSF.

Table 5 Antibodies Used in Western Blotting

Antibody against	Source	Dilution	Manufacturer
Inducible nitric oxide synthase	Rabbit (Polyclonal)	1:500	Transduction Laboratories, USA
Endothelial nitric oxide synthase	Rabbit (Polyclonal)	1:1000	Santa Cruz Biotechnology, CA, USA
Cyclooxygenase-2	Rabbit (Polyclonal)	1:500	Cayman Chemicals, USA
Nitrotyrosine	Mouse (Monoclonal)	1:1000	Zymed Laboratories Inc, USA
Beta-actin	Mouse (Monoclonal)	1:5000	Sigma Bioscience, USA

4. Buffer 4: 10mM Tris-HCl, pH 7.9, 5mM MgCl₂, 1mM EDTA, 10mM KCl, 20% glycerol, 0.1% NP-40.
5. Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Inc.).
6. 2× sample buffer: 0.1M Tris-HCl, pH 6.8, 20% glycerol, 4% sodium dodecyl sulfate [SDS], 0.2% bromophenol blue, 5.25% β-Mercaptoethanol.
7. SDS polyacrylamide gel: for a 10% gel: 0.6M Tris-HCl, pH 8.8, 10% polyacrylamide, 0.05% SDS, 0.05% ammonium persulfate, 0.1% TEMED.
8. Stacking gel: 0.2M Tris-HCl, pH 8.8, 4% polyacrylamide, 0.05% SDS, 0.07% ammonium persulfate, 0.1% TEMED.
9. Tank buffer: 0.025M Tris, 0.192M glycine, 0.1% SDS, pH 8.3.
10. Transfer buffer: 25mM Tris, pH 8.3, 192mM glycine, 20% methanol, 0.1% SDS.
11. Tween Tris-buffered saline (TBST), pH 7.5: 100mM Tris-HCl, 0.9% NaCl, 0.1% Tween-20.
12. Antibodies (Table 5).
13. Secondary antibodies (1:2000 dilution; DAKO Corporation).
14. ECL™ Western Blotting Detection Reagents (Amersham Pharmacia Biotech).

2.12 Thiobarbituric Acid Reactive Substances (TBARS)

1. Tris-KCl buffer: 50mM Tris-HCl, 154mM KCl, pH 7.4.
2. Trichloroacetic acid.
3. Thiobarbituric acid.

2.13 Chymotrypsin (2)

1. 0.1M Tris-HCl, pH 7.5.
2. Stop solution: 1% SDS and 1mL of 0.1M Tris-HCl, pH 9.1.

2.14 Endotoxin

1. Limulus amoebocyte lysate test (Kinetic-QCL).

2.15 CYP2E1

1. CYP2E1 substrate buffer: 0.45 ml of 0.1 M phosphate buffer, pH 7.4, with 8 mM aniline and 1 mM NADPH.
2. 40% Trichloroacetic acid.
3. 10% Na₂CO₃.
4. 2% Phenol.

3 Methods

3.1 Induction of Fatty Liver, Necrosis, and Inflammation

Prepare ethanol and iso-caloric dextrose control diets fresh daily (*see Note 1*). Female Wistar rats are allowed free access to the ethanol-containing diet or pair-fed isocaloric dextrose control diets for 8 wk (*see Note 2*).

3.1.1 Measurement of Urine Alcohol (*see Note 3*)

1. A urine sample is collected between 2100 and 0900 h in a 50-mL tube with 1 mL of mineral oil to prevent evaporation.
2. The cotton-tipped end of the collector is placed into the urine sample until fully soaked.
3. The collector is inserted into the entry port with gentle and steady pressure until the pink fluid flows past the QA Spot™ at the end of the QED device.
4. After the bar stops moving on the scale bar, the alcohol concentration of the sample can be read.

3.1.2 Assessment and Processing of Blood, Liver Samples, and Histological Sections

3.1.2.1 Processing of Blood, Liver Samples, and Histological Sections

1. Blood samples are incubated at room temperature for 30 min.
2. The samples are centrifuged at 17,000 g for 15 min at 4°C.

