Preface

Alcohol consumption is often characterized as an environmental stress to the organism. In response to the stress of alcohol exposure, complex cellular and organismal adaptations occur to manage this insult. In most individuals, modest alcohol consumption over the course of a lifetime does not result in substantive health risks, and in the pathophysiology of some diseases, such as type 2 diabetes, modest alcohol consumption may actually be protective. Yet, chronic and heavy alcohol consumption poses significant health risks. Scientists who study the effects of acute and chronic alcohol consumption know little about what marks the transition from a benign or even protective effect of alcohol to its pathophysiological effect in the development of tissue injury and disease.

Understanding the transition to injury is an important clinical and public health issue, as excessive alcohol consumption can impact nearly every tissue in the body, contributing to more than 60 different medical conditions. The effects of excessive alcohol occur both in the developing organism as well as in the adult. Although the liver, as the site of ethanol metabolism, is particularly sensitive to chronic alcohol exposure, alcohol consumption also leads to damage of other tissues, including brain, heart, and cardiovascular system, as well as disruptions in regulation of endocrine and immune systems. The contribution of alcohol to the development of chronic diseases, such as osteoporosis, heart disease, and diabetes, is particularly relevant today, given the increased incidence of these diseases in our aging population.

Recent advances in understanding the pleiotropic effects of ethanol have been possible because of the development of relevant and rigorously controlled animal and cell culture models of acute and chronic ethanol exposure. Although each of the various models for ethanol exposure may not model perfectly the exposure of humans to alcohol, many model systems have now been developed that can mimic particular conditions of ethanol exposure in target tissues and organs. One of the primary goals of this volume is to provide detailed procedures for several of the more common models of acute and chronic ethanol exposure, enabling studies on the effects of ethanol in both the developing organism and in the adult. Use of these clearly defined models of ethanol exposure, presented in the first section of this volume, will allow for comparison of results among different laboratories, as well as among multiple tissue and organ targets of acute and chronic ethanol exposure.

One of the themes arising in recent studies that investigate the mechanisms of ethanol action on target tissues is the commonality in the impact of ethanol on regulation of cellular metabolism. Thus, in addition to the effects of acute and chronic

alcohol on the complex physiology of the intact organism, alcohol exposure also has a profound impact on the biology of individual cells. As with studies in whole animals, investigations to study the impact of ethanol on cellular biology must be rigorously controlled and designed. Recent advances in the development of specific methodologies to mimic the impact of ethanol metabolism in cultured cells, detailed in the second section of this volume, have furthered our understanding of the molecular mechanisms by which ethanol disrupts cellular function.

Although there are common mechanisms of ethanol action on a variety of cell types, studies of the effects of ethanol on cellular function must also take into consideration the complex differentiated function of individual cells and tissues. Thus, expertise in the use of models of ethanol exposure, as well as in the design and analysis of experiments to ascertain the effects of ethanol on the highly regulated function of each differentiated cell and tissue type, must be combined to finely dissect the mechanisms of ethanol action. Therefore, an additional theme of this volume embraces the methodologies to investigate a variety of cells and tissues that are known to be disrupted by ethanol, from intestinal epithelial cells, to cells in the liver, including hepatocytes and Kupffer cells, to cells in the periphery, including skeletal muscle, adipose and bone. Specific methodologies to investigate the effects of ethanol on neuronal function, including the use of neuronal cell lines and organotypic cultures, are also presented.

It is likely that the effects of ethanol on cell, tissue, and organismal function are fundamentally based on the impact of ethanol on transcriptional and post-transcriptional regulation of gene expression. Novel methodologies to study the molecular mechanisms of ethanol action include the use of gene arrays, as well as proteomic analysis of the post-translational modifications of proteins in organelles and cells exposed to ethanol. Chapters providing the specific expertise required for the design and analysis of gene array and proteomic studies are included in this volume to enable investigators new to these data-rich approaches to successfully "mine" the vast amount of data that can be obtained by these approaches.

In the final analysis, studies into the molecular mechanisms for ethanol action not only result in a further understanding of the pathophysiology of ethanol-induced injury, but also contribute to our understanding of the fundamental mechanisms by which organisms have adapted to subtle changes in their environment. Although excessive alcohol consumption can result in profound impairments in the ability of the organism to develop and function, most organisms can readily handle the subtle insults associated with moderate alcohol consumption. Understanding the genetic, molecular, cellular, and physiological responses to ethanol that "tip the balance" from an adaptive response to a maladaptive/pathological response is critical to the development of therapeutic strategies for the intervention and/or prevention of the effects of ethanol on development and tissue injury. I hope that the very detailed and specific methods presented in this volume will further spur investigators to delve into the complex and fascinating story of the adaptive and maladaptive responses humans have developed to the consumption of alcohol.

