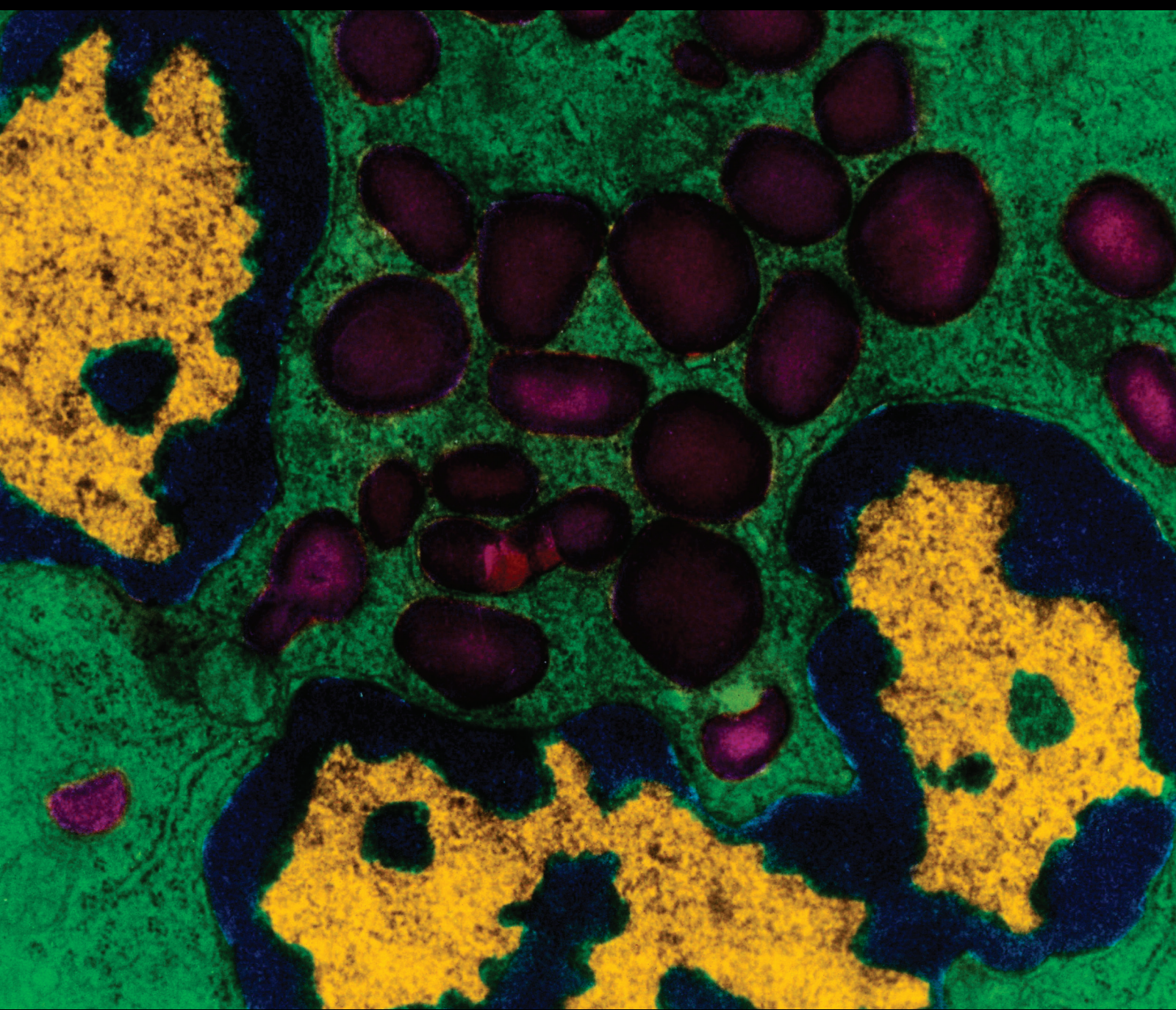
Mediators of Inflammation

Gut Inflammatory Diseases, Infection, and Nutrition

Lead Guest Editor: Helieh S. Oz
Guest Editors: Sung-Ling Yeh and Amedeo Amedei



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Editorial Board

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Hindawi
Mediators of Inflammation
Volume 2018, Article ID 7160859, 4 pages
https://doi.org/10.1155/2018/7160859

Editorial
Gut Inflammatory Diseases, Infection, and Nutrition

Helieh S. Oz,[1] **Sung-Ling Yeh**,[2] **and Amedeo Amedei**[3]

[1]*University of Kentucky Medical Center, Lexington, USA*
[2]*Taipei Medical University, Taipei, Taiwan*
[3]*University of Florence, Florence, Italy*

Correspondence should be addressed to Helieh S. Oz; hoz2@email.uky.edu

Received 24 June 2018; Accepted 24 June 2018; Published 26 July 2018

There is a strong link between inflammation, infectious agents, and nutritional status. Millions of people suffer from chronic inflammatory diseases, and the incidence has significantly increased in recent years. Inflammation is a multifactorial biological and immunological response to different injuries. Inflammation is initiated by several stimuli such as pathogens, chemical irritants, nutritional imbalance, and different cell injures. Inflammation is required in the body process of healing. But, chronic inflammation can cause severe and irreversible complications. Different chronic inflammatory diseases include hepatic, colitis, gastrointestinal, and neurodegenerative. Divers microbial (e.g., *Helicobacter*, *Campylobacter*, *Clostridium*, and *Mycobacterium*), parasitic (e.g., protozoa, helminthes, and flatworms), and viral (HPV, norovirus, and hepatitis B and C) are linked with chronic inflammatory responses. Additionally, nutrition imbalance and specific nutrients may influence immune response, modulating infections, and inflammatory responses.

Crohn's and ulcerative colitis are chronic inflammatory bowel disease (IBD) and progressive complication with a dysregulated gastrointestinal mucosal immune response in patient toward intestinal microbiota. Pediatrics often manifest with more severe Crohn's complication than adults. Crohn's patients may eventually develop strictures and malignancies. IL-17-A-producing T helper cells (Th17) have a key role in provoking progression of disease by production of proinflammatory cytokines which in turn required for regeneration and protection of epithelial cells. Crohn's patients have increased production of IL-17 by T helper cells and higher IL-17 mRNA expression at the mucosal level and increased numbers of Th17 cells are associated with endoscopic definition of disease activity. Unlike previous studies, A. Dige et al. concluded that anti-TNFα antibody therapy has no effect on the mucosal levels of IL-17A, IL-21, and IL-22 or LP T cell production during early treatment (first 4 weeks) in Crohn's disease.

Composition of commensal microbiota can influence autoimmune disease progress and persistence. The intestinal microbiota is involved in triggering the immune system and leading to intestinal inflammation. IBD patients suffer from a dysbiosis, with decrease in diversity and abundance of some beneficial commensal bacteria. For instance, significant reductions have been reported in *Bifidobacteria* and *Lactobacillus* in the IBD patients' fecal content suggesting a therapeutic application to normalize the gut flora in autoimmune patients using probiotics. Recent study report that gut microbial can translocate from gut into the organs as gut barrier compromises and pathogenic T helper cells are evident in the gut, liver, and lymphoid organs. Similarly, microbial are also found in hepatic biopsies of autoimmune patients and not in healthy counterparts. Bifico is a probiotic mixture of *Lactobacillus*, *Bifidobacterium*, and *Enterococcus*. Previous studies have demonstrated Bifico to improve colitis and colitis-associated malignancy, pouchitis, diarrhea, and gastritis in models. In addition, clinical trials have revealed therapeutic application for Bifico in Crohn's and ulcerative colitis patients. Bifico has been shown to elevate the expression of colonic TJs and promote intestinal epithelial barrier function in IL-10-deficient mice. In vitro experiments also showed that Bifico, or single probiotic strains *(Bifidobacterium*, *Lactobacillus*, or *Enterococcus)*, increases transepithelial electrical resistance and the expression of

TJs in *Escherichia coli*-treated Caco-2 monolayers. Bifico significantly inhibited the secretion of proinflammatory cytokines and reduced bacterial invasion and combination of probiotics were more pronounced than single-strain probiotics. Bifico was shown to have anti-inflammatory effect to expand Tregs in mesenteric lymph nodes and disturbance of Th1/Th2 cytokines in the colonic mucosa of TNBS-induced colitis mice. However, the effect of Bifico on the Tregs in intestinal tissue and peripheral blood has not been reported. These experimental studies were performed in active colitis induced with dextran sulfate sodium (DSS) to explore the therapeutic effects of Bifico. Here, Y. Zhang et al. used DSS-active colitis model to investigate Bifico effect associated with local and systemic immune responses. This study explored the protective effect of Bifico pretreating on subsequent intestinal inflammation.

Acute pancreatitis (AP) is an acute and life-threatening inflammatory disease that commonly damages peripancreatic tissues and other distant organs. A consistent percentage (about the 25%) of patients with severe acute pancreatitis (SAP) develops into infected pancreatic necrosis and persistent organ failure, contributing the most to AP mortality. In fact the SAP, due to excessive release of inflammatory factors and increased oxidative stress response, can cause distant organ damage, especially acute lung injury. In addition, currently, there is no effective therapeutic strategy for AP. For this purpose, Y. Li et al. used Cae-induced mild AP (MAP) model and L-arginine-induced SAP model, to investigate the role of naringenin (Nar) in AP and the accompanying organ dysfunctions in mice as well as the underlying mechanisms. Nar is a type of flavonoid, with anti-inflammatory properties, organ-protective effects, and antioxidative functions. They observed that the serum levels of amylase, lipase, and cytokines and the malondialdehyde (MDA) levels of pancreatic tissue were significantly decreased in both MAP and SAP models after Nar treatment. In contrast, glutathione peroxidase, glutathione reductase, glutathione-S-transferase, total sulfhydryl, and nonprotein sulfhydryl were markedly increased both in MAP and SAP after Nar treatment. In addition, the injury in pancreatic and pulmonary tissues was markedly improved as evidenced by the inhibited expression of myeloperoxidase, nod-like receptor protein 3, and interleukin-1β as well as the enhanced expression of nuclear factor erythroid 2-related factor2/heme oxygenase-1 in pancreatic tissues. So, they concluded that Nar exerted protective effects on Cae-induced MAP and L-arginine-induced SAP in mice, suggesting that Nar may be a potential therapeutic intervention for AP.

Gut microbiota (GM) plays several crucial roles in host physiology, influencing different relevant functions. GM diversity is affected by diet and influences metabolic and immune functions of the host's physiology. Consequently, a dysbiosis may be the cause or at least may lead to the progression of various pathologies such as infectious diseases, gastrointestinal cancers, IBD, and even obesity and diabetes. Therefore, GM is an appropriate target for nutritional intervention to improve health and phytochemicals (that can influence GM) have recently been studied as adjuvants for the treatment of obesity and inflammatory diseases. L. Carrera-Quintanar et al. discussed the most recent evidence indicating a relationship between the effects of different phytochemicals and gut microbiota, affecting obesity and/or inflammation. Authors focused on the effect of approximately 40 different phytochemical compounds, candidates for the treatment of obesity and inflammatory diseases. They concluded that several issues need to be resolved before natural products can be effectively translated into the clinic. With regard to the best source of bioactive molecules, the following aspects should be considered: (a) whether it is better to acquire them directly from diet food or from pharmacological sources and (b) whether they should be used alone or as a cotreatment in combination with approved drugs. Therefore, it is urgent to develop specific clinical trials. In addition, disadvantages of commercial nutraceutics' preparations include the high variability in formulations, as well as the dosage quantification and the different means of administration. Finally, critical investigations are required to optimize these phytochemical formulation and dosages for possible future administration.

The digestive system plays an important part in pathogenesis of infection by the human immunodeficiency virus (HIV), which can infect hepatocytes, Kupffer cells, and hepatic stellate cells, inducing the production of inflammatory cytokines and favoring hepatic steatosis. Circulating levels of different hepatic proteins (e.g., albumin, prealbumin, and transferrin) decrease, increasing the mortality risk in AIDS patients. L. Xu et al. evaluated whether the level of butyrylcholinesterase (BchE) could be associated with the progression/prognosis of AIDS patients. Evaluating a cohort of 505 patients, the associations between BChE level and CD4 count, WHO stage, body mass index, and C-reactive protein level, the authors concluded that BChE level is associated with HIV/AIDS severity and is an independent risk factor for increased mortality in AIDS patients.

Surgery is elective treatment for colorectal malignancy, yet the morbidity rate following colorectal resection remains as high as 24%–43%. Some of these postsurgical complications include tissue adhesions at the site of surgery, infections, anastomotic leakage, impaired bowel movement, and malfunction as transient or prolonged postsurgical ileus if not resolved after 5 days or recurrent after recovery. These complications can delay recovery and increase the length of hospitalization and acquired infections and medical expenses. An effective biomarker to predict postsurgical ileus and other complications can be useful for recovery in these patients. G.S.A. Boersema et al. investigated a prospective cohort trail for the association between the inflammatory cytokines and the postoperative complications. The authors studied 47 patients from which 34 (72%) recovered. From 13 patients (28%) who developed postsurgical ileus, 8 (20%) recovered after 5 days and 5 patients (10%) developed recurrent disease. The authors discuss the association of different inflammatory cytokines involvement with postsurgical complications and reason IL-6 changes to predict the infectious complications but not postsurgical ileus after colorectal surgery. They concluded that IL-6 may be promising candidate to assist an early detection of the infections following surgeries.

Maresins, a group of lipid mediators, are biosynthesized from docosahexaenoic acid which displays strong anti-inflammatory and proresolving activity. Resolution of inflammation is an active and highly regulated cellular and biochemical process required to protect against inflammatory complications. S. Tang et al. review the biological actions, pathways, and mechanisms of maresins and their roles in the resolution of inflammation in various disease conditions including lung disease, vascular disease, obesity, diabetes, and IBD. Authors concluded that maresins may prevent neutrophil infiltration, enhance macrophage phagocytosis, inhibit nuclear factor-κB activation, and stimulate tissue regeneration. Similar studies may provide new directions to discover maresin-related stable analogues to control inflammation in the future.

IBD is a multifactorial inflammatory disease of the intestine. Diet has long been suspected to contribute to the IBD development and the Western dietary pattern, which is high fat, high n-6 polyunsaturated fatty acids (PUFA), is associated with an increased IBD risk. N-3 PUFAs contain mostly fish oil and have anti-inflammatory properties. C. Charpentier et al. investigated the dietary influence of fatty acid composition on 2,4,6-trinitrobenzen sulfonic acid- (TNBS-) induced colitis. Rats were fed with diets varying in n-3/n-6/n-9 ratio to reproduce dietary pattern from a pragmatic to a Western diet. There were 4 groups with n-3/n-6/n-9 ratio 1 : 4 : 16, 1 : 1 : 4, 1 : 16 : 16, and 1 : 4 : 24, respectively. n-3/n-6/n-9 ratio 1 : 4 : 16 is recommended as a well-balanced control diet, and 1 : 1 : 4 was a target by dietary advice in a Japanese clinical trial for IBD patients. 1 : 16 : 16 is considered comparable to Western diet, and ratio 1 : 4 : 24 is comparable to the Mediterranean diet. The results showed that compared to the control diet, n-3 polyunsaturated fatty acids-rich diet significantly decreased colon-inducible nitric oxide synthase, cycloxygenase-2 expression, IL-6, and leukotriene B4 production. The authors concluded that n-3 diet group which showed n-3/n-6 ratio equals to 1 attenuated inflammatory markers in the colon that may contribute to partially limit colitis genesis.

Formononetin is an isoflavone compound that has been reported to possess anti-inflammatory properties. D. Wu et al. investigated the effects of formononetin on DSS-induced acute colitis in vivo and in vitro on tumor necrosis factor-α-induced human colonic cell injury models. Mice with colitis were intraperitoneal injected with different dosages of formononetin. The main findings showed that formononetin administration relieved clinical symptoms of colitis, mitigated colonic epithelial cell injury, and upregulated the levels of colonic tight junction proteins ZO-1, claudin-1, and occluding. In the in vitro study, the formononetin prevented acute injury of human colonic cells by increasing colonic tight junction proteins and decreasing inflammatory cytokine expression. The mechanism may partly be associated with NLRP3 inflammasome signaling inhibition, as the NLRP3 pathway protein levels including NLRP3, ASC, and interleukin-1β were downregulated in a dose-dependent manner, in vivo and in vitro, when formononetin was administered. The authors concluded that formononetin could protect colonic epithelial cells from injury to relieve the disease severity of colitis and may have potential to be used in the clinical prevention and treatment of IBD in future.

Angiostrongylosis is an important food-borne diseases and eosinophilic encephalitis in humans, caused by rat gut nematode (rat lungworm). Outbreaks of eosinophilic meningitis have been reported due to the consumption of infected raw snails and vegetables' juice. Angiostrongylus invades the central nervous system and causes neurons' demyelination, eosinophilic encephalitis, and meningoencephalitis. An inflammatory response and surge of cytokines such as IL-17 have been detected in the central nervous system. IL-17 can induce Act1-mediated signaling cascades in CNS resident cells (astrocytes, oligodendrocytes, and neurons) might coordinately mediate CNS inflammation, demyelination, and neurodegeneration. But the mechanisms by which IL-17 involves in the demyelination caused by this nematode is not investigated. F. Ying et al. explore the role of IL-17A on the demyelination and introduce IL-17A-neutralizing antibody to protect against demyelination caused by the parasite as a possible therapeutic option in angiostrongylosis. In addition, iNOS inhibition is the possible mechanism for the therapeutic effect. This study provides a new potential therapeutic alternative for demyelination caused by *Angiostrongylus cantonensis*.

Alcohol consumption in excess causes extensive liver injuries of fatty liver which progresses to hepatitis, fibrosis, cirrhosis, and hepatocarcinoma. Ethanol increases NADH/NAD^+ ratio and promotes fatty acid synthesis and lipid accumulation in liver cells. Further, it causes excessive oxidative stress and increases CYP2E1 activity. In addition, ethanol increases endotoxin bypass from leaky gut which triggers Kupffer's cell activation and inflammatory processes. There is an emerging theory that chronic ethanol abuse damages the tight junction structure in intestinal epithelial cells results in bacterial translocation from the intestines into the in vivo circulation to induce hepatic inflammation. Indeed, patients with alcoholic liver disease have higher levels of endotoxin and intestinal barrier disturbances caused by ethanol are the major source of endotoxemia in these patients. Different source of dietary fat can effect progression of liver injury as diets rich in saturated fatty acids or medium chain triglycerides protect against ethanol-induced liver injury in rodents. In contrast, polyunsaturated fatty acids can provoke liver injury. However, there were some limitations of these previous studies such as only one type of fat was used in each experimental diet, but the effects on other organs or tissues were not explored. Studies have demonstrated that fish oil, which is rich in n-3 polyunsaturated fatty acids, eicosapentaenoic acid, and docosahexaenoic acid, can provide immune regulation, vascular protection, and lipid metabolism modulation. However, the mechanism by which oil can modify intestinal integrity in alcoholic liver disease is not well understood. Further, some studies reveal fish oil and olive oil to improve the fecal microbiota under ethanol exposure, but the effects on intestinal pathological changes in ethanol-fed rats are still unclear. Y.-W. Chien et al. explored whether fish oil can provoke hepatoprotective

effects in ethanol-fed rats by means of maintaining the epithelial barrier function in the intestines and further inhibiting endotoxin in circulation. Authors concluded that the chronic ethanol can elevate plasma endotoxin concentrations and trigger inflammatory responses which can result in liver injury. Substitution of fish oil for olive oil inhibited the appearance of endotoxin in the circulation under ethanol exposure; thus, it decreased inflammatory responses and exerted a hepatoprotective potential in rats under chronic ethanol feeding. However, the mechanism of decreased plasma endotoxin levels by fish oil supplementation alone might not be enough to improve intestinal structural integrity.

About 30–40% of chronic inflammatory disease patients use some form of complementary and alternative medicine, including Chinese traditional herbal therapies. It is estimated that 12 million tons of herbal wastes is produced each year by about 1,500 Chinese traditional herbal medicine enterprises in China. During process, the active ingredients are extracted from plants, and the waste which still contains about 30%–50% of the medicinal active ingredient is buried or burned which becomes major source of environmental pollutant in water and in air. Fermentation by digestive enzymatic reaction utilizing cellulase, protease, pectinase, and lipase can degrade plant cell wall and expose intercellular organelles to assist in extraction of active ingredients. In addition, certain probiotics can improve digestive process and protect against malabsorption, malnutrition, and diarrhea. Antibiotic-associated diarrhea is a frequent side effect caused by altered gut microbiota which supports pathogen growth. F. Meng et al. used probiotics to ferment the herbal residues in Jianweixiaoshi, a mixture of herbal remedy used for diarrhea, and the reason for this compound may become as therapeutic potential against antibiotic-associated diarrhea, as well as to reduce the waste products from herbal residues produced by traditional herbal medicine enterprises.

Flowers of *Osmanthus fragrans (O. fragrans)* and *Chrysanthemum morifolium (C. morifolium)* are commonly used as folk medicine and additives for tea and beverages. The active components isolated from these flowers contain many phenolic compounds that have been shown to have anti-inflammatory and antioxidant properties. Lipotoxicity occurs when excessive harmful lipid accumulates in cells leading to cellular dysfunction and disruption of tissue function. Lipotoxicity plays a critical role in the pathogenesis of nonalcoholic fatty liver disease and renal diseases. P.-J. Tsai et al. investigated the effects of methanolic flower extracts of *O. fragrans* and *C. morifolium* against free fatty acid-induced lipotoxicity in hepatocytes and renal glomerular mesangial cells. The results showed that both extracts inhibited free fatty acid-induced hepatocyte triglyceride accumulation and suppressed mRNA expression of inflammatory cytokines when hepatocytes were stimulated with lipopolysaccharide-treated monocytes. Also, flowers extracts of *O. fragrans* and *C. morifolium* effectively inhibited oleate-induced cellular lipid accumulation and overexpression of fibronectin in mesangial cells. The authors concluded that these flower extracts possess hepato- and renal-protective activity by inhibiting hepatic fat load and inflammation and mesangial extracellular matrix formation. These findings imply that flowers of *O. fragrans* and *C. morifolium* may have potential to protect against nonalcoholic steatohepatitis and renal fibrosis.

Helieh S. Oz
Sung-Ling Yeh
Amedeo Amedei

Hindawi
Mediators of Inflammation
Volume 2018, Article ID 3279607, 7 pages
https://doi.org/10.1155/2018/3279607

Research Article

Effects of Anti-TNFα Treatment on Mucosal Expression of IL-17A, IL-21, and IL-22 and Cytokine-Producing T Cell Subsets in Crohn's Disease

Anders Dige,[1] **Maria K. Magnusson**,[2] **Claus Uhrenholt**,[1] **Tue Kruse Rasmussen**,[3,4] **Tue Kragstrup**,[3,4] **Lena Öhman**,[2,5] **Jens Dahlerup**,[1] **and Jørgen Agnholt**[1]

[1]*Gastro-Immuno Research Laboratory (GIRL), Department of Hepatology and Gastroenterology, Aarhus University Hospital, Aarhus, Denmark*
[2]*Department of Microbiology and Immunology, Institute for Biomedicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden*
[3]*Department of Biomedicine, Aarhus University Hospital, 8000 Aarhus C, Denmark*
[4]*Department of Rheumatology, Aarhus University Hospital, 8000 Aarhus C, Denmark*
[5]*Department of Internal Medicine and Clinical Nutrition, Institute for Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden*

Correspondence should be addressed to Anders Dige; andedige@rm.dk

Received 26 October 2017; Revised 12 March 2018; Accepted 3 April 2018; Published 26 April 2018

Academic Editor: Helieh S. Oz

T helper 17 (Th17) cells produce interleukin (IL) 17-A. In addition, Th17 cells produce IL-21 and IL-22. Th17 cells have a disease-promoting role in Crohn's disease (CD). We investigated the effects of anti-TNFα treatment on mucosal gene expression (qPCR) of IL-17A, IL-21, and IL-22 as well as on the frequency of lamina propria (LP) T cell subsets producing these cytokines (flow cytometry) in 12 active CD patients before and after 4 weeks of anti-TNFα treatment with adalimumab. At baseline, in inflamed mucosa we found increased gene expression of IL-17A and IL-22 but not IL-21 when compared to noninflamed mucosa. There were increased frequencies of IL-21-producing LP T cells but no differences in the frequencies of IL-17A- or IL-22-producing LP T cells when comparing inflamed versus noninflamed mucosa at baseline. There were no changes in the mucosal gene expression of IL-17A, IL-21, and IL-22 or the frequencies of IL-17A-, IL-21- and IL-22-producing LP T cell subsets between baseline and following 4 weeks of adalimumab initiation. Our results do not support the hypothesis that anti-TNFα treatment has an early effect on the mucosal levels of IL-17A, IL-21, and IL-22 or LP T cell production of these cytokines in CD.