3. The upper layer of serum is collected and divided into aliquots and stored at -80°C .
4. The liver tissues are divided into two parts: one portion for snap frozen and stored at -80°C , and another portion is cut into tiny blocks for fixation in formalin.

3.1.2.2 Tissue Fixation

1. Tissue blocks are incubated in 4% neutral buffered formalin for 3 d.
2. The tissues are washed in running water for 3 h.
3. The tissues are immersed in 70% ethanol for overnight.
4. The fixed tissues are dehydrated in ascending percentage of ethanol (80% ethanol for 30 min; 90% ethanol for 30 min; 95% ethanol for 30 min at three exchanges; 100% ethanol for 45 min at three exchanges).
5. The dehydrated tissues are immersed in chloroform overnight at room temperature.
6. Wax is infiltrated into tissues in two different wax-baths with each exchange for 1 h.
7. The tissues are immersed in another wax-bath for another hour under vacuum.
8. The tissues are embedded in paraffin wax.

3.1.2.3 Tissue Sectioning

1. The tissues embedded in paraffin blocks are sectioned at a thickness of $5\text{ }\mu\text{m}$.
2. The tissue sections are flattened in a 40°C waterbath.
3. The tissue sections are mounted on a clean glass slide coated with poly-L-lysine.
4. Tissue sections are dried in a 60°C oven to melt the excess paraffin wax and transferred to a 37°C oven for drying overnight.

3.1.2.4 Hematoxylin and Eosin (H&E) Staining

1. The paraffin sections are dewaxed and rehydrated by immersing the sections in toluene (twice for 5 min each time).
2. The sections are immersed in a series of descending percentages of ethanol (100% ethanol for 1 min at two exchanges; 95% ethanol for 1 min at two exchanges; 70% ethanol for 1 min).
3. The rehydrated sections are washed in running tap water for 5 min and then immersed in distilled water.
4. The rehydrated sections are stained with Harris's hematoxylin for 5 min and then washed in running tap water for 2 min.
5. The overstained hematoxylin sections are differentiated by a quick bath in acid alcohol followed by Scott's tap water for 1 min.
6. The cytoplasm of the cells in the tissues is stained with 1% eosin solution for 3 min and then is given a quick wash in tap water.

7. The sections are dehydrated in ascending percentages of ethanol (70% ethanol for 10 s; 95% ethanol for 15 s; 95% ethanol for 1 min; 100% ethanol for 2 min at two exchanges; 100% ethanol for 5 min).
8. The slides are transferred to toluene (twice at 5 min each) and then mounted with Permount medium.

3.1.2.5 Quantification of the Necrosis Using Image Analysis

1. The percentage of necrotic area was measured by the Leica QWIN Image Analyzer (Leica Microsystems Ltd., Milton Keynes, UK).
2. The H&E-stained sections were captured with a CCD JVC camera connected to a Zeiss Axiophot microscope at 20× objective.
3. One block per animal and one section per block were chosen for quantification. Five fields were chosen randomly and captured from each section.
4. The percentage of necrosis was expressed as the average of the results obtained from each group by dividing the sum of lightly stained necrotic areas with the sum of the reference field multiplied by 100 (3).
5. The severity of liver pathology was assessed as steatosis (the percentage of liver cells containing fat): 1+, ≤25%; 2+, 26–50%; 3+, 51–75%; 4+, >75%. (4).
6. Necrosis was evaluated as the number of necrotic foci per square millimeter, inflammation was scored as the number of inflammatory cells per square millimeter. At least three different sections were examined per sample of liver (4).

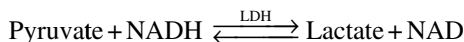
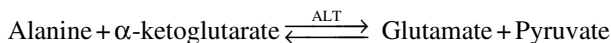
3.1.2.6 Sirius Red Staining and Quantification of Collagen

The collagens are the basic components of connective tissue, and they can be visualized with Sirius Red staining (see **Note 4**).