Laura E. Nagy, PhD
Cleveland Clinic

2
A Voluntary Oral-feeding Rat Model for Pathological Alcoholic Liver Injury

George L. Tipoe, Emily C. Liong, Tung-Ming Leung, and Amin A. Nanji

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.

L. E. Nagy (ed.), *Alcohol: Methods and Protocols*

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 100 mL 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), $NaH_2PO_4 \cdot H_2O$ (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. Permount 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 m*M*), nicotinamide adenine dinucleotide (NADH; 0.2 m*M*) and lactate dehydrogenase (LDH; 2 units) in potassium phosphate buffer (0.1 *M*, pH 7.4)
2. α-ketoglutaric acid (10 m*M*; 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-κB: 5′-AGTTGAGGGGACTTTCCCAGGC-3′
3′-TCAACTCCCCTGAAAGGGTCCG-5′
AP-1: 5′-CGCTTGATGAGTCAGCCGGAA-3′
3′-GCGAACTACTCAGTCGGCCTT-5′

3. [γ^{-32}P]ATP (Amersham Bioscience Ltd, UK).
4. T4 Polynucleotide Kinase (Promega Corporation).
5. TE buffer (10 m*M* Tris-HCl. pH 8.0; 1 m*M* ethylene diamine tetraacetic acid [EDTA]).
6. 10× Reaction buffer: 40% glycerol, 200 m*M* Tris-HCl pH 7.8, 1 *M* NaCl, 50 m*M* $MgCl_2$, 10 m*M* EDTA, 50 m*M* dithiothreitol.
7. Loading dye: 250 m*M* Tris-HCl, pH 7.5, 0.2% bromophenol blue, 40% glycerol.
8. 4% nondenaturing acrylamide gel prepared in TBE buffer (10× TBE buffer (890 m*M* Trisbase, 890 m*M* boric acid, 2 m*M* 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. SuperScript™ First-Stand Synthesis System (Life Technologies).
4. AmpliTaq Gold PCR kit (Roche Applied Science).
5. Primers (Invitrogen Inc.; Tables 3 and 4).
6. 0.05 µg/mL ethidium bromide.
7. Bromophenol Blue and Xylene-Scyanol (Sigma-Aldrich Co.).

2.11 Western Blotting

1. Liver homogenizing Buffer 1: 250 m*M* sucrose, 15 m*M* NaCl, 5 m*M* EDTA, 1 m*M* EGTA, 0.15 m*M* spermine, 0.5 m*M* spermidine, 1 m*M* 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′-CACAGAAGTGGGGGTATGCT-3′
Cyclooxygenase-2	5′-GGAGAGACTATCAAGATAGTGATC-3′
	5′-ATGGTCAGTAGACTTTTACAGCTC-3′
Tumor necrosis factor-α	5′-ATGAGCACAGAAAGCATGATC-3′
	5′-TACAGGCTTGTCACTCGAATT-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 m*M* $MgCl_2$	2.5 µL	95°C	15 min
	10 m*M* dNTP	1.0 µL	95°C	1 min
	10 µ*M* forward primer	0.5 µL	60°C	1 min
	10 µ*M* reverse primer	0.5 µL	72°C	1 min
	Taq Polymerase	0.25 µL		37 cycles
	Milli-Q H_2O	13.75 µL	72°C	10 min
	CDNA	4.0 µL		
ENOS	10× PCR buffer	2.5 µL		
	25 m*M* $MgCl_2$	2.5 µL	95°C	15 min
	10 m*M* dNTP	0.5 µL	95°C	1 min
	10 µ*M* forward primer	0.5 µL	60°C	1 min
	10 µ*M* reverse primer	0.5 µL	72°C	1.5 min
	Taq Polymerase	0.125 µL		35 cycles
	Milli-Q H_2O	17.375 µL	72°C	10 min
	cDNA	1.0 µL		
COX-2	10× PCR Buffer	2.5 µL		
	25 m*M* $MgCl_2$	2.5 µL	95°C	15 min
	10 m*M* dNTP	0.5 µL	95°C	1 min
	10 µ*M* forward primer	0.5 µL	60°C	1 min
	10 µ*M* reverse primer	0.5 µL	72°C	1.5 min
	Taq Polymerase	0.125 µL		37 cycles
	Milli-Q H_2O	17.375 µL	72°C	10 min
	cDNA	1.0 µL		
TNF-α	10× PCR buffer	2.5 µL		
	25 m*M* $MgCl_2$	1.5 µL	95°C	15 min
	10 m*M* dNTP	0.5 µL	95°C	1 min
	10 µ*M* forward primer	0.5 µL	50°C	1 min
	10 µ*M* reverse primer	0.5 µL	72°C	1 min
	Taq Polymerase	0.125 µL		34 cycles
	Milli-Q H_2O	18.375 µL	72°C	10 min
	cDNA	1.0 µL		
GAPDH	10× PCR buffer	2.5 µL		
	25 m*M* $MgCl_2$	1.5 µL	95°C	15 min
	10 m*M* dNTP	0.5 µL	95°C	1 min
	10 µ*M* forward primer	1.25 µL	55°C	1 min
	10 µ*M* reverse primer	1.25 µL	72°C	1 min
	Taq Polymerase	0.15 µL		25 cycles
	Milli-Q H_2O	16.85 µL	72°C	10 min
	cDNA	1.0 µL		