1. Introduction

Crohn's disease (CD) progresses due to a dysregulated mucosal immunological response towards the intestinal microflora in genetically susceptible individuals [1–3]. Interleukin (IL) 17-A-producing T helper (Th17) cells have been reported to play an important disease-promoting role in the progression of CD [4–7] because of their production of proinflammatory cytokines, which besides the hallmark cytokine IL-17A includes IL-21 and IL-22 [8]. However, these cytokines also have protective and regenerative effects on epithelial cells [9–11]. Consequently, the Th17 cells may have contradictory roles in CD, which may explain the inefficiency of anti-IL-17A antibodies as a treatment of CD [12]. Increased frequencies of IL-17-producing T helper cells and higher IL-17 mRNA expression have been observed at the mucosal level in CD patients compared to patients with infectious colitis [13] as well as healthy controls [6, 13–17]. A recent study reported that increased numbers of Th17 cells were associated with endoscopic disease activity in both CD and ulcerative colitis patients, and the Th17 cells were skewed towards concomitant production of interferon-γ [15]. The production of IL-21 and IL-22 is not specific to Th17 cells and has also been attributed to other CD4 T cell

subsets, such as follicular T helper cells [18] and Th22 cells [19], respectively. Increased mucosal IL-21 expression has been observed in patients with active CD compared to ulcerative colitis patients and healthy controls. Increased numbers of IL-21- and IL-22-producing lamina propria (LP) T cells has also been reported in CD patients compared to healthy controls [16, 20].

Treatment with antibodies that neutralize the essential inflammatory cytokine tumor necrosis factor alpha (anti-TNFα) has become a mainstay in the treatment of CD [21]. However, the mechanisms of anti-TNFα efficacy are only partly elucidated. It has been proposed that the induction of apoptosis in LP T cells is important for anti-TNFα efficacy in CD treatment [22–24].

We previously reported that 26 weeks of anti-TNFα treatment was associated with a rise in the frequencies of circulating IL-17A- and IL-21-producing T cells [25]. Two studies from China reported that 10 weeks of anti-TNFα treatment was associated with a decreased mucosal gene expression of IL-17A and IL-21 and reduced frequencies of IL-17A- and IL-21-producing LP cells [26, 27]. However, because the clinical effect of anti-TNFα treatment often occurs one to two weeks following treatment initiation, it is difficult to decipher whether these observations are a bystander phenomenon to a general downregulation of the inflammatory response or a direct treatment mechanism.

We hypothesized that anti-TNFα treatment has an early (i.e., within 4 weeks of treatment initiation) effect on the mucosal IL-17A, IL-21, and IL-22 gene expression and the frequencies of mucosal IL-17A-, IL-21-, and IL-22-producing T cells in active CD. We aimed to test this hypothesis by measuring the mucosal gene expression of IL-17A, IL-21, and IL-22 as well as the cellular protein production of these cytokines in LP T cell subsets before and after 4 weeks of induction treatment with adalimumab. To clarify whether the cytokine levels were specific for the presence of active CD inflammation, we also included observations from areas of noninflamed tissue in the present study.

2. Methods

2.1. Patients and Samples. Twelve patients with active CD were included in this study. The patients had been diagnosed according to clinical, endoscopic, histopathological, and biochemical criteria [28]. Baseline patient characteristics are shown in Table 1. At inclusion, all patients exhibited clinical disease activity, as estimated by a Crohn's Disease Activity Index (CDAI) [29] greater than 150 or a Harvey-Bradshaw index (HBI) [30] of 4 or more. Furthermore, all included patients had biochemical signs of inflammation, for example, either elevated C-reactive protein (CRP) or increased fecal calprotectin levels. All patients displayed endoscopic disease activity at the inclusion endoscopy, which was evaluated using the Simple Endoscopic Score for Crohn's Disease (SES-CD) [31]. No patients were treated with anti-TNFα or corticosteroids or changed immunosuppressant dosing (azathioprine or methotrexate) within the 12 weeks prior to inclusion. Patients received standard induction dosing with subcutaneously administered adalimumab (AbbVie, North Chicago, Illinois), consisting of 160 mg at day 0, 80 mg at 2 weeks, and 40 mg at 4 weeks. All patients underwent a colonoscopy at day 0 and again one week postadministration of the fourth week of adalimumab dose. During the first colonoscopy, pinch biopsies from inflamed and noninflamed areas were obtained, and biopsies were taken from the same anatomical segments during the second colonoscopy. Blood samples were also drawn on the day of each colonoscopy.

2.2. Biochemical Parameters. Biochemical parameters (CRP and fecal calprotectin) were monitored at the times of blood sampling. All blood and fecal samples were analyzed by The Department of Clinical Biochemistry, Aarhus University Hospital, Aarhus, Denmark.

2.3. Analyses of Mucosal IL-17A, IL-21, and IL-22 Gene Expression. RNA was automatically isolated from paraffin-embedded mucosal biopsies using a QIAsymphony according to the manufacturer's protocol. The RNA concentrations and purity were determined using a NanoDrop 2000® 200 NanoQuant (Thermo Scientific). Predesigned primer and probe sets for IL-17A, IL-21, and IL-22 (Life Technologies, Darmstadt, Germany cat. number Hs00174383_m1, Hs00222327_m1, and Hs01574154_m1, resp.) labelled with the FAM-BHQ system as a fluorescence/quencher were used. RT-qPCR was performed on a 96-well StepOnePlus™ Real-Time PCR System (Life Technologies) using a 1-step protocol with TaqMan Gene Expression Assays. Samples were duplicated and the mean cycle threshold (C_T) value was used for statistical analyses. Gene expression was standardized using the housekeeping gene HPRT-1, and data was analyzed using the delta-delta-Ct method as previously described [32].

2.4. Isolation of Lamina Propria Mononuclear Cells (LPMCs). Biopsies were collected in ice-cold PBS and immediately placed on ice. Epithelial cells were removed by incubating the tissue for 15 minutes at 37°C with HBSS-EDTA (CMF HBSS supplemented with 2% AB serum, 1.5 mM Hepes (Gibco Life Technologies, Auckland, New Zealand)) and 2 mM EDTA (Thermo Fischer Scientific/Ambion, Waltham, Massachusetts) at three separate times, followed by a wash in RPMI 1640 that was supplemented with 10% AB serum and 1.5 mM Hepes. LPMCs were prepared via a 45- to 90-minute long incubation at 37°C with 125 μl of collagenase (8 mg/ml) (Sigma-Aldrich, St Louis, Missouri) and 50 U/ml DNase I (Sigma-Aldrich), which were diluted in 5 ml RPMI 1640 supplemented with 10% AB serum and 1.5 mM Hepes. Following digestion, LPMCs were collected by filtration through a 70 μm nylon mesh (BD Biosciences, San Jose, California) and analyzed using flow cytometry.

2.5. Flow Cytometry Staining and Analysis. The freshly isolated LPMCs were adjusted to a final concentration of 2×10^6 LPMCs/ml in culture medium (RPMI 1640 with 10% pooled heat-inactivated human AB serum, 100 U/ml penicillin and 100 μg/ml streptomycin) and incubated overnight at 37°C in a 5% CO_2 atmosphere. The following day, the cells were stimulated with 0.1 μg/ml ionomycin (Sigma-Aldrich, Denmark, cat. number I0634) and 5 μg/ml phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, Denmark,

Table 1: Baseline characteristics.

Patient number	Gender	Immunosuppressant	Behavior	Location	Smoking status	CDAI	HBI	SES-CD	CRP	Fecal calprotectin
1	M	Yes	Stricturing	Ileal	No	301	6	8	8	576
2	F	Yes	Penetrating	Colonic	No	266	10	8	0.6	148
3	M	No	Nonstricturing, nonpenetrating	Colonic	No	153	4	16	3.2	1215
4	F	No	Nonstricturing, nonpenetrating	Colonic	No	299	8	11	51.7	>3600
5	M	Yes	Stricturing	Ileocolonic	No	292	10	17	0.8	405
6	M	Yes	Nonstricturing, nonpenetrating	Ileocolonic	Yes	315	10	7	9.8	941
7	M	No	Nonstricturing, nonpenetrating	Colonic	No	232	12	15	1.4	Missing
8	F	Yes	Nonstricturing, nonpenetrating	Colonic	No	179	7	18	3.8	342
9	F	Yes	Nonstricturing, nonpenetrating	Colonic	No	251	12	14	12.0	495
10	F	Yes	Nonstricturing, nonpenetrating	Colonic	No	296	10	10	5.7	178
11	M	Yes	Nonstricturing, nonpenetrating	Colonic	No	238	11	15	0.9	211
12	M	No	Stricturing	Ileocolonic	No	295	9	15	36.5	>3600

CRP: C-reactive protein in mg/L, reference < 8 mg/L; Fecal calprotectin, reference < 50 mg/kg.

cat. number P1585) in the presence of 10 μg/ml brefeldin A (Sigma-Aldrich, Denmark, cat. number B7651) for 4 hours at 37°C in a 5% CO_2 atmosphere. Then cells were harvested and 0.5×10^6 cells in 100 μl wash buffer (PBS, 2% bovine serum albumin (BSA) and 0.9% azide) were surface-stained with optimized amounts of antibodies against CD4 (anti-CD4-PerCP, BD Biosciences, cat. number 345770) and CD3 (anti-CD3-FITC, Biosciences, cat. number 555492) and Live/Dead Fixable Near-IR Dead cell stain kit (Life Technologies, cat. number L10119) according to the manufacturer's protocol. The surface staining was fixed with 1.5 ml BD FACS Lysing Solution (BD Biosciences, cat. number 349202). The cells were then permeabilized with 0.5 ml FACS Permeabilizing Solution 2 (BD Biosciences, cat. number 340973) and blocked with heat-inactivated mouse serum (Invitrogen, cat. number 10410) before staining with anti-IL-17A Alexa-647 (eBiosciences, cat. number 51-7179-42) and anti-IL-21 PE (eBiosciences, cat. number 12-7219-42) or anti-IL-22 PE (R & D, cat. number IC7821P). Finally, the cells were fixed in 250 μl PBS with 1% formaldehyde. Five-color flow cytometry was performed within 24 hours and 10^5 events in the forward-side scatter lymphocyte gate were recorded. The combination of forward-scatter-height and forward-scatter-area was used to exclude the events without single cell appearances. Live/dead stain was used to exclude nonviable cells from analysis. The stimulation of LPMCs was associated with a distinct downregulation of CD4, thereby prohibiting the possible identification of $CD4^+$ CD3 cells. Instead, we only gated the $CD3^+$ events for the analyses of intracellular IL-17A, IL-21, and IL-22 production. The gating for IL-17A, IL-21, and IL-22 was based on combined isotype and fluorescence-minus-one controls (Supplementary Figure 1). Flow cytometry was performed using a FACSCanto flow cytometer (BD Biosciences), and data was analyzed using FACS Diva 5.1 software (BD Biosciences). The staining procedure failed in four of the included patients at baseline and in three patients at the 4-week follow-up appointment. Paired samples (baseline/week 4) were therefore only available in seven of the included patients, who all responded to anti-TNFα treatment.

2.6. Statistical Analyses. Data is presented as medians with interquartile ranges (IQR). A Wilcoxon signed-rank test was used to evaluate the differences between two sets of paired samples using GraphPad Prism 6.0 (GraphPad Software, La Jolla, USA). p values < 0.05 were considered statistically significant.

2.7. Ethical Considerations. This study conformed to the Declaration of Helsinki. All participants provided a written, informed consent. The study protocol was approved by the Central Denmark Region Committee on Biomedical Research Ethics (journal number M-20100216).

3. Results

3.1. Clinical Effects of Adalimumab Treatment. Adalimumab treatment improved endoscopic disease activity scores. The SES-CD scores decreased from 15 (9–16) at baseline to 5 (2–8) at week 4 ($p = 0.003$). Two patients exhibited normal mucosa (SES-CD value = 0) at week 4. The disease activity scores reduced from a CDAI level of 279 (235–298) at baseline to 102 (61–133) at week 4 ($p = 0.002$). The level of HBI decreased from 10 (8–11) at baseline to 3 (1–5) at week

4 ($p = 0.002$). Furthermore, CRP levels decreased from 4.8 mg/L (1.2–11) at baseline to 0.9 mg/L (0.6–4.1) at week 4 ($p = 0.01$). Fecal calprotectin levels decreased from 495 mg/kg at baseline to 138 mg/kg (30–770) at week 4 ($p = 0.10$). One of the included patients (patient number 7) did not respond to adalimumab treatment and experienced an increased SES-CD score (1 point) at week 4. This patient experienced only a slight reduction in disease activity from baseline to week 4 (CDAI-score: 232–213; HBI-score: 12–10).

3.2. Increased Gene Expression of IL-17A and IL-22 and Increased Frequency of IL-21-Producing T Cells in Inflamed CD Mucosa. All samples from inflamed mucosal areas had detectable IL-17A, IL-21, and IL-22 gene expression. However, this was not true for all noninflamed tissue samples. Therefore, we censored below the detection IL-17A expression data from 1 patient and IL-21 and IL-22 expression data from 3 patients from the comparison graphs and statistical analyses presented in Figure 1(a). Baseline gene expressions of IL-17A and IL-22 were higher in mucosal areas with active inflammation compared to noninflamed mucosal areas in the same individual ($p = 0.008$ and $p = 0.03$, resp., Figure 1(a)). However, the mucosal gene expression of IL-21 only tended to be higher ($p = 0.07$) in inflamed mucosal areas compared to noninflamed mucosal areas (Figure 1(a)).

Flow cytometry analyses were performed in eight patients at baseline. The frequencies of IL-17A- and IL-22-producing T cells among LP cells were 6.3% (4–12) and 7.8% [5.2–10.8], respectively, in areas with active inflammation. These frequencies did not differ from the baseline levels in noninflamed areas of the intestine {(IL-17A 5.5% [2.8–14%], $p = 0.57$), (IL-22 (7.4% [4.3–16%] $p = 0.94$)}. There was an increased frequency of IL-21-producing LP T cells in inflamed areas of the intestines versus the frequencies of these cells in noninflamed areas of the intestines at baseline (10.3% [6.7–13%] versus 6.2% [3.2.–8.8%], $p = 0.02$) (Figure 1(b)). Flow cytometry analyses revealed that the production of IL-17A, IL-21, and IL-22 among LPMC was only present in CD3-expressing cells within the applied lymphocyte gate. There were no differences in the frequencies of LP $CD3^+$ T cells between areas with active inflammation (54% [48–59%]) compared to noninflamed area (51% [49–53%]) ($p = 0.33$).

3.3. Four Weeks of Adalimumab Treatment Does Not Change Mucosal Gene Expression or LP T Cell Production of IL-17A, IL-21, and IL-22. After 4 weeks of adalimumab treatment, new biopsies were obtained from the same areas of the intestine that were inflamed at inclusion. Gene expression of IL-17A was detectable in all obtained samples, whereas the IL-21 and IL-22 expression levels were below the detection limit in one sample, which was censored from subsequent analyses. There were no changes in mucosal gene expression of IL-17A ($p = 0.88$) or IL-21 ($p = 1.0$) compared to gene expression in the biopsies obtained from inflamed areas at baseline. Following adalimumab treatment, the IL-22 gene expression ($p = 0.08$) trended lower compared to baseline (Figure 2(a)).

Moreover, there were no changes in the frequencies of IL-17A-, IL-21-, or IL-22-producing LP T cells after 4 weeks of adalimumab treatment [3.0% (2.3–5.8%) IL-17A ($p = 0.20$), 4.5% (1.4–10.9%) IL-21 ($p = 0.18$), and 5.4% (4.2–7.9%) IL-22 ($p = 0.24$)] compared to the inflamed area at baseline (Figure 2(b)) (paired flow cytometry data were only available from seven patients). The same comparison revealed no change in the frequency of LP $CD3^+$ T cells at week 4 of adalimumab treatment [52% [46–57%] ($p = 0.61$)]. Post hoc censoring of the single patient (patient number 7) who did not respond to the adalimumab treatment did not affect the statistical interpretations of the results.

4. Discussion

Our study investigated the effects of anti-TNFα treatment on mucosal gene expression of IL-17A, IL-21, and IL-22 and LP T cell production of these cytokines in active CD. The data showed that clinical response to anti-TNFα treatment did not change these parameters. However, when comparing mucosal areas with active inflammation to noninflamed mucosal areas at baseline, we did observe increased gene expression of IL-17A and IL-22 as well as increased frequencies of IL-21-producing LP T cells.

The presence of mucosal Th17 cells and the gene expression of IL-17A are associated with CD inflammation. In general, Th17 cells have been considered as disease promoting in the progression of CD [4–6, 33]. Several studies have substantiated this by reporting increased Th17 cell levels and IL-17A gene expression levels in CD patients compared to healthy controls [6, 13–17]. A recent study also reported increased levels of mucosal Th17 cells to be associated with the endoscopic disease activity [15]. However, Hueber et al. found that anti-IL-17A treatment was associated with disease deterioration in some patients, suggesting that Th17 cells may also have an anti-inflammatory role in CD [12]. In agreement with findings by others, we observed increased IL-17A and IL-22 gene expression in inflamed CD mucosa compared to noninflamed mucosa at baseline [6, 9, 13, 14, 17]. However, this was not reflected at the cellular level as there were no differences in the frequencies of IL-17A- or IL-22-producing LP T cells between inflamed and noninflamed areas. This observation indicates that even though the same frequency of T cells expressing IL-17A and IL-22 are present in inflamed and noninflamed mucosa, the relative gene expression is higher during inflammation. This could reflect cytokine production in non-T cells such as innate lymphoid cell type 3 (ILC3), although our analyses indicated that the cytokine expression was limited to CD3 expressing lymphocytes. Globig et al. [15] and Jiang et al. [16] each demonstrated an increase in IL-17A-producing LP T cells in active CD versus mild/quiescent CD versus healthy controls. However, these studies did not include an individual comparison between inflamed and noninflamed CD as performed here. Furthermore, neither of these studies measured longitudinal changes as reported here. In contrast to Globig et al. and Jiang et al., yet similar to our findings, Leung et al. did not observe a difference in the frequency of IL-17-producing cells

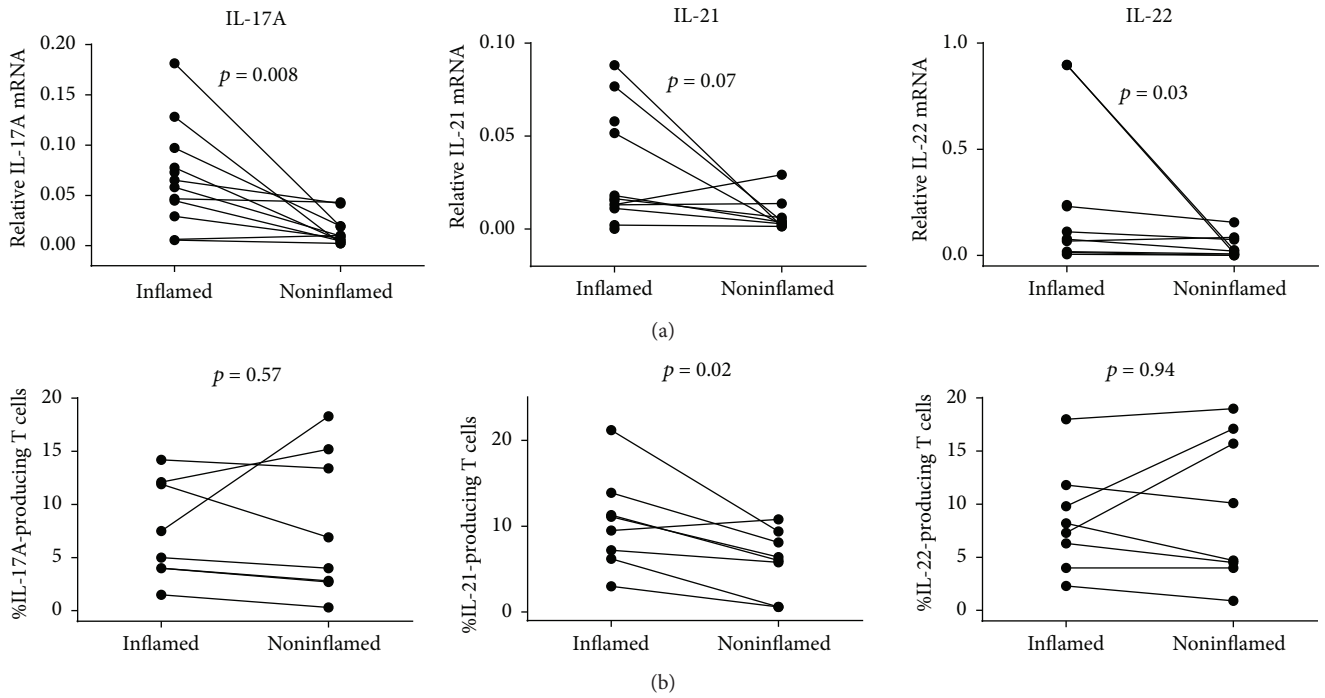

Figure 1: Mucosal gene expression and LP T cell producing IL-17A, IL-21, and IL-22 at baseline in inflamed and noninflamed mucosa. Gene expression was determined by rtPCR (a) and frequencies of IL-17A-, IL-21-, and IL-22-producing cells among LP $CD3^+$ T cells were determined by flow cytometry (b). Gene expression data is displayed as the normalized ratios between the relative expression of the gene of interest and the housekeeping gene *HPRT-1*. Wilcoxon signed-rank test for comparison was applied on paired samples (rtPCR: IL-17A $n = 11$; IL-21 $n = 9$; IL-22 $n = 9$; flow cytometry: $n = 8$ for each cytokine).