1. The fixed and sectioned tissues are dewaxed and rehydrated.
2. The tissues are stained with 0.1% picro-Sirius Red in saturated aqueous picric acid for 1 h.
3. The tissue sections are then differentiated in 0.01% hydrochloric acid for 30 min.
4. The stained slides are immediately dehydrated and mounted.

3.2 *Biochemical assay of ALT*

The degree of necrosis in the liver tissue is assessed by the amount of serum level of ALT. ALT is a biochemical marker of the liver. A high level of circulating ALT indicates a high degree of liver necrosis and injury.



1. 50 μL of serum is added to a cuvette containing 1 mL of ALT substrate.
2. The mixture is incubated at 37°C for 3 min in a thermostated cuvette compartment.
3. The basal rate of NADH consumption is recorded at the wavelength of 340 nm for 2 min.
4. The rate of utilization of NADH by serum alanine aminotransferase was initiated by adding the α -ketoglutaric acid (10 mM).
5. The change in absorbance was measured for a period of 2 min.
6. The activity of alanine aminotransferase was calculated by applying the following equation:

$$\text{ALT activity (U/L)} = [\text{Abs}_{340\text{nm}}/\text{min} \times 1000 \times d \times l] / 6.22 \times 0.05$$

where $\text{Abs}_{340}/\text{min}$ = change of absorbance at 340 nm per minute, 1000 = conversion factor, d = dilution factor, l = light path length of cuvette = 1 cm, $6.22 \text{ mM}^{-1}\text{cm}^{-1}$ = extinction coefficient of NADH at 340 nm, 0.05 mL = volume of serum sample, and U/L = international unit/liter.

3.3 Markers of Oxidative Stress

3.3.1 Assay on Serum total 8-Isoprostane Level

The serum level of total 8-isoprostane was determined by a Competitive Enzyme Immunoassay kit (*see Note 5*).

1. Total (free and esterified) 8-isoprostane is obtained from the serum samples by mixing 3 μL of sample with 12 μL EIA buffer and then a 15 μL of 15% wt/vol potassium hydroxide (KOH) is added.
2. The mixture is incubated in a 40°C water bath for 1 h.
3. 30 μL of potassium dihydrogen phosphate (KH_2PO_4) (1 M) is added, followed by the addition of 90 μL of EIA buffer to a final dilution of 1:50.
4. To prepare the standards for assay, 100 μL of 8-isoprostane standard is transferred with an ethanol-equilibrated pipet tip to a clean Eppendorf as stock.
5. The stock is then diluted with 900 μL of ultrapure water to a final concentration of 5 ng/mL.
6. A series of eight standards is prepared by transferring 900 μL of EIA buffer to the tube labeled with #1 and 500 μL of EIA buffer to tubes #2 to #8.
7. 100 μL of standard from stock is transferred to tube #1 and mixed thoroughly.

8. A 500- μ L aliquot is transferred from tube #1 to #2 and then from tube #2 to tube #3. This step was repeated until tube #8.
9. To wells marked with NSB (nonspecific binding) of the 96-well plate coated with mouse anti-rabbit IgG provided with the kit, 100 μ L of EIA buffer is added and 50 μ L of EIA buffer is added to wells labeled with B0 (maximum binding).
10. 50 μ L of standards and samples are added to the wells accordingly.
11. To the wells labeled with total activity (TA) and blank (Blk), 50 μ L of 8-isoprostane AchE tracer and 50 μ L of 8-isoprostane antiserum is added, whereas only 50 μ L of 8-isoprostane Antiserum is added to the wells labeled NSB.
12. The plate is then covered and incubated at room temperature for 18 h.
13. The content is then emptied and rinsed five times with wash buffer.
14. 200 μ L of Ellman's Reagent is added to each well and 5 μ L of tracer is added to, T. A.
15. The plate is then covered with a plastic film and allowed to develop in the dark with gentle agitation for 60 min.
16. The final absorbance at 405 nm is measured by a microplate reader.