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

2. Buffer 2: 10 m*M* Hepes, pH 7.9, 1.5 m*M* $MgCl_2$, 10 m*M* KCl, 0.1% Leu, 0.1% Apr; 0.5% PMSF.
3. Buffer 3: 0.5 m*M* Hepes, pH 7.9, 0.75 m*M* $MgCl_2$, 0.5 m*M* EDTA, 0.5 m*M* 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: 10 m*M* Tris-HCl, pH 7.9, 5 m*M* $MgCl_2$, 1 m*M* EDTA, 10 m*M* KCl, 20% glycerol, 0.1% NP-40.
5. Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Inc.).
6. 2× sample buffer: 0.1 *M* 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: 0.6 *M* Tris-HCl, pH 8.8, 10% polyacrylamide, 0.05% SDS, 0.05% ammonium persulfate, 0.1% TEMED.
8. Stacking gel: 0.2 *M* Tris-HCl, pH 8.8, 4% polyacrylamide, 0.05% SDS, 0.07% ammonium persulfate, 0.1% TEMED.
9. Tank buffer: 0.025 *M* Tris, 0.192 *M* glycine, 0.1% SDS, pH 8.3.
10. Transfer buffer: 25 m*M* Tris, pH 8.3, 192 m*M* glycine, 20% methanol, 0.1% SDS.
11. Tween Tris-buffered saline (TBST), pH 7.5: 100 m*M* 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: 50 m*M* Tris-HCl, 154 m*M* KCl, pH 7.4.
2. Trichloroacetic acid.
3. Thiobarbituric acid.

2.13 Chymotrypsin (2)

1. 0.1 *M* Tris-HCl, pH 7.5.
2. Stop solution: 1% SDS and 1 mL of 0.1 *M* 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 m*M* aniline and 1 m*M* NADPH.
2. 40% Trichloroacetic acid.
3. 10% Na_2CO_3.
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 μm.
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.

$$\text{Alanine} + \alpha\text{-ketoglutarate} \xrightleftharpoons{\text{ALT}} \text{Glutamate} + \text{Pyruvate}$$

$$\text{Pyruvate} + \text{NADH} \xrightleftharpoons{\text{LDH}} \text{Lactate} + \text{NAD}$$

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 m*M*).
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:

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

where Abs_{340}/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-µL 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 µL of EIA buffer is added and 50 µL of EIA buffer is added to wells labeled with B0 (maximum binding).
10. 50 µL of standards and samples are added to the wells accordingly.
11. To the wells labeled with total activity (TA) and blank (Blk), 50 µL of 8-isoprostane AchE tracer and 50 µL of 8-isoprostane antiserum is added, whereas only 50 µL 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 µL of Ellman's Reagent is added to each well and 5 µL 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:

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

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 $1.56 \times 10^5 cm^2/mmol$.