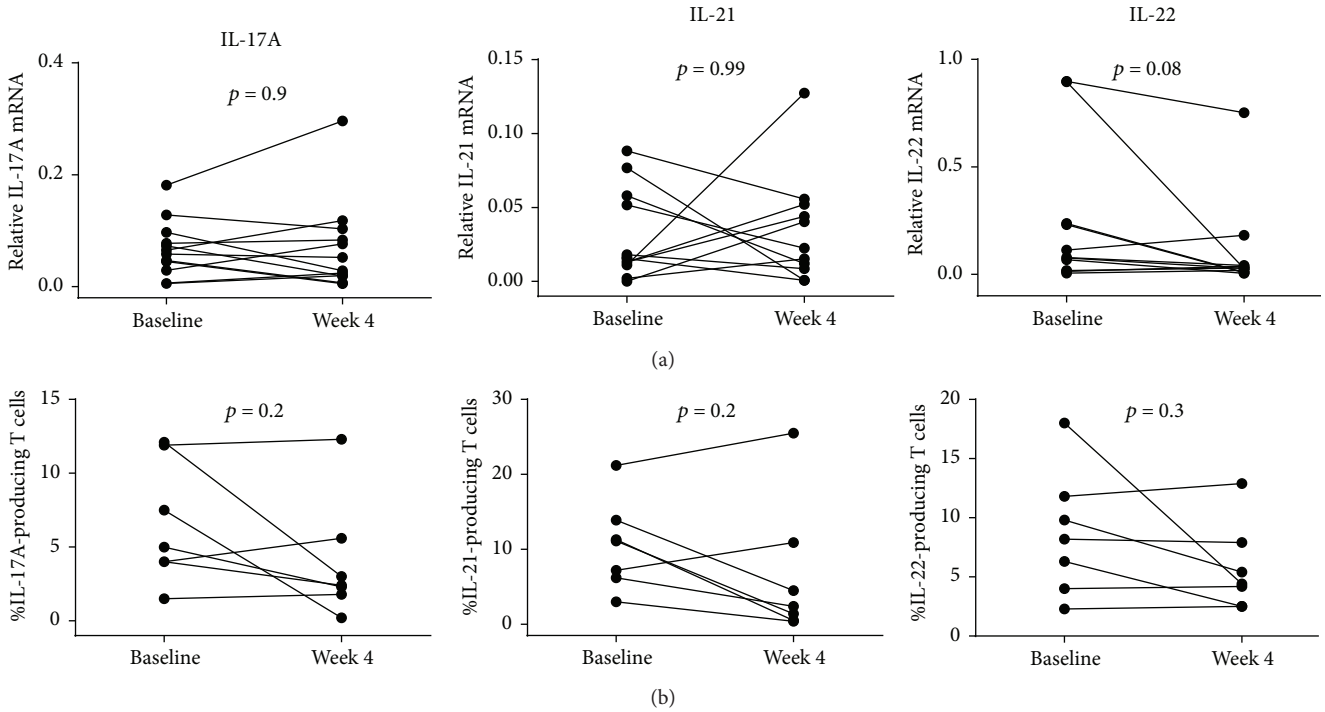

Figure 2: Comparison of mucosal gene expression and LP T cells expressing IL-17A, IL-21, and IL-22 between baseline and week 4 of adalimumab treatment. Gene expression was determined by rtPCR (a) and frequencies of IL-17A-, IL-21-, and IL-22-producing cells among LP $CD3^+$ T cells were determined by flow cytometry (b). Gene expression data is displayed as the normalized ratios between the relative expression of the gene of interest and the housekeeping gene *HPRT-1*. Wilcoxon signed-rank test for comparison was applied on paired samples (rtPCR: IL-17A $n = 12$; IL-21 $n = 11$; IL-22 $n = 11$; flow cytometry: $n = 7$ for each cytokine).

when comparing individual levels of inflamed and noninflamed CD mucosa [34]. Additionally, we did not detect any difference in the frequency of IL-22-producing LP T cells when comparing the levels of inflamed with noninflamed mucosa. One study demonstrated that the frequency of IL-22-producing LP T cells correlates with endoscopic disease activity in CD [16]; however, this study did not include an intraindividual comparison as performed here. In accordance with findings in other studies, we observed a higher level of IL-21-producing LP T cells in inflamed versus noninflamed mucosa at baseline [34, 35].

In contrast to our hypothesis, we did not observe any changes in the gene expression, in inflamed mucosa, of IL-17A, IL-21, or IL-22 as a result of anti-TNFα treatment. Neither did we observe any differences at the cellular level with respect to protein expression of these cytokines in LP $CD3^+$ cells during anti-TNFα treatment despite a marked visual improvement upon endoscopic examination and reduced clinical disease activity during treatment. However, the conclusion regarding the cellular level is based on seven patients. We investigated the frequency and not the absolute cell number of cytokine-producing $CD3^+$ LP T cells to detect any specific effects of anti-TNFα treatment on these cells. If we had studied the absolute numbers, however, we would expect to find a marked difference between inflamed and noninflamed tissue as the absolute numbers of inflammatory cells expands greatly with inflammation.

Our results are in contrast to the data reported by two studies demonstrating that 10 weeks of anti-TNFα treatment in Asian CD patients were associated with decreased gene expression of IL-17A and IL-21 [26, 27]. Furthermore, one of these studies reported decreased frequencies of IL-17A- and IL-21-producing LP cells from anti-TNFα treatment, without specifically limiting their analyses to the T cells [26]. This discrepancy can be related to genetic differences between Asian patients and the Caucasian patients included in the present study; however, a plausible explanation is the varying time points for follow-up examination in the studies (10 versus 4 weeks, resp.). We observed a marked endoscopic improvement following 4 weeks of treatment, which supports that the anti-inflammatory effects of this treatment are well established at this time point. Thus, a 10-week interval makes it intrinsically difficulty to decipher between ameliorated inflammation and the effects of anti-TNFα when examining mucosal IL17-A and IL-21 production. Consistent with needing to perform examinations earlier in the treatment course, we recently reported changes in innate immune responses from 4 weeks of anti-TNFα treatment using this same study cohort and investigational time points [36]. In that analysis, we observed reduced numbers of mucosal macrophages with intermediate HLA-DR expression and increased numbers of $CD103^+$ dendritic cells [36]. Our previous and current results together suggest that anti-TNFα treatment has a more prominent effect on innate immunity at a short interval as compared to adaptive immunity.

In conclusion, if modulation of IL-17A, IL-21, and IL-22 were mechanistically responsible for the clinical efficacy of anti-TNFα treatment, our analyses should have revealed differences between inflamed mucosa before and after 4 weeks of anti-TNFα. Since this was not the case, our data do not support this hypothesis.

Disclosure

Some of the data of this paper were presented at the Danish Society for Gastroenterology and Hepatology meeting 2017.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Acknowledgments

This work was supported by an unrestricted grant from AbbVie, Denmark.

Supplementary Materials

Figure 1: gating strategy for identification of cytokine-producing LP T cells. Isolated LP cells were identified by their forward- and side-scatter appearance (a). Events without single cell appearances were excluded by the combination of forward-scatter-height and forward-scatter-area. Live/dead stain was used to exclude nonviable cells from analysis (b). T cells were identified by their expression of CD3 (c). The lymphocyte gate for cytokine-production was set by a combination of isotype- and fluorescent-minus-one controls (d). This gate was then applied on LP cells stained for cytokine expression (in this example IL-17A and IL-22) (e). *(Supplementary Materials)*

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Hindawi
Mediators of Inflammation
Volume 2018, Article ID 9416391, 11 pages
https://doi.org/10.1155/2018/9416391

Research Article

Probiotic Mixture Protects Dextran Sulfate Sodium-Induced Colitis by Altering Tight Junction Protein Expressions and Increasing Tregs

Yingdi Zhang, Xiaojing Zhao, Yunjuan Zhu, Jingjing Ma, Haiqin Ma, and Hongjie Zhang

Department of Gastroenterology, The First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu Province 210029, China

Correspondence should be addressed to Hongjie Zhang; hjzhang06@163.com

Received 8 August 2017; Revised 27 December 2017; Accepted 15 March 2018; Published 15 April 2018

Academic Editor: Helieh S. Oz

Bifico is a probiotic mixture containing *Bifidobacterium*, *Lactobacillus acidophilus*, and *Enterococcus*. Studies support that *Bifico* has a protective effect in experimental colitis (IL-10-deficient and TNBS) models and in patients with inflammatory bowel disease (IBD). However, the mechanism underlying the protective effects of this mixture of probiotic bacteria remains incompletely clear. Here, we investigated the effect of *Bifico* on intestinal inflammation. In an *in vivo* experiment, dextran sulfate sodium was used to induce colitis. *Bifico* treatment significantly attenuated the severity of colitis in this model. *Bifico* increased the expression of tight junction proteins (TJs). In addition, *Bifico* increased the number of Tregs, but reduced the number of total $CD4^+$ T cells in the peripheral blood. Furthermore, the expression of colonic CD4 protein was decreased while the level of forkhead box P3 (Foxp3) was upregulated. These results suggested that *Bifico* exerts beneficial effects on experimental colitis by increasing the expressions of TJs, upregulating the number of Tregs, and reducing the total $CD4^+$ T cell number in both colon and peripheral blood. The intestinal damage in the pretreated + treated-*Bifico-colitis* group was more severe than that in only the pretreated-*Bifico*-colitis group. This suggested that *Bifico* might aggravate intestinal damage when the mucosal barrier is impaired.

1. Introduction

The intestinal microbiota play a role in triggering the immune system and leading to intestinal inflammation [1]. IBD patients suffer from dysbiosis, which is characterized by a decrease in diversity and abundance of some dominant commensal bacteria [2]. Some studies showed that the amounts of *Lactobacillus* and *bifidobacteria* were significantly reduced in the feces of IBD patients [3, 4]. This suggests that gut flora normalization may serve as a therapeutic option for IBD patients. *Bifico* is a probiotic mixture, containing *Bifidobacterium*, *Lactobacillus*, and *Enterococcus*. In fact, evidences from both experimental studies and clinical trials have demonstrated the various therapeutic effects of *Bifico* on Crohn's disease, ulcerative colitis, colitis-induced and colitis-associated cancer in mice, pouchitis, diarrhea, and gastritis [5–12]. A previous study demonstrated that *Bifico* can increase the expression of colonic TJs and promote intestinal epithelial barrier function in interleukin-10-deficient (IL-10 KO) mice [5]. *In vitro* experiments also showed that the *Bifico*, or single probiotic strains (*Bifidobacterium*, *Lactobacillus*, or *Enterococcus*), increase transepithelial electrical resistance (TER) and the expression of TJs in enteroinvasive *Escherichia coli*- (EIEC-) treated Caco-2 monolayers. *Bifico* significantly inhibited the secretion of proinflammatory cytokines in EIEC-treated Caco-2 monolayers. *Bifico* exposure in vitro reduced bacterial invasion. Moreover, the effects of combined probiotics were more pronounced than those of single-strain probiotics. Another study reported that the anti-inflammatory effects of *Bifico* were related to the expansion of Tregs in mesenteric lymph nodes and disturbance of Th1/Th2 cytokines in the colonic mucosa of TNBS-induced colitis mice. However, the effect of *Bifico* on the Treg cells in intestinal tissue and peripheral blood has not been

reported. In addition, previous animal experimental studies mainly observed the effect of *Bifico* as a therapeutic in active colitis [6].

The aims of this study were to observe the beneficial effect of *Bifico* on intestinal inflammation in dextran sodium sulfate (DSS) experimental colitis and to investigate whether the beneficial effect is associated with local and systemic immune responses. This study explored the protective effect of *Bifico* pretreatment on subsequent intestinal inflammation.

2. Materials and Methods

2.1. Animals. A total of 44 female specific-pathogen-free BALB/c mice (aged 8 to 10 weeks, weighing 20–24 g) were purchased from the Laboratory Animal Center of Nanjing Medical University (Jiangsu, China), maintained in clean cages under a 12 h light-dark cycle and conventional housing conditions, and fed with standard mouse chow. All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the protocol was approved by the Animal Ethics Committee of Nanjing Medical University (Approval ID: NJMU20110312). The *Bifico* capsule contains *Bifidobacterium*, *Lactobacillus*, and *Enterococcus* living bacteria not less than 3×10^7 CFU (Shanghai Sine Pharmaceutical).

2.2. DSS-Induced Colitis and Experimental Design. 4% DSS (0216011080, M.W 36–50 kDa, MP Biomedicals) in drinking water was used to induce acute colitis models as reported before [13, 14]. The DSS was replaced every 2 days. Female BALB/c mice were randomly divided and treated as follows: group 1 (normal, $n = 11$): mice received sham (saline, days 1 to 14); group 2 (*Bifico*, $n = 8$): *Bifico* (days 1 to 14); group 3 (colitis, $n = 8$): DSS start on days 8–14 (saline days 1–14); group 4 (pretreated-*Bifico*-colitis, $n = 9$): *Bifico* (days 1–7) then DSS + sham (days 8–14); and group 5 (pretreated + treated-*Bifico*-colitis, $n = 8$): *Bifico* (days 1–14) and DSS added (days 8–14). At day 15, all animals were euthanatized (Figure 1). All treatments except DSS were given by oral gavage.

2.3. Disease Activity Index (DAI). The DAI was determined by grading on a scale of 0–4, according to previous reports [15]: briefly, weight loss (0, ≤1%; 1, 1–5%; 2, 5–10%; 3, 10–20%; and 4, >20%), intestinal bleeding (0, negative; 2, hemoccult; 4, gross bleeding), and stool consistency (0, normal; 2, loose stools; 4, diarrhea).

2.4. Histological Scores. The colons were excised from the colon-cecal junction to the anus; the lengths of the colon were measured [16]. The distal colon was fixed with 10% formalin [17] and embedded in paraffin. Paraffin sections (4 μm) were stained with hematoxylin-eosin (H&E). Histological scores were evaluated as previously reported [18]: inflammation (none = 0, slight = 1, moderate = 2, and severe = 3), inflamed area/extent (mucosa = 1, mucosa and submucosa = 2, and transmural = 3), crypt damage (none = 0, basal 1/3 damaged = 1, basal 2/3 damaged = 2, only the surface epithelium is intact = 3, and entire crypt and epithelium are lost = 4), and percent involvement (1–25% = 1, 26–50% = 2, 51–75% = 3, and 76–100% = 4).

2.5. Measurement of Tumor Necrosis Factor (TNFα) Levels in Colonic Tissues. Colonic tissues were homogenized in cold PBS containing a cocktail of protease inhibitors supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF). The levels of TNFα in colonic tissues were measured with an enzyme-linked immunoassay kit (ExCell Biology, Shanghai, China), according to the manufacturer's instructions.

2.6. Transmission Electron Microscopy (TEM). Colonic tissue samples were cut into 1 mm × 1 mm × 2 mm sections and were fixed in 2.5% glutaraldehyde for 2 h at 4°C. The sections were then postfixed in 1% osmic acid for 2 h at 4°C, washed with 0.1 M PBS, dehydrated in a graded series of acetone concentrations, embedded in a mounting medium at 37°C for 3 h, and then polymerized at 60°C for 36 h. After ultrasectioning with an ultrathin slice machine (Leica, Germany), the ultrathin sections were viewed and photographed with a JEOL-1010 TEM (Japan).

2.7. Flow Cytometric Analysis of $CD4^+CD25^+Foxp3^+$ T Cells. Single-cell suspensions were prepared from the peripheral blood and spleen, according to the manufacturer's protocols. Red blood cells in peripheral blood and spleen were lysed using Red Blood Cell Lysis Buffer (Beyotime, China). This step was repeated 2-3 times until no more red blood cells were visible. After that, mononuclear cells were resuspended in RPMI 1640 serum-free medium at a final cell density of 1×10^7/mL. Fluorescein isothiocyanate- (FITC-) anti-mouse CD4 (RM4-5, 0.125 μg/test, eBioscience) and allophycocyanin-anti-mouse CD25 (PC61.5, 0.06 μg/test, eBioscience) were added to 100 μL of the cell suspension for 30 min, followed by the addition of 1 mL of fixation/permeabilization working solution (eBioscience) for 10 h at 4°C. Finally, the cells were incubated with phycoerythrin- (PE-) anti-mouse/rat Foxp3 (FJK, 0.5 μg/test, eBioscience) for 30 min at 4°C in the dark. Cells labeled with rat IgG2a PE were used as the isotype negative control. All flow cytometric measurements were performed on a flow cytometer (Beckman Coulter, Krefeld, Germany).

2.8. Immunofluorescence and Immunohistochemical Staining. Immunofluorescence staining of paraffin-embedded sections of colonic tissues was performed in accordance with routine procedures. Slides were incubated with the primary rabbit anti-Foxp3 antibody (1 : 400, Abcam, USA) overnight at 4°C, followed by FITC-labeled secondary goat anti-rabbit IgG antibody (Jackson ImmunoResearch, USA). The sections were then covered with anti-fade mounting medium (Beyotime, China) and were visualized by fluorescence microscopy. Immunohistochemical staining for TJs (JAM-1, 1 : 100, Abcam; claudin-4, 1 : 200, Abcam; occludin, 1 : 100, Proteintech) was performed as described previously [19].

2.9. Western Blotting. Colonic tissues were homogenized, and a total 50 μg of protein was blotted onto a polyvinylidene difluoride membrane (Roche, Germany). The membranes were then incubated with specific polyclonal rabbit antibodies: anti-Foxp3 (1 : 2000, Abcam, USA), anti-IL-17

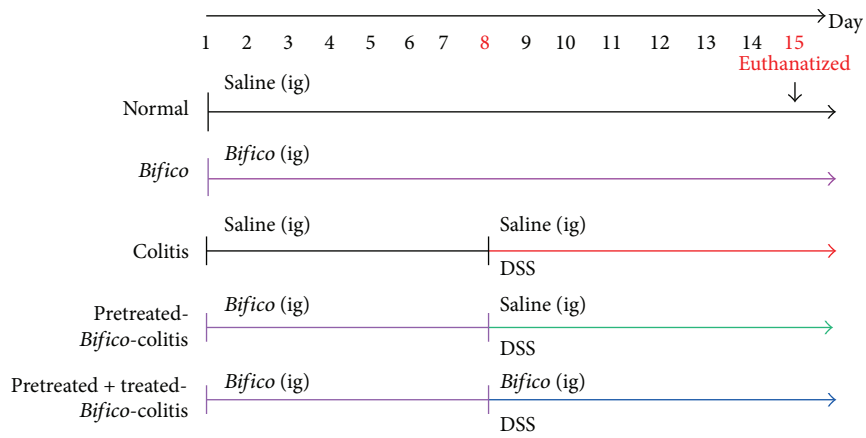


Figure 1: Schematic illustrations of the experimental protocols used in this study. A total of 44 female BALB/c mice were randomly assigned into 5 groups. Normal ($n = 11$), *Bifico* ($n = 8$), colitis ($n = 8$), pretreated-*Bifico*-colitis ($n = 9$), and pretreated + treated-*Bifico*-colitis ($n = 8$). ig: intragastric administration.

(1 : 1000, Abcam, USA), anti-JAM-1 (1 : 1000, Abcam, USA), anti-claudin-4 (1 : 300, Abcam, USA), anti-occludin (1 : 600, Proteintech, China), and monoclonal mouse anti-CD4 (1 : 100, Abcam, USA). The secondary antibodies were incubated at room temperature for 1 h. Data were analyzed by ImageLab2.0.1 software and normalized to GAPDH expression.

2.10. RNA Isolation and Quantitative RT-PCR. Total RNA from the colonic tissues was extracted using TRIzol® reagent (Life Technologies). The total RNA concentration was measured with a BioPhotometer (Eppendorf, Hamburg, Germany). A total of 1 μg total RNA was reverse-transcribed into cDNA using oligi (dT) 18 primers (TaKaRa). The primers were as follows: mouse IL-17 sense, 5′-CTCCAGA AGGCCCTCAGACTAC-3′; antisense, 5′-AGCTTTCCCTC CGCATTGACACAG-3′; and mouse β-actin sense, 5′-AC CACCATGTACCCAGGCATT-3′; antisense, 5′-CCACAC AGAGTACTTGCGCTCA-3′. Following the reactions, cycle threshold values were determined by setting a fixed threshold. The relative amount of IL-17 mRNA was normalized to the reference gene β-actin.

2.11. Statistical Analysis. All images are representative of at least three independent experiments. Data are presented as the mean ± standard error of the mean (SEM). P values were calculated by one-way ANOVA followed by the Tukey post hoc test (SPSS version 21.0). P value < 0.05 was considered significant.

3. Results

3.1. Bifico Attenuated Colitis in Mice. The DAI scores in pretreated-*Bifico*-colitis and pretreated + treated-*Bifico*-colitis groups dramatically decreased compared with the colitis group ($P_1 < 0.001$ and $P_2 < 0.01$) (Figure 2(a)). The mean lengths of the colon were significantly improved in pretreated-*Bifico*-colitis and pretreated + treated-*Bifico*-colitis groups compared with the colitis group ($P_1 < 0.01$ and $P_2 < 0.05$, resp.) (Figures 2(b) and 2(c)). Furthermore, the villus necrosis, hemorrhage, and inflammatory cell infiltrates in the lamina propria were shown in colonic tissues of colitis mice. *Bifico* treatment drastically alleviated inflammatory cell infiltrates in the colon. Meanwhile, the values of colonic histological score in pretreated-*Bifico*-colitis and pretreated + treated-*Bifico*-colitis groups drastically decreased compared with the colitis group ($P < 0.001$ for both) (Figures 2(d) and 2(e)).

3.2. Administration of Bifico Reduced Colonic Levels of TNFα in Colitis Mice. As reported previously, the colonic levels of TNFα were increased in the colitis group ($P < 0.01$). Meanwhile, the level of TNFα in the pretreated-*Bifico*-colitis group was decreased compared to the colitis group ($P < 0.05$). However, there was no significant difference between the colitis group and the pretreated + treated-*Bifico*-colitis group. Notably, the colonic levels of TNFα were slightly increased in the *Bifico* group, but there was no significance compared with the normal group ($P = 0.934$) (Figure 3).

3.3. Alterations in Microstructures of the Colonic Epithelial Barrier in Colitis Mice Observed by TEM. The morphology of the colonic epithelium and the cell membrane was intact, the cell junction was tight, and the villi on the cell surfaces were well-arranged in both the normal group and *Bifico* groups. However, in the colitis group, the integrity of the epithelial membranes was compromised, cell-cell junctions were loose, with an obvious intercellular space broadening, and villi on the cell surfaces were decreased (Figure 4(a)). The microstructure of the cell-cell junction was improved in pretreated-*Bifico*-colitis and pretreated + treated-*Bifico*-colitis groups, and some villi on the surfaces were reserved.