The amount of tracer detected is inversely proportional to the amount of free 8-isoprostane.

$$\text{Absorbance} \propto [\text{Bound 8-isoprostane Tracer}] \propto 1/[\text{8-isoprostane}]$$

A standard curve is plotted with the percentage of tracer binding ($\%B/B_0$) against the concentration of 8-isoprostane. The percentage of tracer binding is obtained by the following equation:

$$\%B/B_0 = [(x - \text{NSB})/B_0] \times 100$$

where x = Absorbance of individual standards or serum samples, NSB = nonspecific binding, and B_0 = maximum binding.

The concentration of total 8-isoprostane expressed as picogram per milliliter (pg/ml) was calculated by substituting the absorbance of each sample into the equation and determined the concentration by applying the standard curve.

3.3.2 Thiobarbituric Acid Reactive Substances (TBARS)

1. The liver tissue is homogenized in 5 mL of Tris-KCl buffer.
2. 1 mL of 20% trichloroacetic acid and 2 mL of 0.67% (w/v) thiobarbituric acid are added to 2 ml of liver homogenate.
3. The mixture is heated in boiling water bath for 10 min.
4. The mixture is centrifuged to precipitate the protein.

The absorbance of the supernatant at 530 nm is recorded by a spectrophotometer using a molar extinction coefficient of malonaldehyde of $1.56 \times 10^5 \text{ cm}^2/\text{mmol}$.

3.3.3 Endotoxin

1. A blood sample is collected in endotoxin-free vials.
2. The blood is centrifuged at 400g for 15 min at 4°C.
3. The sample is diluted 1:10 in pyrogen-free water and heated at 75°C for 30 min to remove the inhibitors of endotoxin from plasma.
4. The sample is incubated at 37°C for 10 min with limulus amoebocyte lysate. (Limulus amoebocyte lysate test)
5. The substrate solution is added to the mixture and incubated for 20 min.
6. The reaction is stopped by adding 25% acetic acid.
7. The absorbance of the sample at 410 nm is read by a spectrophotometer.

3.3.4 Chymotrypsin (2)

1. Liver tissue is homogenized to obtain the cytosolic protein fraction. Protein sample of 20 to 200 µg and a peptide substrate concentration of 40 µM are incubated in 0.1 M Tris-HCl (pH 7.5) (total reaction volume is 0.2 mL) at 37°C with continuous shaking for 60 min.
2. The assay is stopped by adding 300 µL of stop solution.
3. The fluorescence of the leaving group, 4-amino-methyl-coumarin (AMC) for N-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (LLVY-AMC) and N-t-Boc-Leu-Ser-Thr-Arg-7-Amido-4-methylcoumarin (LSTR-AMC) is measured at the excitation and emission wavelengths of 390 nm and 440 nm. The fluorescence of β-Naphthylamine (LLE-NA hydrolysis) is measured at the excitation and emission wavelengths of 335 nm and 410 nm.

3.3.5 CYP2E1 (Aniline Hydroxylase Assay) (5,6)

1. Liver microsomes (25 µg) are incubated at 37°C for 1 h in CYP2E1 substrate buffer.
2. The reaction is stopped by adding 90 µL of 40% trichloroacetic acid.
3. The samples are incubated on ice for 10 min and then centrifuged for 10 min.
4. 0.36 mL of the supernatant is mixed with 0.24 mL of 10% Na₂CO₃ and 0.36 mL of 2% phenol.
5. The mixture is incubated in the dark for 45 min.
6. The absorbance of the samples is recorded at 630 nm.
7. The activity of the samples is deduced from a standard curve.

3.3.6 Immunohistochemical Analysis

Protein expression of iNOS, eNOS, COX-2, and the formation of nitrotyrosine in the liver sections are detected by immunohistochemistry. A DAKO EnVision™+ System, Peroxidase (DAB) kit (DakoCytomation Denmark A/S, Glostrup, Denmark) is used.