3.3.3 Endotoxin

1. A blood sample is collected in endotoxin-free vials.
2. The blood is centrifuged at 400*g* 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 µ*M* 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-scuuinyl-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% Na_2CO_3 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 13,000 rpm for 1 min and the flow through is discarded.
6. The DNA in the lysate is removed by adding 95 µl of diluted DNase-I reaction mixture and incubated at room temperature for 15 min.
7. The reaction is stopped by adding 500 µl of Buffer RA2 and then centrifuged at 13,000 rpm for 1 min.
8. After the flow through was discarded, the column is washed by adding Buffer RA3 twice (600 µL of RA3, centrifuged at 13,000 rpm for 1 min; 250 µL of RA3, centrifuged at 13,000 rpm for 2 min).
9. The RNA is then eluted twice by adding 30 µL of RNase-free water and stood at room temperature for 15 min.
10. The RNA is collected in the collection tube by centrifuging at 13,000 rpm 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 µg of RNA is diluted with RNase-free water to a final volume of 10 µL in a microtube.
3. 1 µL of oligo(dT) and 1 µL 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 µL of reaction mixture (10× First-Strand RT Buffer, 2 µL; 25 mM MgCl2, 4 µL; 0.1 M DTT, 2 µL; RNaseOUT Recombinant RNase Inhibitor, 1 µL) is added to the tube and mixed thoroughly.
7. The tube is then incubated at 42°C for 2 min.
8. 1 µL (50 units) if 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 μg/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 (100 mg) is placed in a 5-mL tube containing 500 μL of ice-cold Buffer 1
2. The tissue is then homogenized with a homogenizer and followed by centrifugation at 5,000 rpm 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 μL 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 μL 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-Blot™ 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 ECL™ 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-κB 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-κB for the binding of the corresponding transcription factor is firstly labeled with [γ^{-32}P]ATP through a phosphorylation reaction (2 µl of consensus oligonucleotide (1.75 pmol/µL), 1 µL of T4 Polynucleotide Kinase 10× Buffer, 1 µL of [γ^{-32}P]ATP (3000 Ci/mmol at 10 mCi/mL), 5 µL of Nuclease-free water, and 1 µL of T4 Polynucleotide Kinase (5–10 U/µL).
3. The mixture is incubated at 37°C for 10 min.
4. The labeling process is stopped by adding 1 µL of 0.5 *M* EDTA and diluted with 89 µL 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-κB p50	Rabbit (Polyclonal)	Santa Cruz Biotechnology, CA, USA
NF-κB 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 1100 g 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× 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 persulgate and 49.7 mL of distilled water, the gel is pre-run at 100 V in 0.5× 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× Reaction Buffer, 2 µL of ssDNA, and 1 µL of antibody (NF-κB p50 or NF-κB p65; 200 µg/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.

Acknowledgments The authors gratefully acknowledge the support of the National Institutes of Health (grant to A.A.N.) and grants from the Committee of Research and Conference Grants, The University of Hong Kong.

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.

References

1. Nanji, A. A., Zhao S, Sadrzadeh, S. M., Dannenberg, A. J., Tahan, S. R., and Waxman, D. J. (1994) Markedly enhanced cytochrome P450 2E1 induction and lipid peroxidation is associated with severe liver injury in fish oil-ethanol-fed rats. *Alcohol Clin. Exp. Re*s. **18,** 1280–1285.

2. Donohue, T. M., Jr., Kharbanda, K. K., Casey, C. A., and Nanji, A. A. (2004) Decreased proteasome activity is associated with increased severity of liver pathology and oxidative stress in experimental alcoholic liver disease. *Alcohol. Clin. Exp. Res.* **28,** 1257–1263.
3. Nanji, A. A., Jokelainen K, Tipoe, G. L., Rahemtulla A, and Dannenberg, A. J. (2001) Dietary saturated fatty acids reverse inflammatory and fibrotic changes in rat liver despite continued ethanol administration. *J. Pharmacol. Exp. Ther.* **299,** 638–644.
4. Tipoe, G. L., Leung, T. M., Liong E, So H, Leung, K. M., Lau, T. Y., Tom, W. M., Fung, M. L., Fan, S. T., and Nanji, A. A. (2006) Inhibitors of inducible nitric oxide (NO) synthase are more effective than an NO donor in reducing carbon-tetrachloride induced acute liver injury. *Histol. Histopathol*. **21,** 1157–1165.
5. Nanji, A. A., Sadrzadeh, S. M., Yang, E. K., Fogt, F., Meydani, M., and Dannenberg, A. J. (1995) Dietary saturated fatty acids: a novel treatment for alcoholic liver disease. Gastroenterology. **109,** 547–554.
6. Imai, Y., Ito, A., and Sato, R. (1966) Evidence for biochemically different types of vesicles in the hepatic microsomal fraction. *J. Biochem. (Tokyo).* **60,** 417–428.