3.4. Expressions and Distribution of TJs in Colonic Tissues and Epithelium Barrier. Compared with the normal group, the expressions of the TJs were significantly reduced in the colitis group ($P < 0.01$ for JAM-1, $P < 0.001$ for occludin, and $P < 0.001$ for claudin-4). The expression levels of these TJs in pretreated-*Bifico*-colitis and pretreated + treated-*Bifico*-colitis groups were extensively increased compared with the

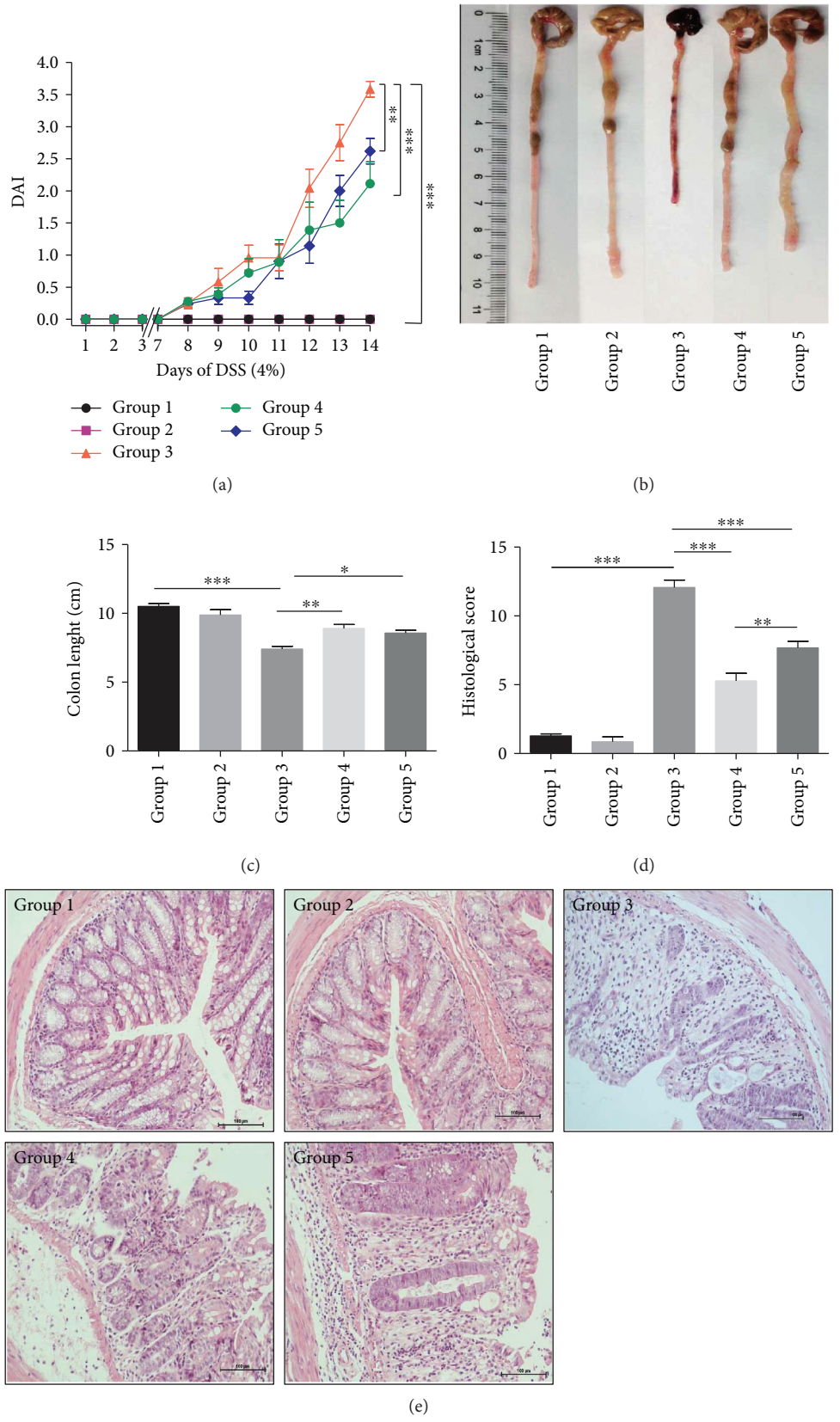


Figure 2: Evaluation of treatment efficacy in colitis mice. (a) DAI was determined in each group of mice. ($n = 8$–11/group). (b) and (c) Macroscopic observation of the colon. Colon length measured in cm. (d) Histological scores were evaluated in colons. (e) Typical histological images (200x magnification). Data are means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Group 1: normal; group 2: *Bifico*; group 3: colitis; group 4: pretreated-*Bifico*-colitis; group 5: pretreated + treated-*Bifico*-colitis.

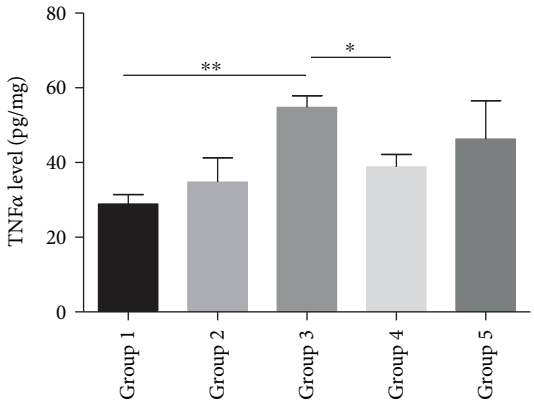


Figure 3: Administration of *Bifico* and concentration of TNF*α*. The colonic levels of TNF*α* were increased in the colitis group (3) compared to the normal group (1) ($P < 0.01$) and decreased in the Pretreated-*Bifico*-colitis group (4) and pretreated + treated-*Bifico*-colitis group (5) ($P_1 < 0.05$ and $P_2 = 1.00$, resp.). Data are means ± SEM ($n = 8$–11/group). $^*P < 0.05$ and $^{**}P < 0.01$.

colitis group ($P_1 < 0.05$ and $P_2 < 0.05$ for JAM-1, $P_1 = 0.073$ and $P_2 < 0.01$ for occludin, and $P_1 = 0.598$ and $P_2 < 0.05$ for claudin-4). In addition, the expression levels of occludin and claudin-4 in the pretreated + treated-*Bifico*-colitis group were slightly higher than those in the pretreated-*Bifico*-colitis group with no significant difference ($P = 0.246$ for occludin and $P = 0.345$ for claudin-4) (Figure 4(b)). There was no difference between the *Bifico* group and the normal group, but a scattered distribution and decreased expressions of TJs were found in colitis mice. However, the intensity and the percentage of cells stained for TJs were improved in pretreated-*Bifico*-colitis and pretreated + treated-*Bifico*-colitis groups, in comparison with mice in the colitis group (Figure 4(c)).

3.5. Bifico Effects on Total CD4$^+$ T Cells, CD4 Protein Expression, Tregs, and Foxp3$^+$ Cells. The proportion of total CD4$^+$ T cells in the peripheral blood in the colitis group was increased (colitis versus normal group: 55.10 ± 4.73 versus 34.19 ± 1.49, $P < 0.01$); however, compared with the colitis group, the increased proportion of total CD4$^+$ T cells was partially reversed both in pretreated-*Bifico*-colitis (36.04 ± 3.99 versus 55.10 ± 4.73, $P < 0.01$) and pretreated + treated-*Bifico*-colitis groups (47.90 ± 3.66 versus 55.10 ± 4.73, $P = 0.556$) (Figure 5(a)). There was no significant difference in the number of splenic CD4$^+$ T cells among different groups (Figure 5(b)). In addition, there was a decreasing trend of the colonic CD4 protein level in the pretreated-*Bifico*-colitis group compared with the colitis group ($P = 0.639$). Meanwhile, a significant difference was shown between pretreated + treated-*Bifico*-colitis and colitis groups ($P < 0.01$) (Figure 5(c)). In addition, the population of CD4$^+$CD25$^+$Foxp3$^+$ Tregs in the peripheral blood was significantly upregulated in the pretreated-*Bifico*-colitis and pretreated + treated-*Bifico*-colitis groups compared with the colitis group ($P < 0.05$ for both) (Figure 6(a)). However, no significant difference in the amount of CD4$^+$CD25$^+$Foxp3$^+$ Tregs in the spleen was detected among groups (Figure 6(b)). In fact, the number of Foxp3$^+$ Tregs in colonic tissue was increased by the administration of *Bifico* in pretreated-*Bifico*-colitis and pretreated + treated-*Bifico*-colitis groups compared with the colitis group (Figure 6(c)). The expression of Foxp3 protein was significantly decreased in the colitis group compared with the normal group ($P < 0.001$) as well as the pretreated-*Bifico*-colitis group ($P < 0.05$). Notably, the expressions of colonic Foxp3 protein were slightly increased in the pretreated + treated-*Bifico*-colitis group, but was not significant ($P = 0.224$) (Figure 3 versus (Figure 6(d)). However, compared with the normal group, the expression of Foxp3 protein in the *Bifico* group was decreased ($P < 0.05$).

3.6. Bifico Modulated the Expression of IL-17. There was an increasing trend for the colonic IL-17 mRNA expression in the pretreated-*Bifico*-colitis and pretreated + treated-*Bifico*-colitis groups ($P_1 < 0.01$ and $P_2 < 0.05$, resp.) and a decrease in the colitis group (Figures 7(a)–7(c)) ($P = 0.780$).

4. Discussion

Some studies suggested that *Bifico* supplements are able to prevent colitis development in human IBD and experimental colitis [5–7, 20]. The current study shows that the intestinal damage in the pretreated + treated-*Bifico*-colitis group was more severe compared with that in the pretreated-*Bifico*-colitis group, suggesting that *Bifico* might have a protective effect on colonic tissue in the intact intestinal mucosal barrier. In contrast, when the mucosal barrier is impaired, probiotics might aggravate colonic tissue damage, and this needs further research. The TJs consist of transmembrane proteins such as occludin, claudins, junctional adhesion molecules, and adaptor proteins like Zos [21]. An oral administration of *Bifico* has been shown to reduce colon inflammation and to protect epithelial barrier function in IL-10 KO mice [5]. Consistent with these findings, this study shows that *Bifico* increased the expressions of TJs and improved the microstructures in colitis mice. Previous investigators showed that *Bifico* treatment significantly reduced the levels of TNF*α* in the colon of experimental colitis mice [5, 6]. Our data support these findings that *Bifico* may ameliorate intestinal inflammation by inhibiting TNF*α* production.

Foxp3 is required for induction of the immune suppressive function of CD4$^+$Foxp3$^+$Tregs and maintenance of mucosal immune homeostasis by regulating the balance between CD4$^+$Foxp3$^+$Tregs and helper effector T cells. Tregs are critically involved in the prevention of human IBD and experimental colitis [6, 22]. A previous study has shown that the anti-inflammatory effects of *Bifico* were related to the expansion of Tregs in mesenteric lymph nodes of colitis mice and the ratio of Th1/Th2 might be regulated by Tregs [6]. We found that *Bifico* increased the number of Tregs in the peripheral blood. However, the numbers of Tregs in the spleen among groups showed no significant differences. Meanwhile, *Bifico* could increase the colonic Foxp3 protein level, but decrease the colonic CD4 protein level in colitis mice. These results may indicate that the decreased frequency of Treg subsets in the peripheral blood and Foxp3 expression in the colonic tissues might destroy the intestinal immune

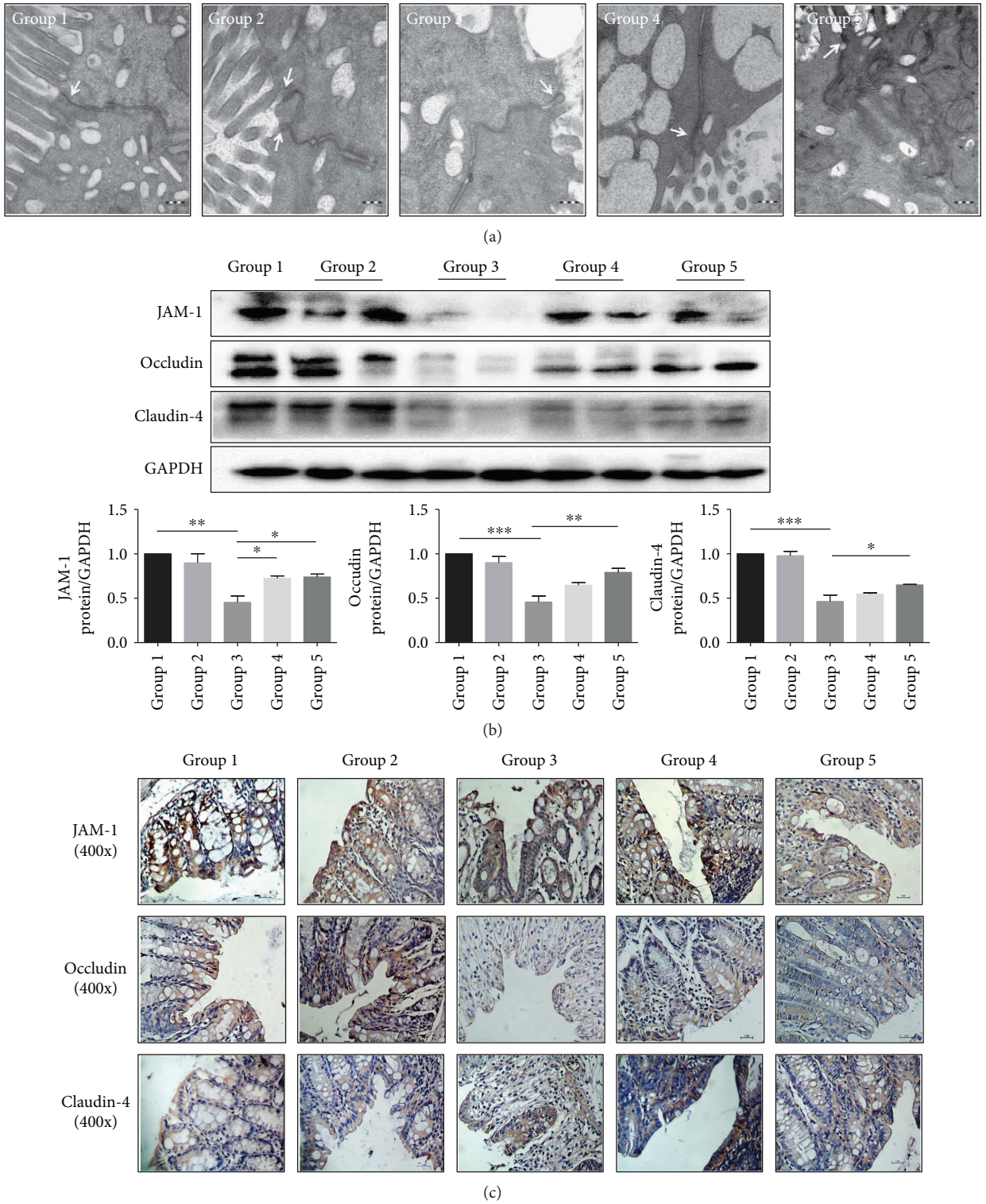


FIGURE 4: The effect of *Bifico* on the expression and distribution of TJs (a). Microstructural changes in colonic tissues of each group by TEM (80000x magnification) (b) The expressions of JAM-1, occludin, and claudin-4 were detected by Western blot. Group 1 ($n = 3$), group 2 ($n = 6$), group 3 ($n = 6$), group 4 ($n = 6$), and group 5 ($n = 6$). (c) The expressions and distribution of TJs (JAM-1, occludin, and claudin-4) were analyzed by immunohistochemistry (400x magnification). Data are means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. Group 1: normal; group 2: *Bifico*; group 3: colitis; group 4: pretreated-*Bifico*-colitis; group 5: pretreated + treated-*Bifico*-colitis.

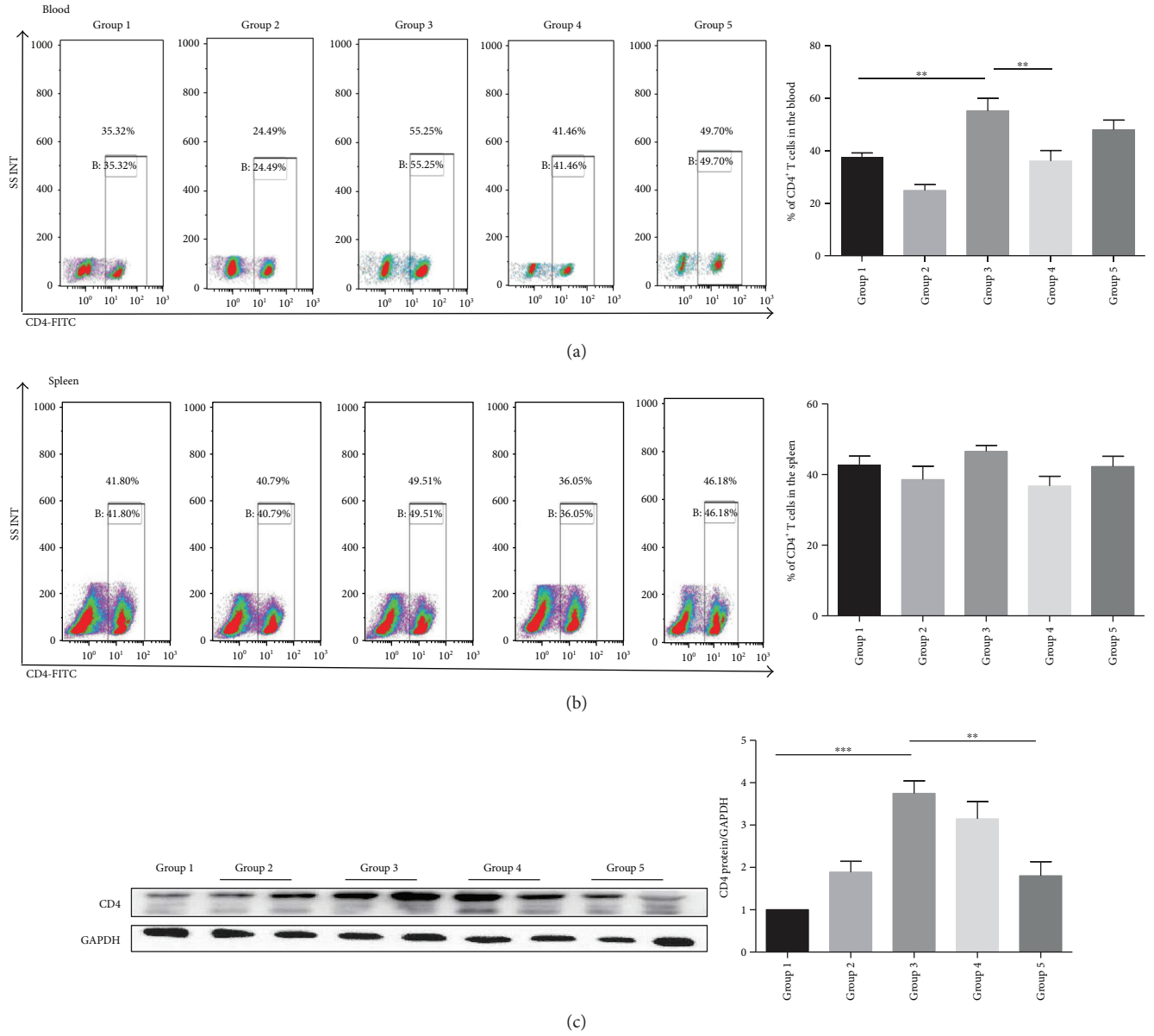
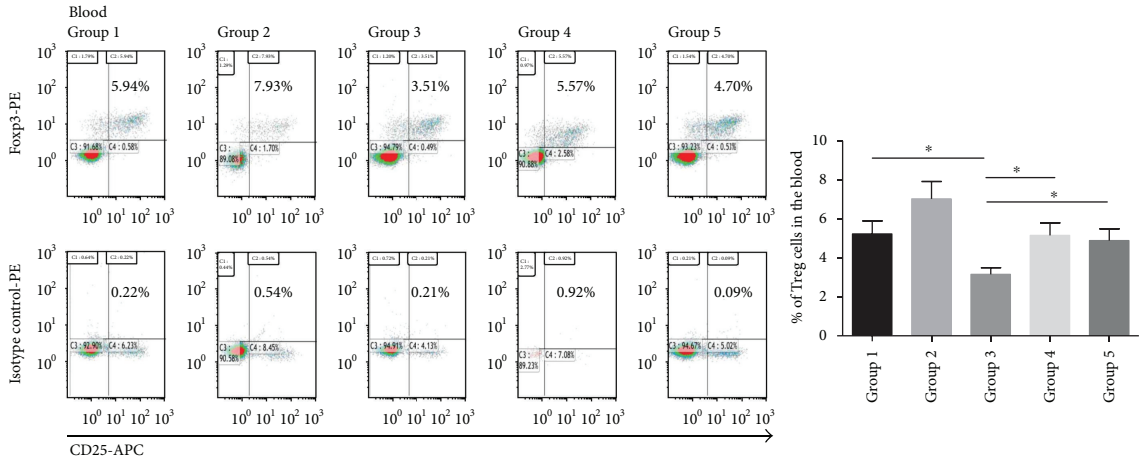


FIGURE 5: The proportion of total $CD4^+$ T cells in the peripheral blood and spleens in each group. (a) The frequency of $CD4^+$ T cells in the blood. (b) The proportion of total $CD4^+$ T cells in the spleen ($n = 6$–9/group). The number of $Foxp3^+$ cells in colonic tissues (the white arrows indicate $Fox3^+$ cells) ($n = 6$–9/group). (c) Western blot analysis of CD4 protein expression levels. Group 1 normal ($n = 3$); group 2 *Bifico* ($n = 6$); group 3 colitis ($n = 6$); group 4 pretreated-*Bifico*-colitis ($n = 6$); group 5 pretreated + treated-*Bifico*-colitis ($n = 6$). Data are means ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$.

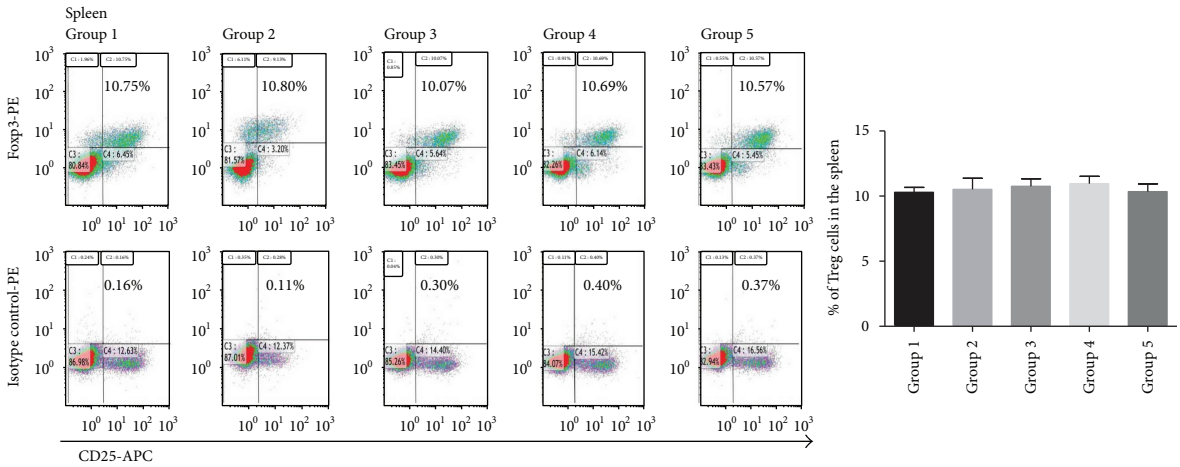
tolerance and activate inflammation. However, a significant investigation is required to prove these explanations.

Of note, the normal mice treated with *Bifico* demonstrated a slightly increased TNFα level, a decreased Foxp3 protein level, and an increased CD4 protein expression in colonic tissues as well as an increased number of Tregs in the peripheral blood. It is possible that *Bifico* acts as a foreign antigen and triggers weak immune responses, yet this immune response may not lead to pathological inflammatory damage. This suggested that proper immune activation may enhance the mucosal defense and thus be beneficial for the promotion of the host intestinal immunity [23]. These also need to be investigated.