1. The sectioned tissues are dewaxed by immersing the sections in toluene (5 min for two exchanges).
2. The tissue sections are rehydrated in a series of descending percentage of ethanol (100% ethanol for 1 min at two exchanges; 95% ethanol for 1 min at two exchanges; 70% ethanol for 1 min).
3. The sections are rinsed in running tap water.
4. Sections are then rinsed with TBS buffer.
5. Proteolytic digestion is carried out by incubating the sections in 0.05 M TBS with 0.1% Trypsin and 0.1% CaCl_2 for 5 min at room temperature.
6. Sections are washed with TBS three times.
7. Peroxidase is added to each section and incubated for 5 min and then washed.
8. Blocking solution of 10% v/v normal serum in BSA solution is applied to each section and incubated for 1 h at room temperature.
9. Primary antibody is added to each section except the negative control and incubated at 4°C overnight. (All the primary antibodies were diluted with 1% BSA to the optimum concentration. The primary antibodies and the dilutions used in immunohistochemistry are listed in Table 1).
10. Negative control sections are incubated with normal serum.
11. Sections are washed with TBS for three times, 5 min each.
12. Peroxidase-labeled polymer HRP is added to each section and incubated at room temperature for 30 min.
13. The unbound polymer on the sections is washed with TBS for three times, 5 min each.
14. DAB+ substrate-chromogen solution is added and allowed to develop.
15. The sections are then washed in distilled water and counterstained with Harris's Hematoxylin for 5 s.
16. The stained sections are dehydrated in ascending percentage of ethanol from 70% to 100% and finally in toluene.
17. The slides were mounted with Permount medium.

3.3.7 RT-PCR and Western Blotting of Tissue Samples

3.3.7.1 Semiquantitative Analysis on Gene Expression

3.3.7.1.1 Total RNA Extraction

1. Total RNA is extracted from the liver tissues by using the NucleoSpin RNA II extraction kit.
2. 10 mg of tissue is placed in 400 µl lysis buffer (Buffer RA1) containing 4 µl of β -mercaptoethanol.
3. The tissue is homogenized with a homogenizer on ice.
4. 250 µl of 95% ethanol is added to the lysate and mixed by vortexing and added to a NucleoSpin column.

5. The column with lysate is centrifuged at 13,000rpm for 1 min and the flow through is discarded.
6. The DNA in the lysate is removed by adding 95 μ L of diluted DNase-I reaction mixture and incubated at room temperature for 15 min.
7. The reaction is stopped by adding 500 μ L of Buffer RA2 and then centrifuged at 13,000rpm for 1 min.
8. After the flow through was discarded, the column is washed by adding Buffer RA3 twice (600 μ L of RA3, centrifuged at 13,000rpm for 1 min; 250 μ L of RA3, centrifuged at 13,000rpm for 2 min).
9. The RNA is then eluted twice by adding 30 μ L of RNase-free water and stood at room temperature for 15 min.
10. The RNA is collected in the collection tube by centrifuging at 13,000rpm for 1 min.
11. The concentration of the RNA samples is measured by Gene Quant II Spectrophotometer.
12. The RNA samples are stored at -80°C until use.

3.3.7.1.2 RT-PCR

This is a semiquantitative analysis method to study the expression of messenger RNA level of the genes by amplifying the complementary DNA made from the corresponding RNA. In this part of experiment, total RNA was extracted from the liver tissue and based on the RNA obtained, a complementary DNA was prepared for polymerase chain reaction. The manufacturing of the mRNA of a gene was caused by the trigger of that specific gene and the subsequent making of protein. Thus, the level of expression of mRNA revealed the transcription level of the gene.

3.3.7.1.3 Preparation of Complementary DNA From RNA

1. The complementary DNA is prepared based on the corresponding RNA by SuperScript™ First-Strand Synthesis System for RT-PCR kit.
2. For each sample, 2 μ g of RNA is diluted with RNase-free water to a final volume of 10 μ L in a microtube.
3. 1 μ L of oligo(dT) and 1 μ L of 10 mM dNTP are added to the tube.
4. The mixture is incubated at 65°C for 5 min.
5. The mixture is chilled on ice for 3 min.
6. 9 μ L of reaction mixture (10 \times First-Strand RT Buffer, 2 μ L; 25 mM MgCl_2 , 4 μ L; 0.1 M DTT, 2 μ L; RNaseOUT Recombinant RNase Inhibitor, 1 μ L) is added to the tube and mixed thoroughly.
7. The tube is then incubated at 42°C for 2 min.
8. 1 μ L (50 units) of SuperScript II RT is added to the mixture and incubated at 42°C for 50 min.
9. The reaction is terminated by incubating at 70°C for 15 min.
10. The final cDNA is stored at -80°C .

3.3.7.1.4 Polymerase Chain Reaction

After obtaining the complementary DNA, the genes of interest are amplified by polymerase chain reaction on the specific primers. Sense and antisense primers for the genes (GAPDH, iNOS, eNOS, COX-2, and tumor necrosis factor- α) are designed by the online software provided by Whitehead Institute for Biomedical Research and are manufactured by Invitrogen Inc. The detailed panel for reaction mixture and thermal cycle for PCR and the specific primers used in RT-PCR are shown in Tables 3 and 4.

The final PCR products are loaded to a 2% agarose gel containing 0.05 μ g/mL ethidium bromide. The intensity of the PCR products was quantified by an image analyzer software Image J (National Institutes of Health, Bethesda, MD).

3.3.7.2 Protein Expression Analysis by Western Blotting

3.3.7.2.1 Total Protein Extraction

3.3.7.2.1.1 Cytosolic Protein Extraction

1. Liver tissue (100mg) is placed in a 5-mL tube containing 500 μ L of ice-cold Buffer 1
2. The tissue is then homogenized with a homogenizer and followed by centrifugation at 5,000rpm for 10 min at 4°C.
3. The pellet is saved for nuclear protein extraction.
4. The supernatant is transferred to a new 1.5 ml tube and centrifuged at 13,000 rpm for 20 min at 4°C.
5. The supernatant is divided into aliquots and stored at -80°C.

3.3.7.2.1.2 Nuclear Protein Extraction

1. The pellet saved from cytosolic protein extraction is resuspended in 500 μ L of Buffer 2.
2. The mixture is mixed thoroughly before centrifuged at 6000 rpm for 10 min at 4°C.
3. The pellet is resuspended in 400 μ L of nuclear Buffer 3 and mixed.
4. The suspension is then rocked on a rotor for 30 min at 4°C.
5. The samples are then centrifuged at 13,000 rpm for 30 min at 4°C.
6. The supernatant is then transferred to a dialysis tubing and dialyzed against 500 mL of Buffer 4 for three changes with continuous stirring at 4°C overnight.
7. The nuclear protein is collected and stored in aliquots at -80°C.

3.3.7.2.1.3 Protein Assay

The concentration of the cytosolic and nuclear protein samples were measured by Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Inc., Hercules, CA).

1. Protein standard is prepared by dissolving BSA in distilled water.
2. Serial dilution is performed to give a set of protein standards (0 mg/mL, 0.015625 mg/mL, 0.03125 mg/mL, 0.0625 mg/mL, 0.125 mg/mL, 0.25 mg/mL, 0.5 mg/mL, and 1 mg/mL).
3. The concentrated (5×) protein assay reagent is diluted with distilled water to working concentration (1×) before used.
4. The protein samples are diluted with distilled water to an optimum concentration which lay within the range of the protein standards.
5. 20 µL of standards or samples are added to a well of a 96-well plate in duplicate followed by adding 200 µL of diluted protein assay reagent.
6. The absorbance of each well is measured by a microplate reader at the wavelength of 570 nm.

3.3.7.2.1.4 Measurement of Protein Expression With Western Blotting

Western blotting is the analysis of the expression of a protein by the interaction between the protein and the corresponding antibody.

1. A fixed amount of protein (Table 6) of each sample is diluted with distilled water to a final volume of 10 µL.
2. 10 µL of 2× sample buffer is added to each sample.
3. The mixtures are mixed and then boiled at 99°C for 5 min before chilled on ice.
4. SDS polyacrylamide separation gel is prepared for the separation of different sizes of protein from the total protein extract.
5. A layer of stacking gel is prepared and settled on top of the separating gel.
6. The gel is placed in a tank filled with tank buffer.