Some studies have demonstrated that IL-17 is an important proinflammatory cytokine and is highly expressed in the inflamed gut in IBD patients and colitis mice [24, 25]. In contrast, other studies have suggested that IL-17A has a protective role in a T-cell transfer model of colitis [26, 27]. Furthermore, the severity of murine colitis was enhanced with anti-IL-17 neutralizing antibody treatment or IL-17A knockout [28, 29]. In our study, the expressions of IL-17 mRNA and protein were decreased in colitis mice, but were increased after *Bifico* intervention. This is in agreement with a previous study, which showed that the expression of IL-17 in colitis mice was increased treated with *Bifidobacterium breve* [16]. The production of splenic IL-17 is reduced by



(a)



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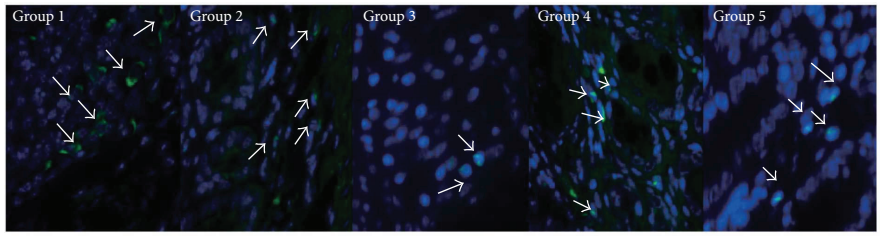

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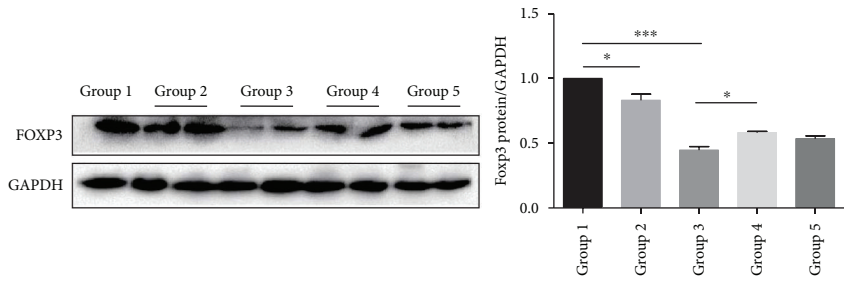

(d)

FIGURE 6: The proportion of total $CD4^+CD25^+Foxp3^+$ Tregs cells in the peripheral blood and spleen. (a) The frequency of Treg cells in the blood ($n = 6$–9/group) (b) and in the spleen ($n = 6$–9/group). (c) The number of $Foxp3^+$ cells in colonic tissue (the white arrows indicate $Fox3^+$ cells). (d) Western blot analysis of mice colonic Foxp3 protein expression level in each group. Group 1 normal ($n = 3$); group 2 *Bifico* ($n = 6$); group 3 colitis ($n = 6$); group 4 pretreated-*Bifico*-colitis ($n = 6$); group 5 pretreated + treated-*Bifico*-colitis ($n = 6$). Data represent means ± SEM. $^*P < 0.05$ and $^{***}P < 0.001$.

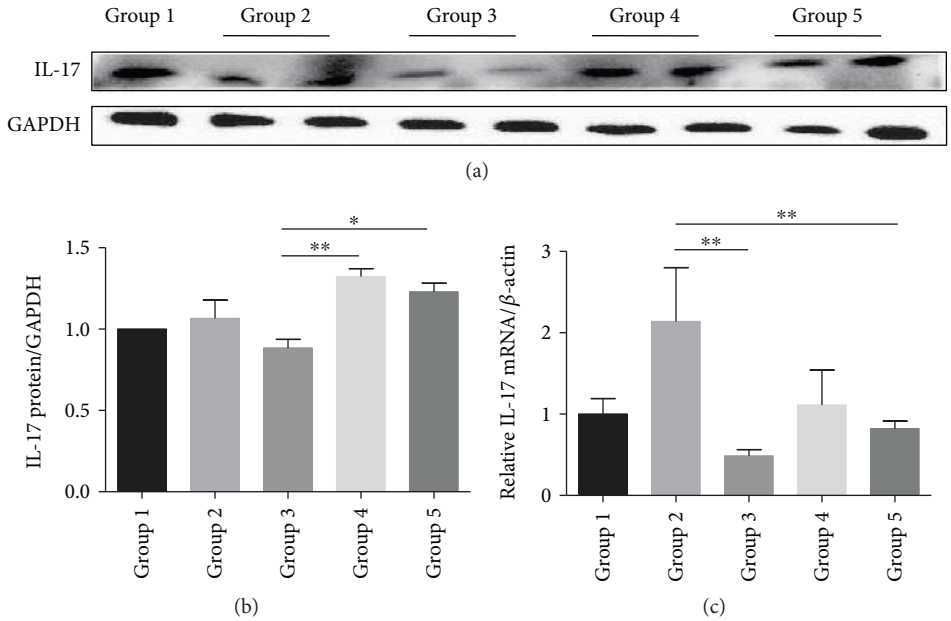

Figure 7: *Bifico* modulated the expression of IL-17. (a, b) Western blot analysis of IL-17 protein expression levels in colonic tissues. Group 1 normal ($n = 3$); group 2 *Bifico* ($n = 6$); group 3 colitis ($n = 6$); group 4 pretreated-*Bifico*-colitis ($n = 6$); group 5 pretreated + treated-*Bifico*-colitis ($n = 6$). (c) The levels of IL-17 mRNA ($n = 8$–11/group). Data are means ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$.

DSS [30]; however, the precise roles of IL-17 in the development of IBD need to be elucidated in future studies.

Our study has some strengths, as *Bifico* might have a protective effect on colonic tissue when the intestinal mucosal barrier is intact. In contrast, when the mucosal barrier is impaired, probiotics might aggravate colonic tissue damage. *Bifico* could increase the number of Tregs in the peripheral blood and have no influence in the spleen among different groups. Our study has several limitations. First, TJ expression is only an indirect reflection of the barrier function. To exactly measure the barrier function, physiologic measurements like absorption of orally administered dextran or bacteria-size particles should be performed. Second, this study explored the effects of *Bifico* as a whole, but functions of single probiotic strains contained in *Bifico* (*Bifidobacterium*, *Lactobacillus*, *or Enterococcus*) were not studied separately. Third, these results suggested that *Bifico* might exert beneficial effects on experimental colitis by upregulating the number of Tregs and reducing total $CD4^+$ T cells in both colonic tissue and peripheral blood. Further, pathways through which Tregs and $CD4^+$ T cells ameliorate the inflammation need to be explored. Finally, this study only explored the preventive effects of *Bifico* (with no major clinical application) while no study was performed to explore therapeutic effects in this model. Therefore, extensive exploration in this field is needed.

5. Conclusions

This study demonstrated some beneficial effect of *Bifico* on colitis. The potential mechanism involved in improving the expression of TJs is increasing the number of Tregs in colonic tissues and the peripheral blood, while decreasing the proportions of total $CD4^+$ T cells in colonic tissues and the peripheral blood.

Abbreviations

IBD: Inflammatory bowel disease
DSS: Dextran sulfate sodium
TJs: Tight junction proteins
JAM-1: Junctional adhesion molecule 1
Foxp3: Forkhead box P3
IL-17: Interleukin-17
IL-10: Interleukin-10
DAI: Disease activity index
H&E: Hematoxylin-eosin
TNFα: Tumor necrosis factor
PMSF: Phenylmethylsulfonyl fluoride
TEM: Transmission electron microscopy
FITC: Fluorescein isothiocyanate
PE: Phycoerythrin
SEM: Standard error of the mean.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Authors' Contributions

Yingdi Zhang and Hongjie Zhang conceived and designed the experiments; Yingdi Zhang, Xiaojing Zhao, and Yunjaun Zhu performed the experiments; Yingdi Zhang analyzed the data; Jingjing Ma and Haiqin Ma contributed materials/analysis tools; and Yingdi Zhang, Hongjie Zhang, and

Xiaojing Zhao wrote the paper. Yingdi Zhang, Xiaojing Zhao, and Yunjuan Zhu contributed equally to this work.

Acknowledgments

This study was supported by grants from the National Natural Science Foundation of China (81470827).

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Hindawi
Mediators of Inflammation
Volume 2018, Article ID 3232491, 13 pages
https://doi.org/10.1155/2018/3232491

Research Article

Naringenin Protects against Acute Pancreatitis in Two Experimental Models in Mice by NLRP3 and Nrf2/HO-1 Pathways

Yong Li,[1] Yiyuan Pan,[1] Lin Gao,[1] Jingzhu Zhang,[1] Xiaochun Xie,[1] Zhihui Tong,[1] Baiqiang Li,[1] Gang Li,[1] Guotao Lu,[1,2] and Weiqin Li[1]

[1]*Surgical Intensive Care Unit (SICU), Department of General Surgery, Jinling Clinical Medical College of Nanjing Medical University, No. 305 Zhongshan East Road, Nanjing, Jiangsu Province 210002, China*
[2]*Department of Gastroenterology, The Affiliated Hospital of Yangzhou University, Yangzhou University, Yangzhou, China*

Correspondence should be addressed to Guotao Lu; gtlu@yzu.edu.cn and Weiqin Li; lwqsaplab@163.com

Received 12 October 2017; Revised 28 January 2018; Accepted 5 February 2018; Published 8 April 2018

Academic Editor: Amedeo Amedei

Background. Naringenin (Nar) is a type of flavonoid and has been shown to have anti-inflammatory and antioxidative properties. However, the effects of Nar on acute pancreatitis (AP) have not been well studied. In this study, we aimed to investigate the function of Nar in a mouse model of AP. *Methods*. Mild acute pancreatitis (MAP) was induced by caerulein (Cae), and severe acute pancreatitis (SAP) was induced by L-arginine in mice. Nar was administered intraperitoneally at doses of 25, 50, or 100 mg/kg following MAP induction and at a dose of 100 mg/kg following SAP induction. The serum levels of cytokines, lipase, and amylase were determined, and pancreatic and pulmonary tissues were harvested. *Results*. The serum levels of amylase, lipase, and cytokines were significantly decreased in both MAP and SAP models after Nar treatment. The malondialdehyde (MDA) levels of the pancreatic tissue was significantly reduced in both MAP and SAP after Nar treatment. In contrast, glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferase (GST), total sulfhydryl (T-SH), and non-proteinsulthydryl (NP-SH) were markedly increased in both MAP and SAP after Nar treatment. The injury in pancreatic and pulmonary tissues was markedly improved as evidenced by the inhibited expression of myeloperoxidase, nod-like receptor protein 3, and interleukin 1 beta as well as the enhanced expression of nuclear factor erythroid 2-related factor 2/heme oxygenase-1 in pancreatic tissues. *Conclusions*. Nar exerted protective effects on Cae-induced MAP and L-arginine-induced SAP in mice, suggesting that Nar may be a potential therapeutic intervention for AP.

1. Introduction

Acute pancreatitis (AP) is an acute and life-threatening inflammatory disease that commonly damages peripancreatic tissues and other distant organs, leading to nearly 250,000 inpatient admissions at a cost of approximately $2.2 billion in the United States annually [1]. Pathophysiological characteristics of AP include local pancreatic tissue injury, systemic inflammatory responses, and multiorgan dysfunctions. Although most of the patients with AP have a mild course of the disease, 15% to 25% of patients with severe acute pancreatitis (SAP) develop into infected pancreatic necrosis and persistent organ failure [2], contributing mostly to AP mortality [3]. There is limited understanding of how the pancreatic acinar cell injury caused by the initial triggering events progresses into local tissue damage and even systemic inflammation. SAP, due to excessive release of inflammatory factors and increased oxidative stress response, can cause distant organ damage, especially acute lung injury. In addition, there is no effective therapeutic strategy for acute pancreatitis so far. It is well known that AP is a typical acute inflammatory response disease involving a variety of inflammatory cytokines, the activation of inflammasome, and the oxidative stress [4].

Naringenin (Nar) is a type of flavonoid, the predominant flavanone in grapefruit. Nar has been shown to have anti-inflammatory properties, organ-protective effects [5], and antioxidative functions [6]. Nar is involved in the regulation of many metabolic and signal transduction pathways such as the nuclear factor signaling pathway [7]. A recent study

indicated that naringenin significantly protected against lipopolysaccharide-induced acute lung injury in rats [8]. Other studies have shown that oxidative stress plays a key role in the pathogenesis of acute pancreatitis induced by caerulein (Cae) [9, 10]. Free radicals have been found to participate in the development of the necrotic types of SAP induced by L-arginine (L-arg) [11]. Hence, in order to investigate the role of Nar in AP and the accompanying organ dysfunctions in mice as well as the underlying mechanisms, we used two animal models, Cae-induced mild acute pancreatitis (MAP) model and L-arg-induced SAP model. Moreover, we examined the features of pancreas and lung injury under the pathological condition.

2. Materials and Methods

2.1. Animals and Diets. Male mice in the Institute of Cancer Research (ICR) background weighing approximately 25–30 g were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China) and were allowed to acclimatize for a minimum of 1 week prior to the experimentation. All mice were housed in a specific pathogen-free room under a 12/12 h light-dark cycle at 24°C with free access to water and fed standard laboratory chow. All the animal procedures were approved by the Animal Care and Use Committee of Nanjing University (number 20151008) and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals issued by the National Institutes of Health.

2.2. Experimental Design and Procedures. In the Cae-induced MAP model, mice were randomly divided into 5 subgroups ($n = 8$–12 each group) as follows: control group, MAP model group, MAP + low-dose Nar (25 mg/kg) group, MAP + medium-dose Nar (50 mg/kg) group, and MAP + high-dose Nar (100 mg/kg) group. MAP was induced by 10 intraperitoneal injections of Cae (AnaSpec Inc., Fremont, USA) at a dose of 50 μg/kg body weight (BW) in phosphate-buffered saline (PBS) at hourly intervals, and the control group was injected with PBS in the same way. In the Nar group, Nar (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) was dissolved in 5% DMSO and injected intraperitoneally following AP induction immediately. The MAP model group was administered with the same volume of vehicle solution at the same time point as described above.

In the L-arg-induced SAP model, mice were randomly divided into 3 subgroups ($n = 10$ each group) as follows: control group, SAP model group, and SAP + Nar (100 mg/kg) group. SAP was induced by 2 intraperitoneal injections of 8% L-arg (pH = 7.4; Sigma-Aldrich Chemical Co., St. Louis, MO, USA) in PBS at a dose of 2 g/kg BW at hourly intervals, and the control group was injected with PBS in the same way. In the Nar (100 mg/kg) group, mice received intraperitoneal injections immediately following SAP model induction, and the SAP model group was administered with the same volume of vehicle solution at the same time point.

2.3. Biochemical Assays. Blood samples were obtained from the tail veins of sevoflurane-anesthetized mice at 0, 6, and 12 hours after the initial Cae injection and at 0, 24, 48, and 72 hours after initial L-arg injection. Blood samples were collected using heparinized syringes and centrifuged at 4000 rpm for 10 minutes at 4°C to separate the upper plasma from the lower cells for amylase and lipase measurements.

Amylase activity was measured using 5 ethylidene-G7PNP as a substrate with a commercial kit (Beijing Zhongsheng Beikong Biochemistry Company, China), and lipase activity was also measured with a commercial kit (Nanjing Jiancheng Biochemistry Company, China) according to the manufacturer's manual. The plasma levels of interleukin 1 beta (IL-1β), interleukin 6 (IL-6), and tumor necrosis factor alpha (TNF-α) were determined by enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, San Diego, CA, USA) according to the manufacturer's manual.

2.4. Histological Examinations. Mice were anesthetized with an intraperitoneal administration of sodium pentobarbital (50 mg/kg) and then sacrificed. Pancreatic and pulmonary tissues were harvested and fixed in 4% paraformaldehyde and embedded in paraffin. Small parts of the pancreatic and pulmonary tissues were quickly frozen using liquid nitrogen and stored at −80°C until use.

The paraffin sections of the pancreas and lung tissue were stained with hematoxylin and eosin (H&E). Two pathologists who were blind to the experimental treatments evaluated the degrees of pancreatic injury by light microscopy by scoring the severity of edema, inflammation, and necrosis [12, 13]. We also evaluated the degree of pulmonary injury by scoring the severity of neutrophil infiltration, thickness of alveolar, and alveolar congestions [14, 15].

2.5. Immunofluorescence Examinations. Paraffin-embedded pancreatic and pulmonary tissue sections (5 μm) were rehydrated in xylene and then in descending concentrations of ethanol solutions followed by high-temperature antigen retrieval in citrate buffer (10 mM, pH 6.0) for 20 minutes. Then these slides were cooled down at room temperature, rinsed with PBS, and treated with 0.3% H_2O_2 for 10 minutes to block endogenous peroxidase activity. Nonspecific binding was blocked with 10% goat serum albumin in PBS for 30 minutes at 37°C. Then these slides were incubated overnight at 4°C in a humidified chamber with rabbit polyclonal antibody anti-myeloperoxidase (MPO; 1 : 100 dilution) and incubated with fluorescein-labeled secondary antibody (1 : 200 dilution) for 1 h at room temperature. The slides were observed under a fluorescence microscope, and the photographs were captured using an Olympus CKX41 camera (Olympus Company, Japan). When evaluating MPO expression, ten fields across each slide were randomly selected for the analysis of the mean fluorescence intensity.

2.6. Measurement of Pancreatic MDA. Pancreatic tissue lipid peroxidation was determined by measuring MDA using thiobarbituric acid reactive substances. The pancreatic tissue was weighed and homogenized in potassium phosphate buffer (50 mmol/L, pH 7.4) containing butylated hydroxytoluene (12.6 mmol/L). Aliquots of the homogenate were incubated with thiobarbituric acid (0.37%) in an acidic solution (15%

trichloroacetic acid and hydrochloric acid at 0.25 mol/L) at 90°C for 45 min. The homogenates were centrifuged (5 min, 8000 ×g), and aliquots from the supernatants were extracted using n-butanol, followed by vortexing for 30 s and centrifugation (2 min, 8000 ×g). The absorbance was measured at 535 nm in a microplate reader and calibrated at 572 nm. The results were calculated using a molar extinction coefficient of 1.55×10^5 mol/L/cm and expressed as nmols of MDA per mg of tissue.

2.7. Measurement of Antioxidant Enzyme Activity. The GPx activity was determined as described previously [16]. The absorbance was monitored at 340 nm at 37°C for 10 min, and the results were expressed as μmol of reduced glutathione (GSH)/min/mg of protein. The GR activity was measured as described previously [17] by measuring the consumption of nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor in the reduction of oxidized glutathione to reduced GSH. The results were expressed as U of GR/mg of protein. One U of enzyme activity was defined as the amount of GR that oxidizes 1 μmol of NADPH per min. The GST activity was measured as described previously [18] using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. The results were expressed as U of GST/mg of protein. One U of enzyme activity was defined as the amount of GST that produces 1 μmol of the conjugate of GSH with CDNB per min. The total protein concentration in the homogenate was measured using the method of Bradford [19]. The levels of sulfhydryl compound, pancreatic T-SH, and NP-SH were determined by Ellman's reaction using 5′5′-dithio-bis-2-nitrobenzoic acid (DTNB). Aliquots of 4 mL homogenates in ice-cold ethylenediaminetetraacetic acid (0.02 mol/L; pH 8.9) were mixed with 3.2 mL of distilled water and 0.8 mL of 50% trichloroacetic acid. The tubes were centrifuged at 3000 ×g for 15 min. The supernatant (2 mL) was mixed with 4 mL Tris buffer (0.4 mol/L; pH 8.9) and 0.1 mL DTNB (0.01 mol/L). The absorbance was measured within 5 min after the addition of DTNB at 412 nm. The absorbance was extrapolated from a glutathione standard curve. Data were expressed as μg/g of the tissue.

2.8. Western Blot Analysis. Pancreatic tissues were homogenized on ice and centrifuged at 4°C (13000g, 15 min). Then the cytoplasmic proteins in tissue homogenate were extracted using cytoplasmic extraction reagents (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. The supernatants were collected, and the protein concentrations were determined. Equal amounts of protein (40 μg/lane) were separated on 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked in 5% (w/v) skimmed milk and incubated with antibodies against mouse nod-like receptor protein 3 (NLRP3; 1 : 1000 dilution; Abcam, Cambridge, UK), IL-1β (1 : 1000 dilution; Cell Signaling Technology, Danvers, MA, USA), heme oxygenase-1 (HO-1; 1 : 1000 dilution; Abcam), nuclear factor erythroid 2-related factor 2 (Nrf2; 1 : 1000 dilution; Abcam), and β-actin (1 : 1000 dilution; Sigma-Aldrich Chemical Co.), followed by incubation with secondary goat anti-rabbit antibody (1 : 10,000 dilution) or secondary goat anti-mouse antibody (1 : 10,000 dilution) conjugated to horseradish peroxidase for 1 h at room temperature. The protein bands were quantified by the mean ratios of integral optical density normalized to the housekeeping gene β-actin expression.

2.9. Quantitative Reverse Transcription PCR (qRT-PCR). The mRNA expression of NLRP3, IL-1β, Nrf2, and HO-1 in the pancreatic tissues of experimental mice was determined using qRT-PCR. Pancreatic tissues were homogenized in TRIzol® reagent (Life Technologies, Carlsbad, CA, USA), and total RNA was extracted according to the manufacturer's instructions. Then 6–8 ovaries from each group were transferred to 1.5 mL tubes and washed twice with RNase-free PBS. 350 mL of RNA extraction lysis buffer was added into each tube. The experiment was repeated three times. Total RNA was extracted using a RNA prep Pure Micro Kit (DP420; Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. RNA concentrations were measured by a spectrophotometer (NanoDrop 2000c, Thermo Fisher Scientific, Waltham, MA, USA). Equal amounts of samples (100 ng/reaction) were reverse-transcribed using a FastQuant RT Kit (KR-106-02; Tiangen). A SYBR-based qPCR was then performed using Bestar qPCR Mastermix (DBI-2223; DBI Bioscience, Ludwigshafen, Germany) on an ABI StepOnePlus platform (Thermo Fisher Scientific). Quantitation of various mRNAs was performed, and GAPDH was used as the internal control. The relative mRNA expression was measured using the comparative $2^{-(\Delta\Delta Cq)}$ method. The primer sequences used to amplify mRNAs are shown in Table 1.

2.10. Statistical Analysis. Statistical analysis was performed using GraphPad Prism 6 software (GraphPad, San Diego, CA, USA), and data were presented as the mean ± standard deviation (SD). The results were analyzed using one-way analysis of variance, Student-Newman-Keuls test, and Mann–Whitney rank sum test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Naringenin Protected against Cae-Induced MAP. In our study, we found that Nar could alleviate the injuries of pancreatic tissues caused by Cae-induced pancreatitis in a dose-dependent manner. At the standard induction dose of Cae (50 μg/kg), the pancreatic tissues were mainly characterized as obvious edema, inflammatory cell infiltration, and necrosis, while the pancreatic injuries in Nar-treated (100 mg/kg and 50 mg/kg) mice were significantly reduced compared with the MAP group (Figure 1(a)). In addition, the histological scores of pancreatic tissues in Nar-treated mice were remarkably lower than those in the MAP group ($P < 0.001$) (Figure 1(b)).

We also measured the serum parameters and found that, at the standard induction dose of Cae (50 μg/kg), the serum amylase levels in the MAP group were remarkably higher than those in the MAP + high-dose Nar (100 mg/kg) group ($P < 0.001$) and MAP + low-dose Nar (50 mg/kg) group

Table 1: Primer sequences for RT-PCR.

Primer		Sequence (5′ to 3′)
NLRP3	Forward	CGAGACCTCTGGGAAAAAGCT
	Reverse	GCATACCATAGAGGAATGTGATGTACA
IL-1β	Forward	TGTAATGAAAGACGGCACACC
	Reverse	TCTTCTTTGGGTATTGCTTGG
Nrf2	Forward	CAGTGCTCCTATGCGTGAA
	Reverse	GCGGCTTGAATGTTTGTC
HO-1	Forward	ACAGATGGCGTCACTTCG
	Reverse	TGAGGACCCACTGGAGGA
GAPDH	Forward	GGAGCGAGATCCCTCCAAAAT
	Reverse	GGCTGTTGTCATACTTCTCATGG

($P < 0.05$). Similarly, the serum lipase levels in the MAP group were significantly higher than those in the MAP + high-dose Nar (100 mg/kg) group ($P < 0.001$) and MAP + low-dose Nar (50 mg/kg) group ($P < 0.01$) (Figure 1(c)).