Table 6 Conditions for Western Blot Analysis

	iNOS	eNOS	COX-2	Nitrotyrosine
Protein	Cytosolic	Cytosolic	Cytosolic	Cytosolic
Amount of protein (µg)	100	40	50	40
Percentage of acrylamide gel	7%	7%	10%	10%
Blocking time (hr)	1 h	1 h	1 h	6 h
Primary antibody manufacturer	Transduction Laboratories	Transduction Laboratories	Cayman Chemicals	Zymed Laboratories
Primary antibody concentration	1:500	1:1000	1:500	1:1000
Source of primary antibody	Rabbit (Polyclonal)	Rabbit (Polyclonal)	Rabbit (Polyclonal)	Mouse (Monoclonal)
Secondary antibody concentration	1:2000	1:2000	1:2000	1:2000
Film exposure time (min)	10 min	5 min	10 min	5 min

7. The chilled samples are loaded onto the wells on the stacking gel and run down the gel at a constant current of 30 mA.
8. The separated proteins on the gel are transferred onto an Immun-BlotTM PVDF Membrane in transfer buffer at a constant voltage of 20 V at 4°C overnight.
9. The membrane is removed from the transfer set up and washed briefly in TBST and incubated in blocking buffer (5% powdered nonfat milk in TBST).
10. The membrane is washed briefly with TBST followed by incubation with a primary antibody against the specific protein for overnight at 4°C with gentle agitation.
11. The antibody bound membrane is washed thoroughly with TBST for five changes with each change for 5 min.
12. The membrane is then incubated with secondary antibody for 2 h at room temperature with agitation (Table 6).
13. The washing procedure is repeated after incubation with secondary antibody.
14. Excessive washing buffer is removed and 0.5 mL ECLTM Western Blotting Detection Reagents is added to each membrane and incubated at room temperature for 1 min.
15. After removing excessive ECL reagent, the membrane is covered with an X-ray film and exposed in a cassette in the dark room.
16. The exposed X-ray film is developed in developer and fixer solutions.
17. The intensity of the bands were measured and quantified by Image J (National Institutes of Health).

3.3.8 Analysis of the DNA-Binding Activity of the Transcription Factors (NF- κ B and AP-1) With EMSA

The transcription of DNA into RNA requires the binding of transcription factors onto the specific site of the DNA. To assess the activity of these transcription factors, EMSA was performed using the Gel Shift Assay Systems.

1. Nuclear protein is obtained by the methods described in Western Blotting and followed by quantification of the amount of nuclear protein in the extract.
2. Specific consensus oligonucleotide of: NF- κ B for the binding of the corresponding transcription factor is firstly labeled with [γ -³²P]ATP through a phosphorylation reaction (2 μ L of consensus oligonucleotide (1.75 pmol/ μ L), 1 μ L of T4 Polynucleotide Kinase 10 \times Buffer, 1 μ L of [γ -³²P]ATP (3000 Ci/mmol at 10 mCi/mL), 5 μ L of Nuclease-free water, and 1 μ L of T4 Polynucleotide Kinase (5–10 U/ μ L).
3. The mixture is incubated at 37°C for 10 min.
4. The labeling process is stopped by adding 1 μ L of 0.5 M EDTA and diluted with 89 μ L of TE buffer.
5. The labeled oligonucleotide is then purified by passing through a Quick Spin Column for radiolabeled DNA purification and the unincorporated nucleotides are removed.
6. The buffer in the column is drained by gravity and then placed in a collection tube.

Table 7 Antibodies Used in Supershift Assay

Antibody	Source	Manufacturer
NF- κ B p50	Rabbit (Polyclonal)	Santa Cruz Biotechnology, CA, USA
NF- κ B p65	Rabbit (Polyclonal)	Santa Cruz Biotechnology, CA, USA