3.2. Naringenin Protected against L-Arginine-Induced SAP. In the mouse model of Cae-induced AP, we found that Nar protected against AP in a dose-dependent manner. Hence, we selected 100 mg/kg as the intervention dose in the mouse model of L-arg-induced SAP and examined the serum levels of amylase and lipase. Consistently, the serum amylase and lipase levels were significantly higher in the SAP group than those in the SAP + high-dose Nar (100 mg/kg) group ($P < 0.001$) and SAP + low-dose Nar (50 mg/kg) group ($P < 0.01$) (Figures 2(a) and 2(b)).

In the mouse model of L-arg-induced SAP, we also found that pancreatic injuries in Nar- (100 mg/kg) treated mice were significantly alleviated compared with the SAP group. Accordingly, the histological scores were lower than those in the SAP group. These data indicate the protective roles of Nar in both MAP and SAP (Figures 2(c) and 2(d)).

Acute lung injury is one of the most prominent features of organ failures in SAP. According to the pulmonary H&E staining results, alveolar interval inflammatory cell infiltration and expansion of capillary congestion in Nar- (100 mg/kg) treated mice were significantly alleviated compared with those in the SAP group. Consistently, the histological scores of lung tissues in Nar-treated mice were significantly lower than those in the SAP group ($P < 0.001$) (Figures 2(e) and 2(f)).

3.3. Naringenin Reduced Inflammatory Cell Recruitment in Mouse Models of MAP and SAP. In pancreatitis, the inflammatory response is triggered by the production of a series of inflammatory cytokines such as IL-6, IL-1β, and TNF-α. In the MAP model, the administration of a standard dose of Cae (50 μg/kg) resulted in an elevation of serum IL-6, IL-1β, and TNF-α levels compared with the MAP group. In addition, Nar treatment (50 mg/kg) reduced all these parameters compared with the MAP group ($P < 0.05$) (Figure 3(a)). Interestingly, coadministration of Cae and Nar (100 mg/kg) reduced serum IL-6, Il-1β, and TNF-α levels even further ($P < 0.001$) (Figure 3(a)). The similar results were also observed in the SAP model (Figure 3(b)).

MPO is specifically expressed in neutrophils and is released into the circulation under the condition of inflammation. Therefore, MPO activity can reflect the activation of neutrophils. In this study, we performed immunofluorescent staining of MPO in the pancreatic tissues, which was used to reflect the degree of pancreatic inflammation. In the MAP group, the MPO staining of the pancreatic tissue was significantly stronger than that in the Nar- (100 mg/kg) treated group (Figure 3(c)). Nar also had the similar effect on MPO immunostaining in the SAP model (Figure 3(d)).

3.4. Naringenin Reduced the Pancreatic Generation of Oxygen-Free Radicals in MAP and SAP Mice. Oxidative stress is involved in the inflammatory response of acute pancreatitis. We measured the levels of MDA, a lipid peroxidation marker, to reflect the degree of pancreatic injury. The results showed that Nar (100 mg/kg) treatment significantly reduced the MDA levels in the pancreatic tissue contrast with the MAP mice ($P < 0.001$) (Figure 4(a)). In the SAP + Nar 100 mg/kg group, the MDA levels in the pancreatic tissue were markedly decreased (Figure 4(b)). The GPx, GR, GST, TT-SH, and NP-SH were upregulated in both MAP and SAP after Nar treatment (100 mg/kg) (Figures 4(a) and 4(b)).

3.5. Naringenin Impaired NLRP3 Inflammasome Activation and IL-1β Production. We performed Western blot analyses and qRT-PCR to detect the expression of NLRP3 and IL-1β in the pancreatic tissues in both models. Our results showed that the expression of NLRP3 was remarkably elevated in both models, and administration of Nar (100 mg/kg) significantly reduced the NLRP3 expression in pancreatic tissues compared with the MAP and SAP groups ($P < 0.001$ and $P < 0.05$, resp.) (Figures 5(a) and 5(b)). The activation of NLRP3 inflammasome promotes the maturation and release of IL-1β. Our results showed that the expression of IL-1β was remarkably inhibited in the Nar (100 mg/kg) pretreatment group compared with the MAP and SAP groups ($P < 0.001$ and $P < 0.05$, resp.) (Figures 5(a) and 5(b)). In addition, qRT-PCR was performed to detect the mRNA expression of NLRP3 and IL-1β in different groups. Our results indicated that Nar (100 mg/kg) treatment reduced both NLRP3 and IL-1β mRNA expressions in pancreatic tissues compared with the MAP group ($P < 0.05$ and $P < 0.01$, resp.) (Figure 5(c)). The similar results were also observed in the SAP model (Figure 5(c)).

3.6. Naringenin Enhanced Nrf2/HO-1 Expression in the Pancreatic Tissues in Both Models. Oxidative stress has been shown to play a vital role in the pathogenesis of AP, and the Nrf-2/HO-1 pathway is closely associated with oxidative stimulation. Nrf2 can translocate to the nucleus where it interacts with the antioxidant response element (ARE) to induce downstream gene expression, including HO-1. Previous studies indicated that the Nrf2/HO-1 pathway was majorly regulated in AP. In order to investigate whether

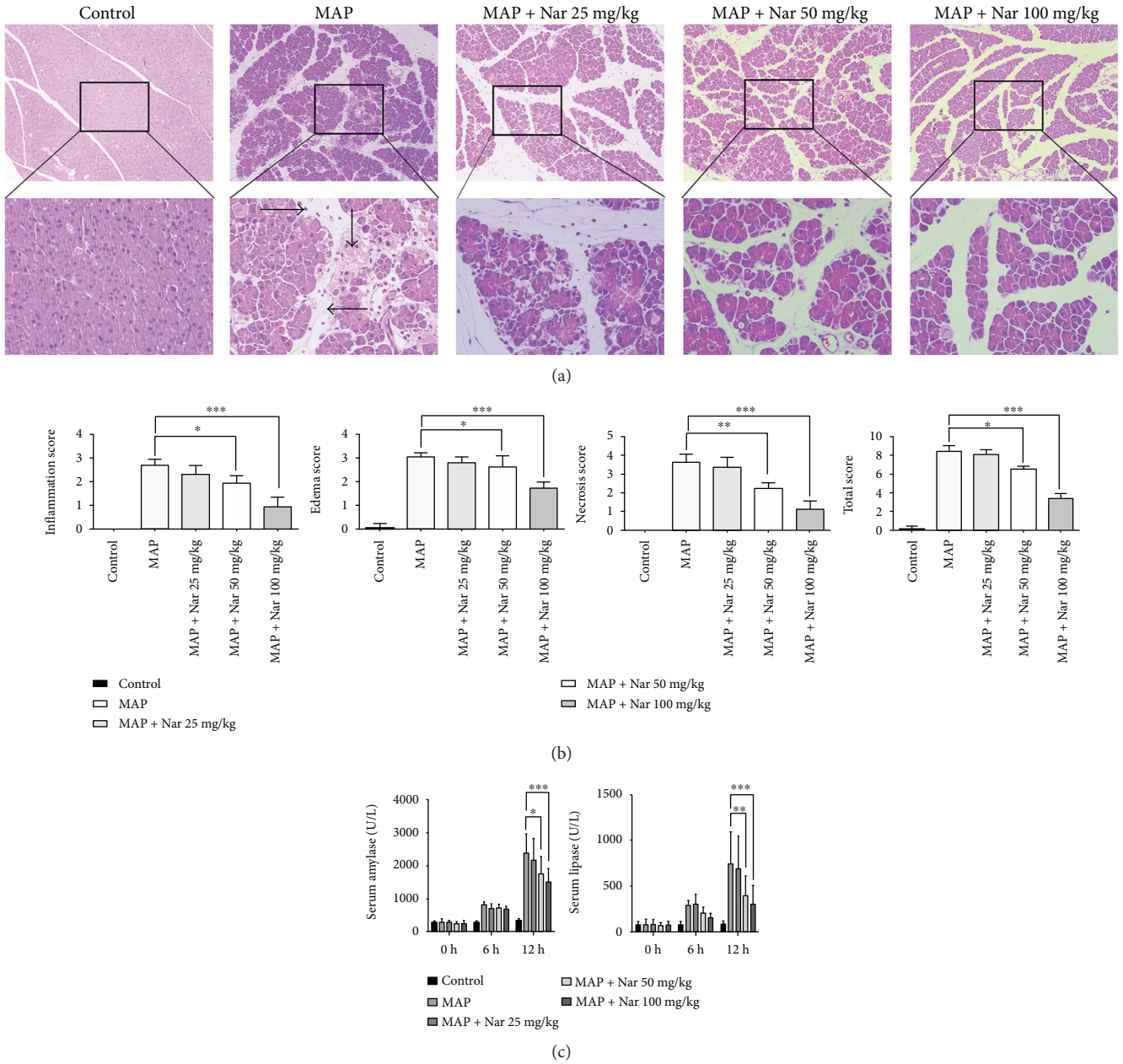


Figure 1: Naringenin alleviated the severity of pancreatic tissue injury in MAP. (a) Representative H&E staining of pancreatic tissues in magnifications 100x and 400x. (b) The pathological scores of pancreatic tissues. (c) Serum amylase and lipase levels of mice. $^{*}P < 0.05$ compared with the MAP group. $^{**}P < 0.01$ compared with the MAP group. $^{***}P < 0.001$ compared with the MAP group. →: inflammation; ↓: acinar necrosis; ←: edema; MAP: mild acute pancreatitis; H&E: hematoxylin and eosin.

Nar exerted antioxidant and anti-inflammatory effects on pancreatic tissues in the MAP and SAP models by inducing HO-1 and Nrf2 expression, we examined protein expression of HO-1 and Nrf2 by Western blotting and qRT-PCR following Nar treatment at different doses. The results demonstrated that Nar increased HO-1 protein level in a dose-dependent manner in the MAP model (Figure 6(a)). Similarly, Nar was also shown to induce HO-1 and Nrf2 expression at a high dose in the SAP model (Figure 6(b)). Collectively, these findings suggest that Nar plays a protective role against MAP and SAP possibly through the induction of Nrf2 and HO-1 (Figures 6(a)–6(c)).

4. Discussion

Our study has revealed that Nar exerted protective effects against both cerulean-induced and L-arginine-induced pancreatitis and distant organ injuries. Furthermore, we have confirmed that prophylactic administration of Nar could reduce pancreatic pathological injuries, inflammatory responses, and the activation of NLRP3 inflammasome as well as relieving the oxidative stress in experimental MAP and SAP mice. To our knowledge, we demonstrate for the first time that Nar may be used as a novel and effective therapeutic agent for AP.

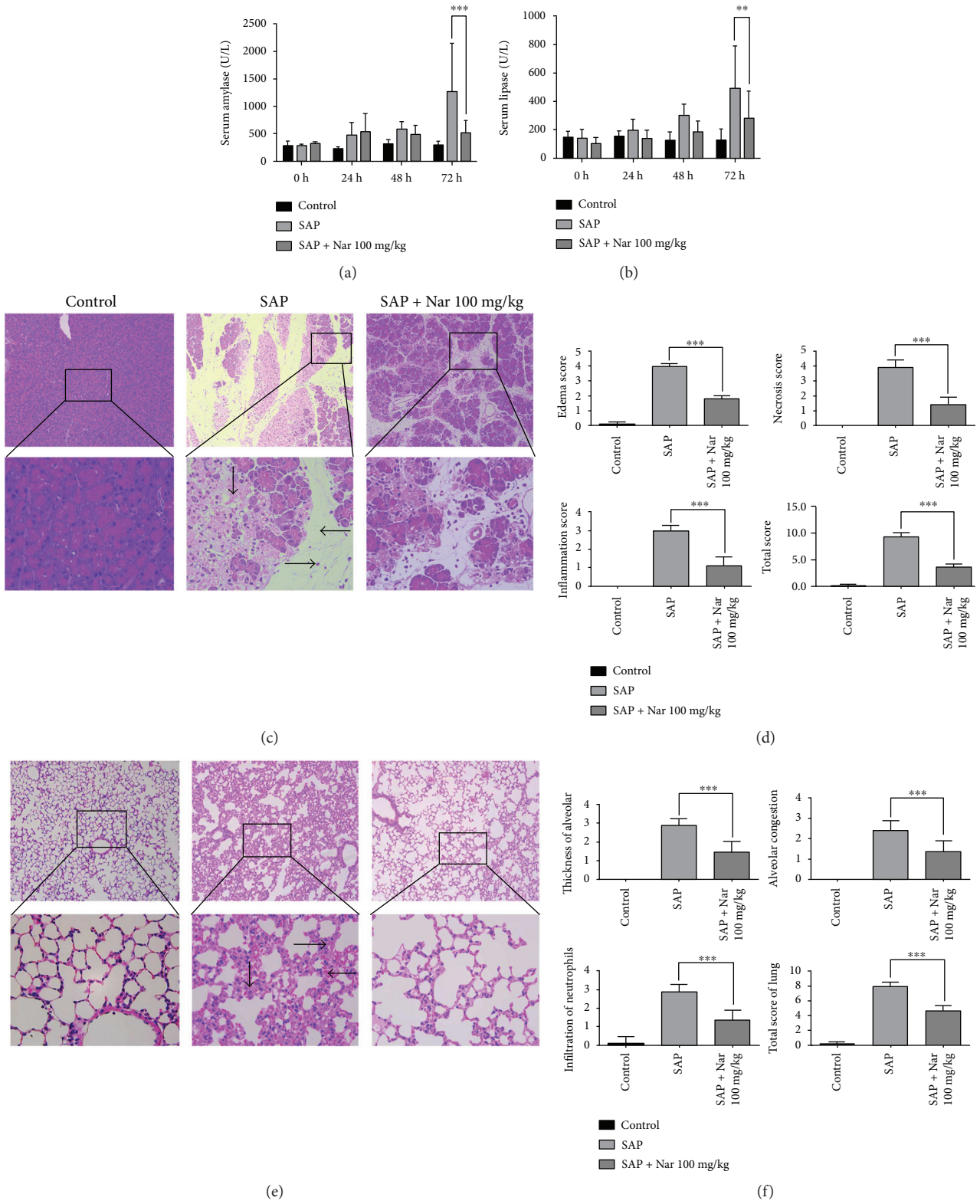
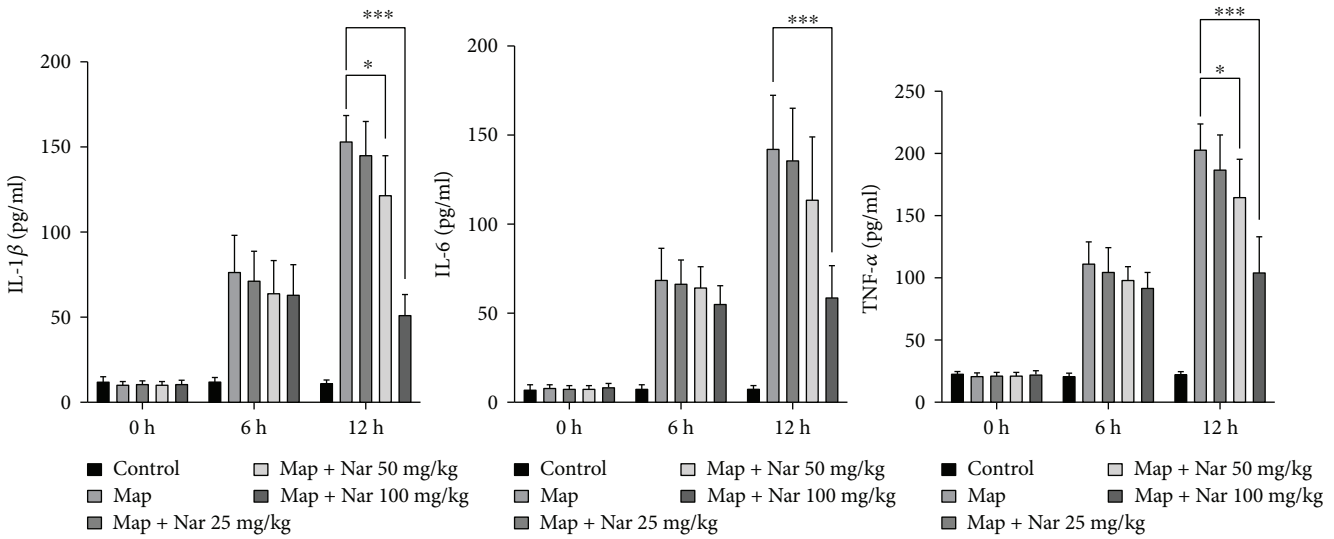
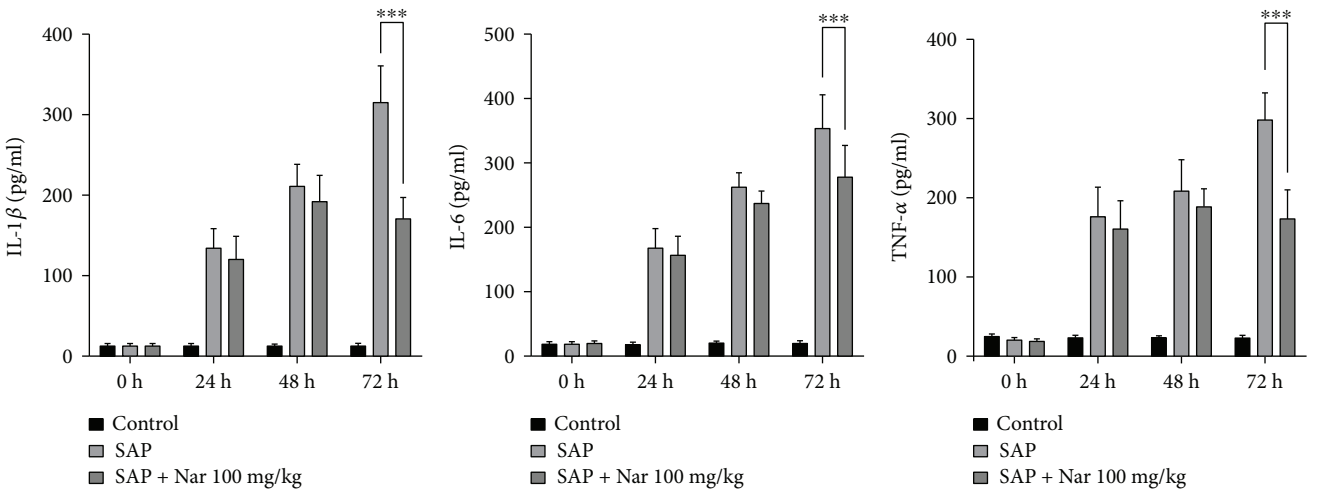


Figure 2: Naringenin alleviated the severity of pancreatic tissue injury in SAP. (a) Time course of serum amylase (a) and lipase (b) levels. (c) Representative H&E staining of pancreatic (c) and lung (e) tissues. Pathological scores of pancreatic (d) and lung (f) tissues. $^{**}P < 0.01$ compared with the SAP group. $^{***}P < 0.001$ compared with the SAP group. (c) →: inflammation; ↓: acinar necrosis; ←: edema. (e) ⟶: thickness of alveolar; ↓: infiltration of neutrophils; ⟵: alveolar congestion. SAP: severe acute pancreatitis; H&E: hematoxylin and eosin.



(a)



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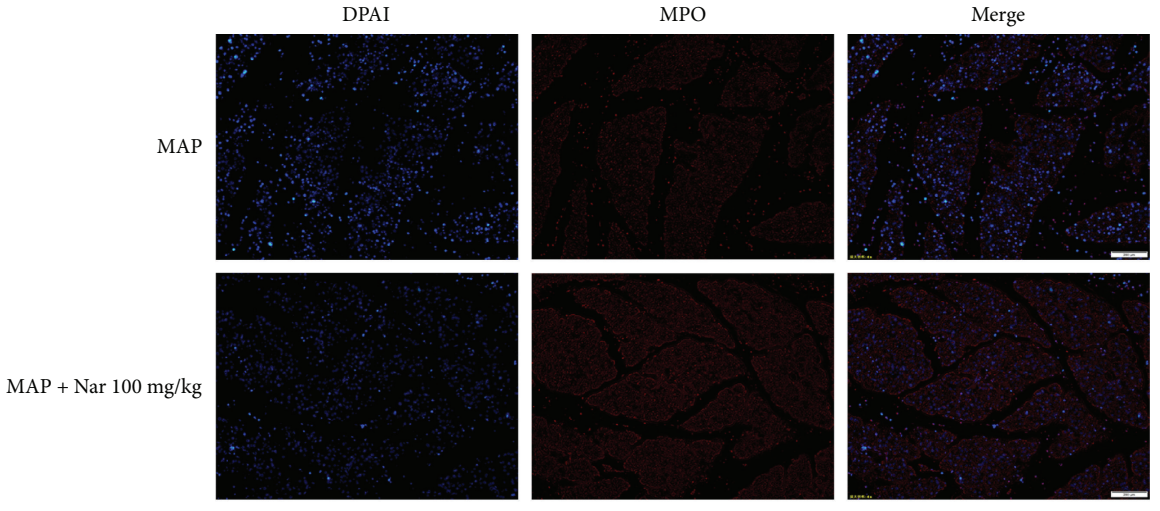

(c)

Figure 3: Continued.

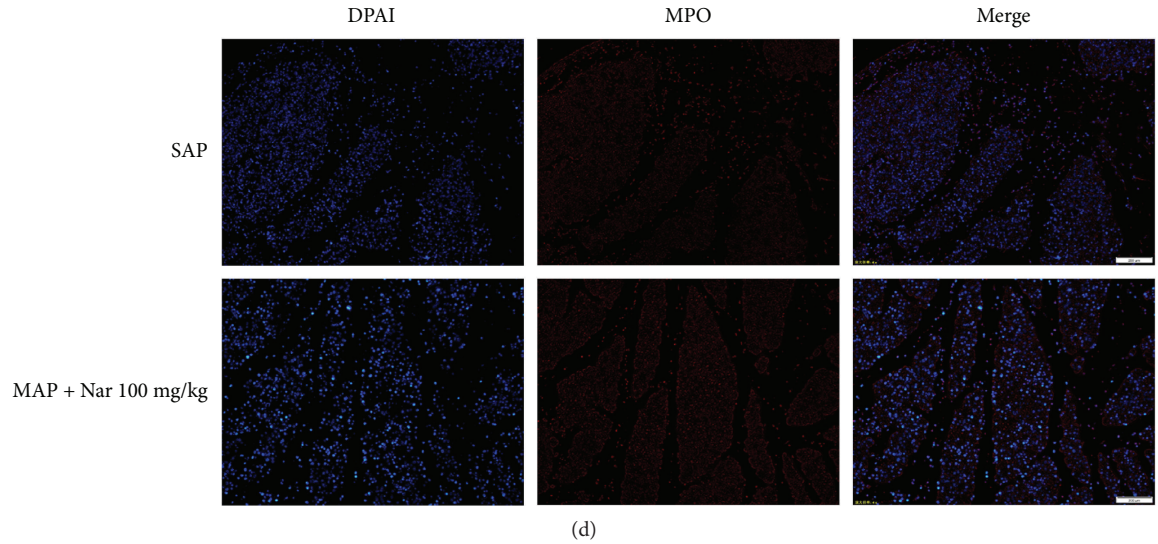

Figure 3: Naringenin inhibited the production of proinflammatory cytokines in MAP and SAP. (a) Serum levels of IL-6, IL-1β, and TNF-α in MAP (a) and SAP (b). Representative immunostaining of pancreatic MPO in MAP (c) and SAP (d) in magnification 40x. MAP: mild acute pancreatitis; SAP: sever acute pancreatitis; IL-6: interleukin 6; IL-1β: interleukin 1 beta; TNF-α: tumor necrosis factor alpha. $^{*}P < 0.05$ compared with the MAP group. $^{***}P < 0.001$ compared with the MAP group. $^{***}P < 0.001$ compared with the SAP group.