7. The column is centrifuged at 1100 g in a swing-bucket rotor for 2 min and the eluted buffer is discarded together with the collection tube.
8. The column is then placed in a new collection tube in an upright position.
9. The connection between the two tubes is sealed with a piece of parafilm.
10. The labeled probe is added to the tube carefully to the centre of the column.
11. The column is placed in a 15-mL centrifuge tube and centrifuged at 1100g for 2 min.
12. The purified probe is collected as the eluate after centrifugation and stored at -20°C afterwards.
13. The radioactivity of the labeled probes is measured by mixing 1 μL labeled oligonucleotide with 1 mL of Aquasol in a vial.
14. The radioactivity is then measured by LS-6500 Multi-purpose Scintillation Counter.
15. DNA binding reaction is then performed by adding 24 μg of each nuclear protein sample with 2 μL of 10 \times Reaction buffer, 2 μL of salmon sperm DNA (ssDNA), and 1 μL of labeled oligonucleotide (at least 50,000 cpm/ μL).
16. The mixture is brought to a brief vortex and incubated at room temperature for 20 min.
17. 2 μL of loading dye is added instead of protein sample as negative control.
18. The mixed samples together with the negative control are added to the wells of a 4% nondenaturing acrylamide gel, 6.8 mL 40% acrylamide solution and 10% ammonium persulfate and 49.7 mL of distilled water, the gel is pre-run at 100 V in 0.5 \times TBE buffer for 30 min).
19. The samples are run down the gel by applying an electric force at 100 V.
20. Supershift is done to verify the correctness of the binding of the nuclear protein in the experiment.
21. 24 μg of the nuclear protein is mixed with 3 μL of 10 \times Reaction Buffer, 2 μL of ssDNA, and 1 μL of antibody (NF- κ B p50 or NF- κ B p65; 200 $\mu\text{g}/\text{mL}$) against the target nuclear protein (Table 7).
22. The whole mixture is incubated at room temperature for 30 min before the addition of labeled oligomer and then incubated for another 20 min.
23. The supershift mixture is loaded to the gel with other samples.
24. The gel is then backed by a filter paper and wrapped with a piece of saran-wrap followed by drying in a gel dryer at 50°C for 30 min.
25. The signal of radioactivity of the binding is detected by exposing the gel to an X-ray film in a cassette at -80°C for 24 h.
26. The film is developed and the expression of the bands is quantified afterwards.

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4 Notes

1. The fish oil diet is kept under nitrogen at 4°C to avoid auto-oxidation of the fatty acids. Auto-oxidation causes a foul odor to develop in the diet. To overcome the reluctance of the animals to feed on the fish oil diet, we flavored the diet with chocolate powder. The chocolate had no effect on the biochemical or pathological alterations induced by ethanol. However, other investigators, using the same dietary regimen, have not used the chocolate flavoring and have not encountered any problems.
2. Because ethanol is a dietary nutrient that displaces other nutrients as a source of calories, the nutrient variables must be rigorously controlled. The pair-feeding regimen used in the oral-ethanol feeding model allowed for isocaloric pair-feeding.
3. Blood ethanol levels are monitored by daily measurement of 24-h urine ethanol content. We have ascertained that urine alcohol levels correlate well with blood alcohol levels. There is no cycling of blood or urine alcohol levels (compare intragastric model).
4. The Sirius Red stained collagen was classified into three parameters: total collagen, collagen along the central vein, and collagen in the pericellular area. The collagen deposited along the central vein was that between the endothelium and the adjacent hepatocytes. The pericellular area collagen was the extracellular matrix accumulated in the hepatic sinusoids away from the central vein. One block was chosen per animal. One section was chosen per block. Five fields from each slide were selected randomly and captured with CCD JVC camera with a Zeiss Axiophot microscope. The collagen stained with Sirius Red was quantified using LEICA Qwin Image Analyzer (Leica Microsystems Ltd., Milton Keynes, UK). The percentage of total collagen was calculated by the sum of the Sirius Red positive area divided by the sum of the reference area multiplied by 100. Similar method was used in the evaluation of the percentage of the collagen in the central vein. By subtracting the average value of the two parameters, the percentage of the collagen in the pericellular region was obtained.
5. This assay is based on the competition between the free 8-isoprostane and the 8-isoprostane acetylcholinesterase (AChE) conjugate (Tracer) for a limited amount of isoprostane-specific rabbit antiserum binding sites.

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