As a type of flavonoid, Nar is the predominant flavanone in grapefruit and is found to have strong anti-inflammatory, antioxidant, and organ-protective activities. A previous study has indicated that Nar alleviates the histopathological changes in the liver and kidney caused by alloxan-induced diabetes in mice [20]. Nar can downregulate TNF-a, IL-1β, IL-6, IL-10, and other inflammatory cytokines in macrophages infected with live *Chlamydia trachomatis* [21]. Our study is consistent with these findings, and we also confirmed that administration of Nar could exert protective effects on both MAP and SAP. Nar has been shown to play protective roles even over a wide range of dosages or concentrations. Our study has further made sure that Nar pretreatment at 100 mg/kg has obvious protective effects. However, the potential role of Nar in inflammatory disease has not been extensively studied, especially in AP, which needs further investigation.

The inflammatory response is a hallmark in the pathogenesis and progression of pancreatitis and pancreatitis-induced distant organ injuries, in which the release of inflammatory cytokines and neutrophil exudation are two critical events. IL-6, IL-1β, and TNF-α are the most important cytokines involved in the inflammatory response, and their serum levels are directly associated with the severity of AP. In our study, we have concluded that Nar could alleviate the cascade activation of these inflammatory cytokines and generate the protective effects on organ injuries. MPO is mainly expressed in neutrophils and could serve as a biomarker of activated neutrophils. Our results were consistent with previous findings that MAP and SAP cause an enhancement in MPO expression in pancreatic tissues while Nar pretreatment leads to a significant decrease in MPO expression. Collectively, the cascade activation of inflammatory mediators and the overreaction of phagocytic cells along with their reciprocal interactions play important roles in the local tissue injury due to exaggerated inflammatory response. Nar appears to serve as a potential therapeutic agent for AP.

The inflammasome is a large multiprotein complex composed of nucleotide-binding domain and leucine-rich repeat-containing proteins or AIM2, adaptor protein ASC, and caspase-1 and plays a critical role in host defense against exogenous pathogens and inflammation [22, 23]. The canonical inflammasomes include NLRP3, NLRP1, NAIP–NLRC4, and AIM2. Among them, NLRP3 inflammasome is the most well-studied one and overactivation of the NLRP3 inflammasome is involved in the pathogenesis of several inflammatory diseases. A previous study indicates that inhibition of NLRP3 inflammasome activation attenuates experimental AP in mice [4]. Our results showed that NLRP3 inflammasome indeed played a vital role in AP and Nar could protect mice against MAP and SAP via inhibiting the activation of NLRP3 inflammasome.

The pathophysiology of AP is very complicated, and oxygen-derived free radicals are found to be involved in the pathogenesis of AP [24]. The pancreatic tissue is more susceptible to oxidative stress than other tissues because of extremely weak expression of antioxidative enzymes in pancreatic islet cells [25]. Oxygen free radicals generated during acute pancreatitis not only cause pancreatitis acinar cell damage but also contribute to the pancreatic damage [26, 27]. In terms of the anatomical location, physiological function, and hemodynamics, the pancreas and liver are closely related. The release of oxygen free radicals in AP reaches the liver through blood circulation and causes damage to the liver, resulting in a decreased ability of the liver to scavenge the free radicals and an increased systemic oxidative stress response. Previous findings have shown that in the rat model of Cae-induced AP, some substances such as tissue MDA are significantly upregulated. Therefore, we hypothesize that in mouse

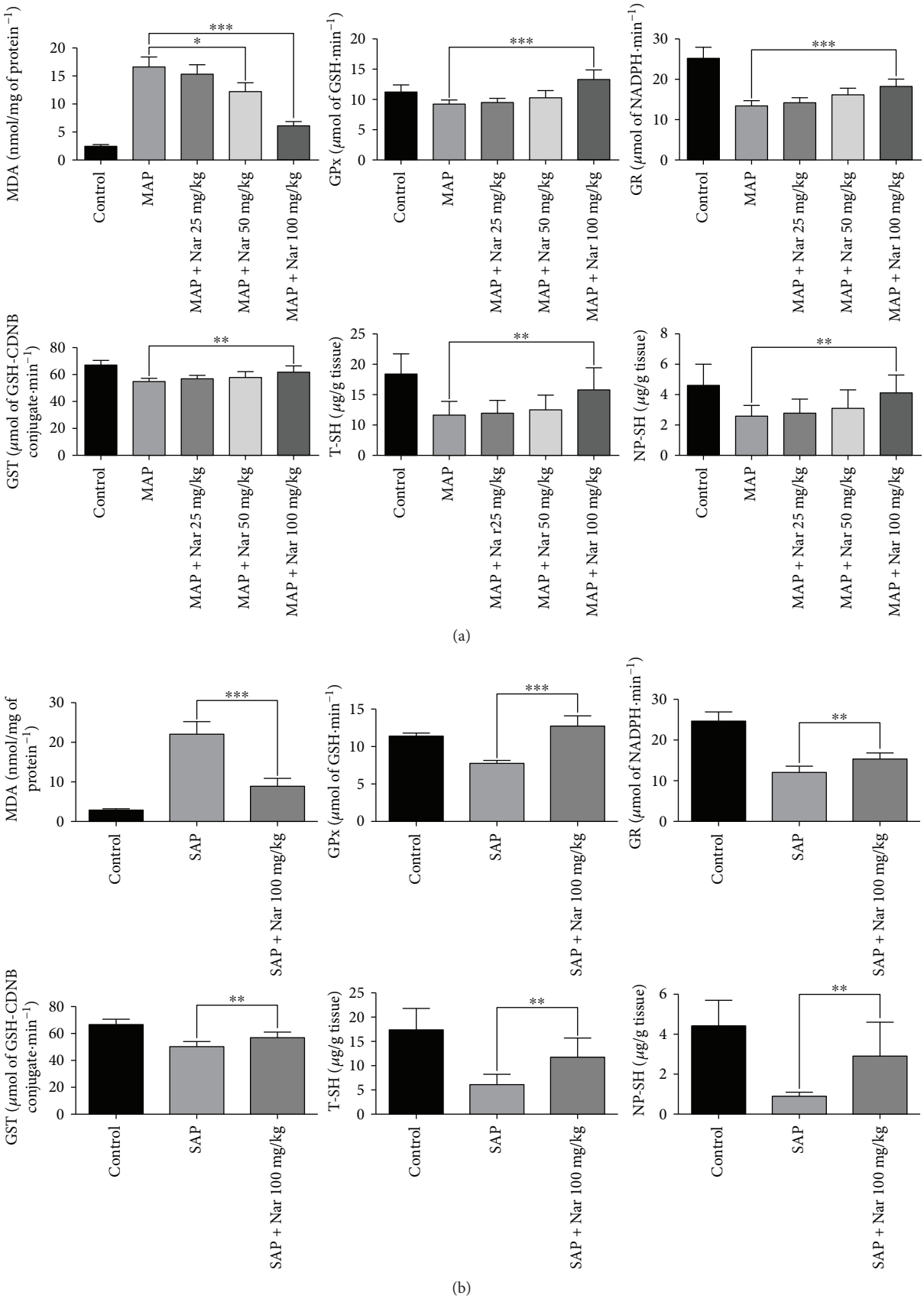

Figure 4: Naringenin inhibits oxidative stress in MAP and SAP. The levels of MDA, GPx, GR, GST, T-SH, and NP-SH were measured in pancreatic tissues in MAP (a) and SAP (b). $^{*}P < 0.05$ compared with the MAP group. $^{**}P < 0.01$ compared with the MAP or SAP group. $^{***}P < 0.001$ compared with the MAP or SAP group.

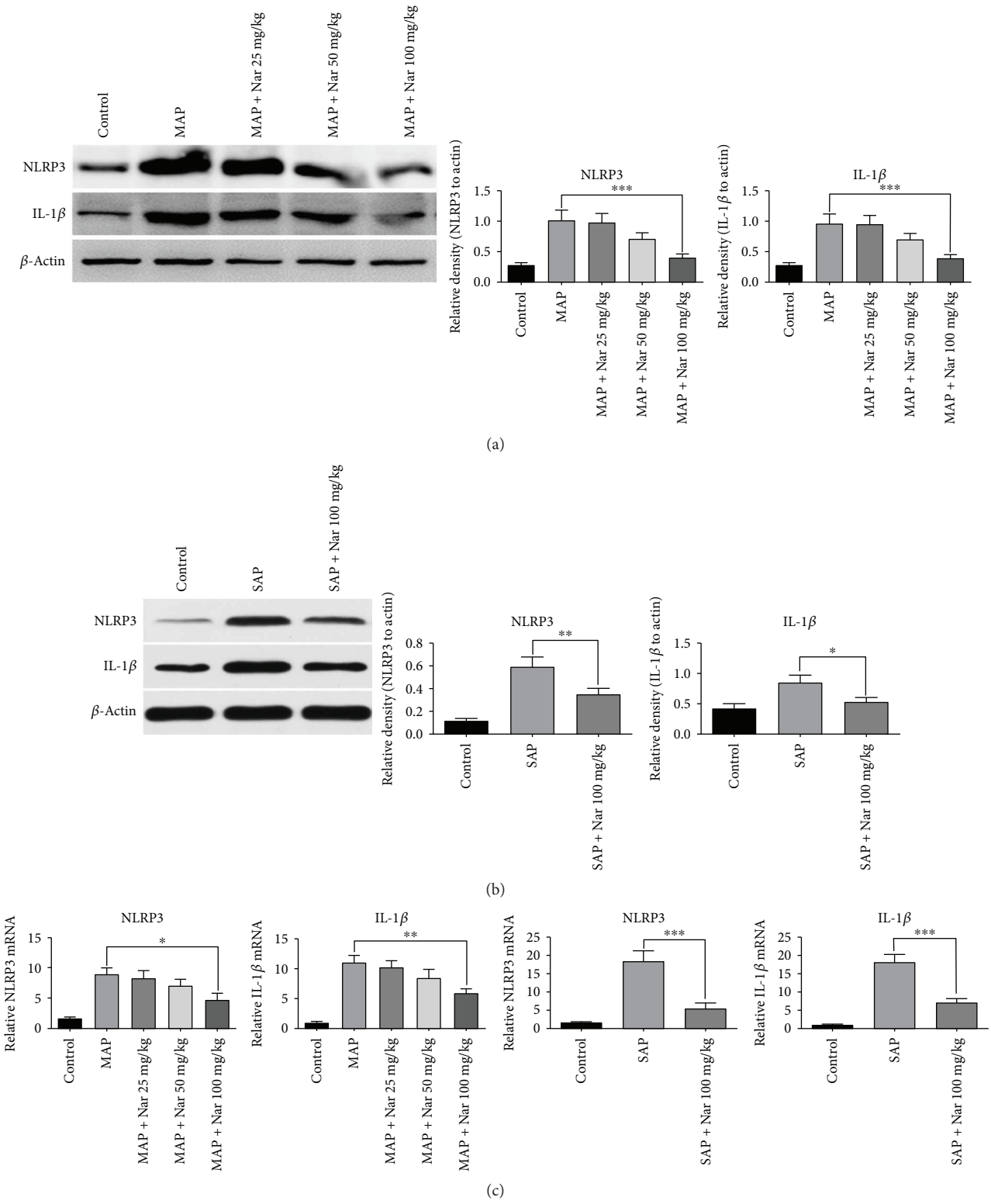

FIGURE 5: Naringenin suppressed expression of NLRP3 and IL-1β in MAP and SAP. Western blot analysis of NLRP3 and IL-1β protein expression in MAP (a) and SAP (b). (c) qRT-PCR analysis of NLRP3 and IL-1β mRNA expression in the pancreatic tissues in MAP and SAP. $^{*}P < 0.05$ compared with the MAP or SAP group. $^{**}P < 0.01$ compared with the MAP group. $^{***}P < 0.001$ compared with the MAP or SAP group. NLRP3: nod-like receptor protein 3; IL-1β: interleukin 1 beta; MAP: mild acute pancreatitis; SAP: sever acute pancreatitis.

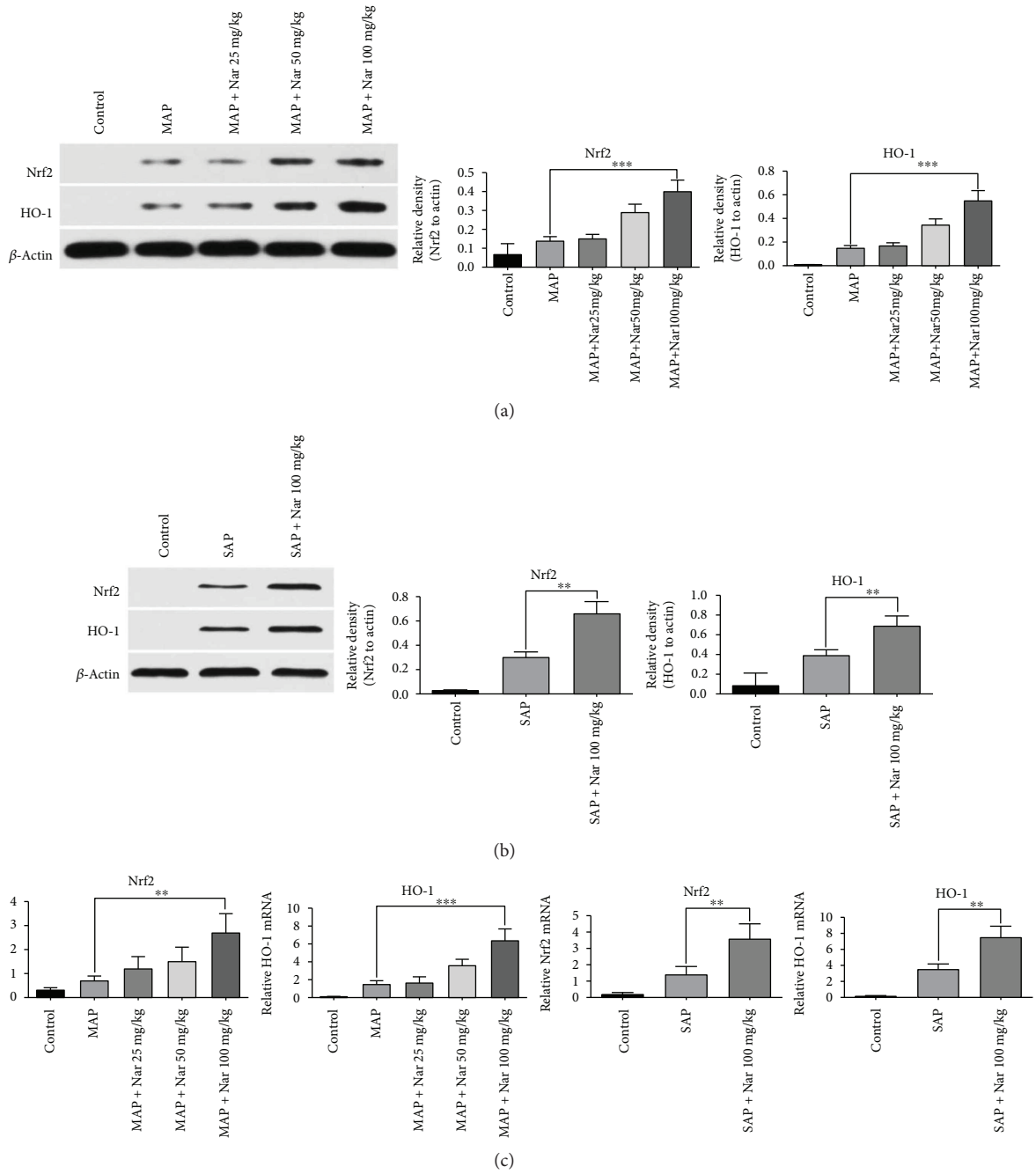

Figure 6: Naringenin enhanced protein expression of Nrf2 and HO-1 in MAP and SAP. Western blot analysis of Nrf2 and HO-1 expression in MAP (a) and SAP (b). (c) mRNA expression of Nrf2 and HO-1 in the pancreatic tissues in MAP and SAP by qRT-PCR. $^{**}P < 0.01$ compared with the SAP group. $^{***}P < 0.001$ compared with the MAP group. Nrf2: nuclear factor erythroid 2-related factor 2; HO-1: heme oxygenase-1; MAP: mild acute pancreatitis; SAP: sever acute pancreatitis.

models of pancreatitis induced by Cae or L-arg, oxidative stress-related molecules like MDA released locally by the pancreatic tissue are transported to the liver, which impairs the liver's free radical-scavenging capacity, resulting in enhanced oxidative stress and pancreotoxic manifestations. The levels of indicators of oxidative stress correlate with the severity of acute pancreatitis. A recent study reveals that proanthocyanidin derived from grape seeds can function as a protective factor in the oxidative stress-mediated pancreatic dysfunction in rats via Nrf-2/HO-1 signaling [28]. The proanthocyanidin/Nrf-2/HO-1 axis also plays a key role in preventing oxidative stress in human bronchial epithelial BEAS-2B cells [29]. Another study has demonstrated that HO-1 and CO exert anti-inflammatory effects via decreasing the expressions of TNF-α, IL-1β, and macrophage inflammatory protein-1 and increasing IL-10 levels [30–32]. Based on these findings, we speculate that inhibition of oxidative stress is a major determinant in reducing the

inflammation and decreasing the severity of pancreatitis. HO-1 can catalyze the degradation of heme to produce CO which acts as an antioxidant and is an important molecule in host defense against oxidative stress. It has anti-inflammatory abilities through downregulating the inflammatory factors such as matrix metalloproteinase 2 and COX-2 [33–35]. Nrf2 acts as an upstream regulator of ARE-dependent phase II enzyme, translocating to the nucleus where it interacts with the ARE to further promote the expression of antioxidant genes, including HO-1 [36, 37]. Our results indicated that Nrf2 was activated and HO-1 was upregulated in Nar-induced immune defense against oxidative stress. These findings provide a potential therapeutic strategy to prevent AP involving oxidative stress and exaggerated inflammatory responses.

In conclusion, Nar exerts protective effects on organ injuries of Cae- and L-arg-induced pancreatitis by inhibiting oxidative stress and inflammatory response via the inactivation of NLRP3 inflammasome and upregulation of Nrf2/HO-1 expression.

Conflicts of Interest

The authors have no conflicts of interest to declare.

Authors' Contributions

Weiqin Li and Guotao Lu formulated the idea of the paper, supervised the research, and reviewed and revised the manuscript. Yong Li, Yiyuan Pan, and Lin Gao performed the research and wrote the manuscript. Zhihui Tong, Guotao Lu, and Baiqiang Li provided comments and technical advice. Jingzhu Zhang and Xiaochun Xie participated in preparing the figures and tables and analyzing the data. Lin Gao revised the manuscript and provided comments. All authors reviewed the manuscript. Yong Li, Yiyuan Pan, and Lin Gao contributed equally to this work.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (no. 81570584), Key Research and Development Program Foundation of Jiangsu Province of China (no. BE2015685 and no. BE2016749), National Natural Science Foundation of Yangzhou City (SQN20140063), and Postdoctoral Science Foundation of China (2014M562664).

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Hindawi
Mediators of Inflammation
Volume 2018, Article ID 9734845, 18 pages
https://doi.org/10.1155/2018/9734845

Review Article

Phytochemicals That Influence Gut Microbiota as Prophylactics and for the Treatment of Obesity and Inflammatory Diseases

Lucrecia Carrera-Quintanar,[1] **Rocío I. López Roa**,[2] **Saray Quintero-Fabián**,[3] **Marina A. Sánchez-Sánchez**,[4,2] **Barbara Vizmanos**,[5] **and Daniel Ortuño-Sahagún**[4]

[1]*Universidad de Guadalajara, Laboratorio de Ciencias de los Alimentos, Departamento de Reproducción Humana, Crecimiento y Desarrollo Infantil, CUCS, Guadalajara, JAL, Mexico*
[2]*Universidad de Guadalajara, Laboratorio de Investigación y Desarrollo Farmacéutico, Departamento de Farmacobiología, CUCEI, Guadalajara, JAL, Mexico*
[3]*Universidad Nacional Autónoma de México, Instituto Nacional de Pediatría, Unidad de Genética de la Nutrición, Instituto de Investigaciones Biomédicas, Mexico City, Mexico*
[4]*Universidad de Guadalajara, Laboratorio de Neuroinmunobiología Molecular, Instituto de Investigación en Ciencias Biomédicas (IICB), CUCS, Guadalajara, JAL, Mexico*
[5]*Universidad de Guadalajara, Laboratorio de Evaluación del Estado Nutricio, Departamento de Reproducción Humana, Crecimiento y Desarrollo Infantil, CUCS, Guadalajara, JAL, Mexico*

Correspondence should be addressed to Daniel Ortuño-Sahagún; daniel.ortuno.sahagun@gmail.com

Received 15 September 2017; Revised 17 January 2018; Accepted 13 February 2018; Published 26 March 2018

Academic Editor: Amedeo Amedei

Gut microbiota (GM) plays several crucial roles in host physiology and influences several relevant functions. In more than one respect, it can be said that you "feed your microbiota and are fed by it." GM diversity is affected by diet and influences metabolic and immune functions of the host's physiology. Consequently, an imbalance of GM, or dysbiosis, may be the cause or at least may lead to the progression of various pathologies such as infectious diseases, gastrointestinal cancers, inflammatory bowel disease, and even obesity and diabetes. Therefore, GM is an appropriate target for nutritional interventions to improve health. For this reason, phytochemicals that can influence GM have recently been studied as adjuvants for the treatment of obesity and inflammatory diseases. Phytochemicals include prebiotics and probiotics, as well as several chemical compounds such as polyphenols and derivatives, carotenoids, and thiosulfates. The largest group of these comprises polyphenols, which can be subclassified into four main groups: flavonoids (including eight subgroups), phenolic acids (such as curcumin), stilbenoids (such as resveratrol), and lignans. Consequently, in this review, we will present, organize, and discuss the most recent evidence indicating a relationship between the effects of different phytochemicals on GM that affect obesity and/or inflammation, focusing on the effect of approximately 40 different phytochemical compounds that have been chemically identified and that constitute some natural reservoir, such as potential prophylactics, as candidates for the treatment of obesity and inflammatory diseases.

1. Introduction

Obesity is a chronic state of low-grade inflammation constituting a well-known risk factor for multiple pathological conditions, including metabolic syndrome and insulin resistance [1], and it has also been implicated as a proactive factor and associated with a nonfavorable disease course of chronic autoimmune inflammatory disorders, such as multiple sclerosis (MS) [2]. Several studies over the last decade report interest in fermentation products from gut microbiota (GM) in the control of obesity and related metabolic disorders [3]. GM denotes an entire ecosystem inhabiting each organism, thus constituting a "superorganism" [4]. GM plays several crucial roles in host physiology and influences several relevant functions: it harvests energy from indigestible food, influences fatty

acid oxidation, fasting, bile acid production, satiety, and lipogenesis, and even influences innate immunity (reviewed in [3]). In more than one respect, we are able to establish that you "feed your microbiota and are fed by it." GM provides signals that promote the production of cytokines, leading to the maturation of immune cells modulating the normal development of immune functions of the host immune system [5, 6]. Consequently, an imbalance of GM, or dysbiosis, can be the cause or at least lead to the progression of several pathologies such as infectious diseases, gastrointestinal cancers, cardiovascular disease, inflammatory bowel disease, and even obesity and diabetes [7, 8]. Additionally, a pathological state can cause an imbalance in this microbial ecosystem. For instance, a dysfunction of the innate immune system may be one of the factors that favor metabolic diseases through alteration of the GM [9].

In terms of immune response, the immune system recognizes conserved structural motifs of microbes, called PAMPs (pathogen-associated molecular patterns), by mean of toll-like receptors (TLR), which are expressed in the membrane of sentinel cells [10]. This interaction induces immune responses against microbes through the activation of inflammatory signaling pathways. Therefore, GM, which interacts with epithelial TLR, critically influences immune homeostasis [9]. Although the complete etiology of inflammatory diseases remains unknown, intestinal gut dysbiosis has been associated with a variety of neonatal and children's diseases [4], in which chronic intestinal inflammation and mucosal damage derives from alteration of GM [11].

Diet provides the nutritional supplies for life and growth, and some components exert valuable effects when consumed regularly. These components are called "functional foods" or "nutraceuticals" [12]. Consequently, functional foods contain bioactive substances, nutraceutics, which can be classified as micronutrients (vitamins and fatty acids) and nonnutrients (phytochemicals and probiotics) (see Table 1 in [13]). These components, with a wide range of chemical structures and functionality, provide different beneficial effects beyond simple nutrition, resulting in improved health.

Gut bacterial diversity is mainly affected by the diet, which may also affect its functional relationships with the host [14–17]. During their gastrointestinal passage, the components of the diet are metabolized by intestinal bacteria [18]. Diets rich in carbohydrates and simple sugars lead to *Firmicutes* and *Proteobacteria* proliferation, while those rich in saturated fat and animal protein favor *Bacteroidetes* and *Actinobacteria* [19]. Microbial diversity of the intestine decreases in diets with higher fat content [16]. Several physiological aspects of the gut environment can be influenced by the diet, then, including absorption of micronutrients, vitamins, and nutraceutics, and changes in pH of the gut environment, which in turn alters the balance of the GM [20]. Therefore, GM influences the biological activity of food compounds but is also a target for nutritional intervention to improve health [18].

On this basis, phytochemicals, like nutraceuticals that can influence GM, are being studied as coadjuvants to treat obesity and inflammatory diseases. In this review, we will present, organize, and discuss the most recent evidence that points to a relationship of the phytochemical effect on GM that affects obesity and/or inflammation, focusing on the effect of phytochemicals as potential prophylactics and candidates for the treatment of these diseases.

2. Phytochemicals Can Influence Obesity and Inflammatory Diseases through Affecting GM

Phytochemicals can be defined as "bioactive nonnutrient plant compounds present in fruits, vegetables, grains, and other plants, whose ingestion has been linked to reductions in the risk of major chronic diseases" [21]. Held to be phytochemicals, prebiotics are nondigestible food components (mainly carbohydrate polymers, such as fructooligosaccharides and mannooligosaccharides) that benefit the human body because they modulate GM through selective stimulation of some bacterial species proliferation in the colon, named "probiotics" [22]. These include endosymbionts such as lactic acid bacteria, bifidobacteria, yeast, and bacilli, which participate in the metabolism of their hosts [13]. Regarded as functional foods, both prebiotics and probiotics have been considered potential constituents of therapeutic interventions that modify GM in an attempt to modulate in turn some inflammatory diseases (comprehensively reviewed in [23]). On the other hand, the remaining phytochemical compounds may be classified on the basis of some common structural features into groups as follows: polyphenols and derivatives, carotenoids, and thiol-sulfides, among others (see Table 1 in [13]). Of the latter, the polyphenols represent the largest group.

Polyphenols are secondary metabolites of plants and represent vastly diverse phytochemicals with complex chemical structures. They are commonly present in plant foods, such as cacao, coffee, dry legumes (seeds), fruits (like apples and berries), nuts, olives, some vegetables (such as lettuce and cabbage), tea, and wine. The daily intake of dietary phenols is estimated to be above 1 g, which is 10 times higher than the vitamin C intake from diet [24]. The interaction between polyphenols and GM has been well established [25]. Polyphenols are frequently conjugated as glycosides, which derive in aglycones when metabolized by GM. Generally, the intestinal metabolism of polyphenols includes hydrolysis of glycosides and esters, reduction of nonaromatic alkenes, and cleavage of the skeletons [26, 27]. Studies have reported that only a low number of polyphenols can be absorbed in the small intestine. The remaining (90–95%) nonabsorbed polyphenols reach the colon in high concentrations (up into the mM range), where they are degradated by microbial enzymes before their absorption [28]. Compared to their parent compounds, the permanence in plasma for metabolites is extended and they are finally eliminated in urine [29, 30]. GM, then, can regulate the health effects of polyphenols, and reciprocally, polyphenols can modulate GM and even interfere with its own bioavailability [31].

Table 1: Effects of different phytochemicals on GM and/or obesity with anti-inflammatory actions.

Phytochemicals	Compound	Model	Effect on gut microbiota	Antioxidant and anti-inflammatory effect	Effect on obesity	Ref
Polyphenols		C57BL/6 J ApcMin mice	Bacterial diversity was higher in the bilberry group than in the other groups	Attenuation of inflammation in cloudberry-fed mice		[183]
Flavonones	Baicalein	C57BL/6 J mice		Suppress activation of NF-κB and decrease expression of iNOS and TGF-β	Activation of AMPK pathway and suppression of fatty acid synthesis, gluconeogenesis, and increased mitochondrial oxidation	[184]
Catechins	Epigallocatechin-3-gallate	C57BL/6 J mice	The *Firmicutes*/*Bacteroidetes* ratio is significantly lower in HFD + EGCG but higher in control diet + EGCG		Potential use for prevention, or therapy, for obesity-related and oxidative stress-induced health risks	[185]
	Epigallocatechin-3-gallate	C57BL/6 J mice	Regulates the dysbiosis and maintains the microbial ecology balance		Significant protective effect against obesity induced by high-fat diet (HFD)	[186]
	Epigallocatechin-3-gallate	Wistar rats	EGCG affects the growth of certain species of GM		Weights of abdominal adipose tissues fed 0.6% EGCG diet were suppressed. Regulated energy metabolism in the body	[187]
	Quercetin	C57BL/6 J mice	An increase in *Firmicutes*/*Bacteroidetes* ratio and in gram-negative bacteria and increased in *Helicobacter* by HFD. Quercetin treatment benefits GM balance	Quercetin reverted dysbiosis-mediated Toll-like receptor 4 (TLR-4) NF-κB signaling pathway activation and related endotoxemia, with subsequent inhibition of inflammasome response and reticulum stress pathway activation	Benefits gut-liver axis activation associated to obesity, leading to the blockage of lipid metabolism gene expression deregulation	[109]
	Quercetin	Wistar rats	Quercetin supplementation attenuates *Firmicutes*/*Bacteroidetes* ratio and inhibiting the growth of bacterial species previously associated to diet-induced obesity (*Erysipelotrichaceae*, *Bacillus*, *Eubacterium cylindroides*). Quercetin was effective in lessening high-fat sucrose diet-induced GM dysbiosis			[188]
	Quercetin	Fischer 344 rats	Exerts prebiotic properties by decreased pH, increased butyrate production, and altered GM		Onion extract increased glutathione reductase (GR) and glutathione peroxidase (GPx1) activities in erythrocytes. In contrast, g-glutamate cysteine ligase catalytic subunit gene expression was upregulated	[189]
	Kaempferol	3 T3-L1 adipocytes		Kaempferol reduced LPS proinflammatory action. Demonstrating the anti-inflammatory and antioxidant effects	Concomitantly, polyphenols increased the production of adiponectin and PPARγ, known as key anti-inflammatory and insulin-sensitizing mediators	[110]

Table 1: Continued.

Phytochemicals	Compound	Model	Effect on gut microbiota	Antioxidant and anti-inflammatory effect	Effect on obesity	Ref
Anthocyanins		C57BL/6 J mice	Feces of GM-deficient mice showed an increase in anthocyanins and a decrease in their phenolic acid metabolites, while a corresponding increase was observed in jejunum tissue		Mice with intact GM reduced body weight gain and improved glucose metabolism	[190]
Anthocyanins		C57BL/6 J mice		Anthocyanins could effectively reduce the expression levels of *IL-6* and *TNFα* genes, markedly increasing SOD and GPx activity	Anthocyanins reduced body weight could also reduce the size of adipocytes, leptin secretion, serum glucose, triglycerides, total cholesterol, LDL-cholesterol, and liver triglycerides	[191]
Phenolic acid	Curcumin	Mice	A direct effect of bioactive metabolites reaching the adipose tissue rather than from changes in GM composition	Nutritional doses of *Curcuma longa* is able to decrease proinflammatory cytokine expression in subcutaneous adipose tissue	An effect independent of adiposity, immune-cell recruitment, angiogenesis, or modulation of GM controlling inflammation	[192]
	Curcumin	LDLR−/− mice	Curcumin improves intestinal barrier function and prevents the development of metabolic diseases	Significantly attenuated the Western diet-induced increase in plasma LPS levels	Significantly reduced WD-induced glucose intolerance and atherosclerosis	[193]
	Curcumin	Human IEC lines Caco-2 and HT-29	Curcumin modulates chronic inflammatory diseases by reducing intestinal barrier dysfunction despite poor bioavailability	Curcumin significantly attenuated LPS-induced secretion of master cytokine IL-1β from IEC and macrophages. Also reduced IL-1β-induced activation of p38 MAPK in IEC and subsequent increase in expression of myosin light-chain kinase	Curcumin attenuates WD-induced development of type 2 diabetes mellitus and atherosclerosis	[194]
Stilbenes	Resveratrol	Kunming mice	HF microbiomes were clearly different from those in CT and HF-RES mice. After treatment, *Lactobacillus* and *Bifidobacterium* were significantly increased, whereas *Enterococcus faecalis* was significantly decreased, resulting in a higher abundance of *Bacteroidetes* and a lower abundance of *Firmicutes*		Treatment inhibited increases in body and fat weight in HF mice. Decreased blood glucose to control levels, decreased blood insulin and serum total cholesterol compared with HF mice. Severe steatosis seen in HF mice was well prevented in treated mice. Treatment significantly suppressed expression of PPAR-γ, Acc1, and Fas, suggesting inhibition of triglyceride storage in adipocytes	[195]
	Resveratrol	Glp1r−/− mice	Treatment modified GM	Decreased the inflammatory status of mice	Glucoregulatory action of RSV in HFD-fed diabetic wild-type mice, in part through modulation of the enteroendocrine axis *in vivo*	[196]

Table 1: Continued.

Phytochemicals	Compound	Model	Effect on gut microbiota	Antioxidant and anti-inflammatory effect	Effect on obesity	Ref
	Resveratrol	Wistar rats	Trans-resveratrol supplementation alone or in combination with quercetin scarcely modified the GM profile but acted at the intestinal level, altering mRNA expression of tight-junction proteins and inflammation-associated genes	Altering mRNA expression of tight-junction proteins and inflammation-associated genes	Administration of resveratrol and quercetin together prevented body weight gain and reduced serum insulin levels. Effectively reduced serum insulin levels and insulin resistance	[188]
	Resveratrol	Adipocytes		Generally, resveratrol opposed the effect induced by LPS, functioning as an ameliorating factor in disease state	LPS altering glycosylation processes of the cell. Resveratrol ameliorates dysfunctioning adipose tissue induced by inflammatory stimulation	[197]
	Resveratrol	Humans	Steroid metabolism of the affected GM should be studied in detail		Subtle but robust effects on several metabolic pathways	[198]
	Piceatannol	C57BL/6 mice	Pic altered the composition of the GM by increasing *Firmicutes* and *Lactobacillus* and decreasing *Bacteroidetes*		Pic significantly reduced mouse body weight in a dose-dependent manner. Significantly decreased the weight of liver, spleen, perigonadal, and retroperitoneal fat compared with the HFD group. Pic significantly reduced adipocyte cell size of perigonadal fat and decreased weight of liver	[199]
	Piceatannol	Zucker obese rats	It did not modify the profusion of the most abundant phyla in GM, though slight changes were observed in the abundance of several *Lactobacillus*, *Clostridium*, and *Bacteroides* species belonging to *Firmicutes* and *Bacteroidetes*	Shows a tendency to reduce plasma LPS by 30%	Pic did not reduce either hyperphagia or fat accumulation. There is a tendency toward the decrease of circulating on-esterified fatty acids, LDL-cholesterol, and lactate. While Pic tended to improve lipid handling, it did not mitigate hyperinsulinemia and cardiac hypertrophy	[155]
Organosulfur compounds	GEO (garlic essential oil) DADS (DiAllyl DiSulfide)	C57BL/6 mice		Significantly decreased the release of proinflammatory cytokines in liver, accompanied by elevated antioxidant capacity via inhibition of cytochrome P450 2E1 expression	GEO and DADS dose-dependently exerted antiobesity and antihyperlipidemic effects by reducing HFD-induced body weight gain, adipose tissue weight, and serum biochemical parameters	[200]

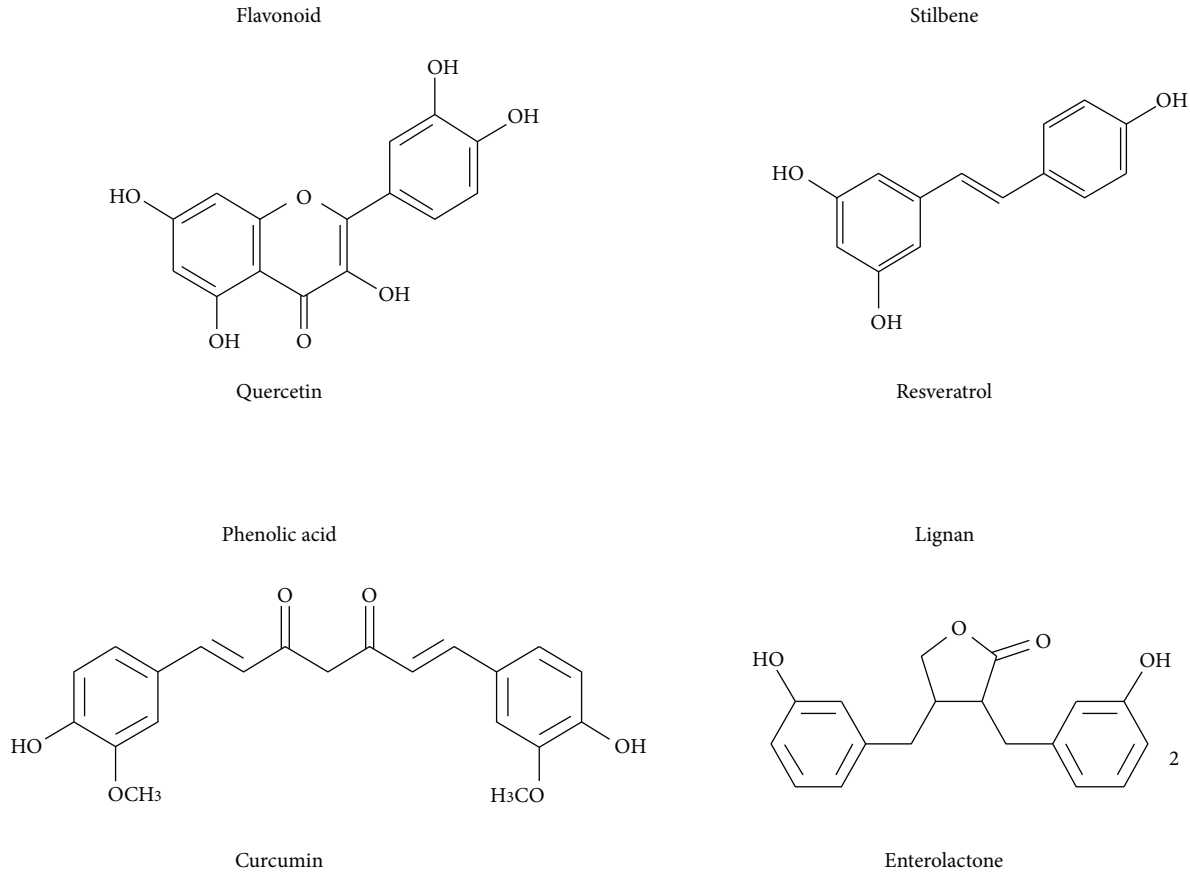

Figure 1: Chemical structure of representative molecules for the four main polyphenol groups.

Approximately 8000 structures of polyphenols have been identified [32], which can be classified into four main groups (Figure 1) as follows: (a) flavonoids (with eight subgroups), (b) phenolic acids (curcumin), (c) stilbenoids (resveratrol), and (d) lignanes. Polyphenols have been extensively studied over the past decade because of their strong antioxidant and anti-inflammatory properties and their possible role in the prevention and cotreatment of several chronic diseases, such as hypertension, diabetes, neurodegenerative diseases, and cancer [33–36]. In addition, polyphenols have recently attracted interest in the media and in the research community because of their potential role in reducing obesity, an increasingly serious health issue in different population age ranges [37, 38]. Polyphenols such as catechins, anthocyanins, curcumin, and resveratrol have been suggested as exerting beneficial effects on lipid and energy metabolism [39–41] and potentially on weight status. Multiple mechanisms of action have been proposed mostly as a result of animal and cell studies, such as inhibition of the differentiation of adipocytes [40], increased fatty acid oxidation [42], decreased fatty acid synthesis, increased thermogenesis, the facilitation of energy metabolism and weight management [43], and the inhibition of digestive enzymes [44].

Phenolic compounds from tea [45], wine [29], olives [46] and berries [47, 48] have demonstrated antimicrobial properties. Depending on their chemical structure, tea phenolics inhibit the growth of several bacterial species, such as *Bacteroides* spp., *Clostridium* spp., *Escherichia coli*, and *Salmonella typhimurium* [29]. Furthermore, tea catechins are able to change the mucin content of the ileum, affecting the bacterial adhesion and therefore their colonization [48]. Another study revealed that (+) catechin favored the growth of the *Clostridium coccoides-Eubacteriumrectale* group and *E. coli* but inhibited that of *Clostridium histolyticum*. In addition, the growth of beneficial bacteria, such as *Bifidobacterium* spp. and *Lactobacillus* spp., was nonaffected or even slightly favored [45, 49]. Both flavonoids and phenolic compounds reduce the adherence of *Lactobacillus rhamnosus* to intestinal epithelial cells [50]. The anthocyanins, a type of flavonoid, inhibit the growth of several pathogenic bacteria, including *Bacillus cereus*, *Helicobacter pylori*, *Salmonella* spp., and *Staphylococcus* spp. [47, 48]. Consequently, phytochemicals that affect the balance of the GM may influence obesity and inflammatory diseases.

Therefore, through the modulation of GM, polyphenols have the potential to generate health benefits. Although there is accumulative evidence concerning the polyphenolic effect on GM, the effects of the interaction between polyphenols and specific GM functions remain mostly uncharacterized; thus, much research remains to

be conducted. We will focus on specific polyphenols that have been reported as able to affect GM and, in addition, influence obesity and/or inflammation.

3. Experimental Nutritional Interventions with Phytochemicals That Modify Gut Microbiota Exert an Effect on Obesity and/or Inflammatory Parameters

According to the United States National Agricultural Library, a "nutritional intervention" is "A clinical trial of diets or dietary supplements customized to one or more specific risk groups, such as cancer patients, pregnant women, Down syndrome children, populations with nutrient deficiencies, etc." [51]. In a broader sense, we review herein the use of phytochemicals in experimental models (mainly polyphenols), which are able to modify GM and exert an effect on obesity and/or inflammatory parameters, in order to analyze and discuss their potential use for the prophylaxis and treatment of obesity and inflammatory diseases by the maintenance and control of GM.

To compile the information from scientific literature on the polyphenols that can be related with GM, we considered the following terms for search in PubMed: "gut microbiota" OR "intestinal microbiota" OR "gut flora" OR "intestinal flora" OR "gut microflora" OR "intestinal microflora," and we added the specific compound (as listed in Figure 2). From this search, we can conclude that there is at least one report that correlates every polyphenol listed with GM. In addition, of the 40 listed compounds, there are 15 that yield at least 10 works that support the relationship between polyphenols and GM. However, there is still much work to be done in this area in terms of exploring in greater detail the specific actions of each compound on GM. Later, we added to these searches the following terms: "anti-inflammatory OR antiinflamatory" on one subsequent search, or "obesity" for another search. In both cases, the numbers of articles were scarce with a total of 116 and 71, respectively, although this number does not represent a real situation, because there are several articles that are repeated, and those that include more than one compound. From these articles, we extracted information that led to the indication of a relationship among the effects of different phytochemicals on the GM that affects obesity and/or the immune response (Table 1).

3.1. Flavonoids. The first and largest subgroup of polyphenols is integrated by flavonoids, with >6000 compounds identified and isolated from different plant sources [52], a large family of chemical compounds that constitutes plant and flower pigments and that shares the common function of being free radical scavengers. Due to the thousands of structurally different compounds, it becomes quite difficult to analyze all of them. Therefore, we performed a wide search of different specific compounds that have been reported in the literature and compiled them into eight subgroups, including the most representative compounds within each group (Figure 2). Essentially, all of these are widely recognized by their antioxidant [32, 53, 54] and anti-inflammatory [34, 55, 56] properties. Indeed, they inhibit reactive oxygen species (ROS) synthesis and hypoxia-signaling cascades, modulate cyclooxygenase 2 (COX-2), and block epidermal growth factor receptor (EGFR), insulin-like growth factor receptor-1 (IGFR-1), and nuclear factor-kappa B (NF-κB) signaling pathways. In addition, flavonoids are able to modulate the angiogenic process [57], and the majority of these have been recently involved with obesity [58, 59].

3.1.1. Flavones. Numerous studies have been undertaken on the influence of GM on the intestinal absorption and metabolism of particular flavones, such as apigenin, luteolin, and chrysin, both in rodents and in human cells [60–63]. On the other hand, there are multiple studies that associate different flavones with anti-inflammatory effects. This is the case for apigenin [64–67], luteolin [68, 69], and chrysin [34]. Furthermore, recent studies involve apigenin with the amelioration of obesity-related inflammation [70] and regulating lipid and glucose metabolism [71], luteolin with the amelioration of obesity-associated insulin resistance, hepatic steatosis and fat-diet-induced cognitive deficits [72–75], and chrysin, which inhibits peroxisome proliferator-activated receptor-γ (PPAR-γ) and CCAAT/enhancer binding protein A (C/EBPα), major adipogenic transcription factors in preadipocytes [75] and which also modulate enhanced lipid metabolism [76]. However, to the best of our knowledge, there is still no study that considers together these following three aspects: GM, inflammation, and obesity as positively affected by these flavones. Consequently, this constitutes a whole new avenue for studying these interactions.

3.1.2. Flavanones. Like the previous subgroup, flavanones also influence and interact with GM [28, 77, 78]. The main compounds included here also exhibit anti-inflammatory properties, such as hesperetin [79, 80], naringenin [81], morin [82–84], and eriodictyol [85–87]. Additionally, they influence lipid metabolism as a potential preventive strategy for obesity. For instance, hesperetin exhibits lipid-lowering efficacy [88, 89]; naringenin regulates lipid and glucose metabolism [71] and also prevents hepatic steatosis and glucose intolerance [90] by suppressing macrophage infiltration into the adipose tissue [91]. In addition, both compounds improve membrane lipid composition [92]. Furthermore, morin exhibits antihyperlipidemic potential by reducing lipid accumulation [31, 93]. Finally, eriodictyol ameliorates lipid disorders and suppresses lipogenesis [94]. Taken together, all of this evidence strongly indicates that these compounds can be usefully applied to prevent or treat obesity and its associated inflammation, but it is relevant to take GM into account in order to incorporate it into the organism's metabolism. Again, there are to our knowledge no studies that correlate all three of these aspects.

3.1.3. Flavonones. In this case, nomenclature represents a problem in the literature search, because the term "flavonones" is usually substituted by "flavanones," which in fact represent a different subgroup. Due to this, compounds included in this subgroup were individually searched in databases. Three compounds were considered: hesperidin,

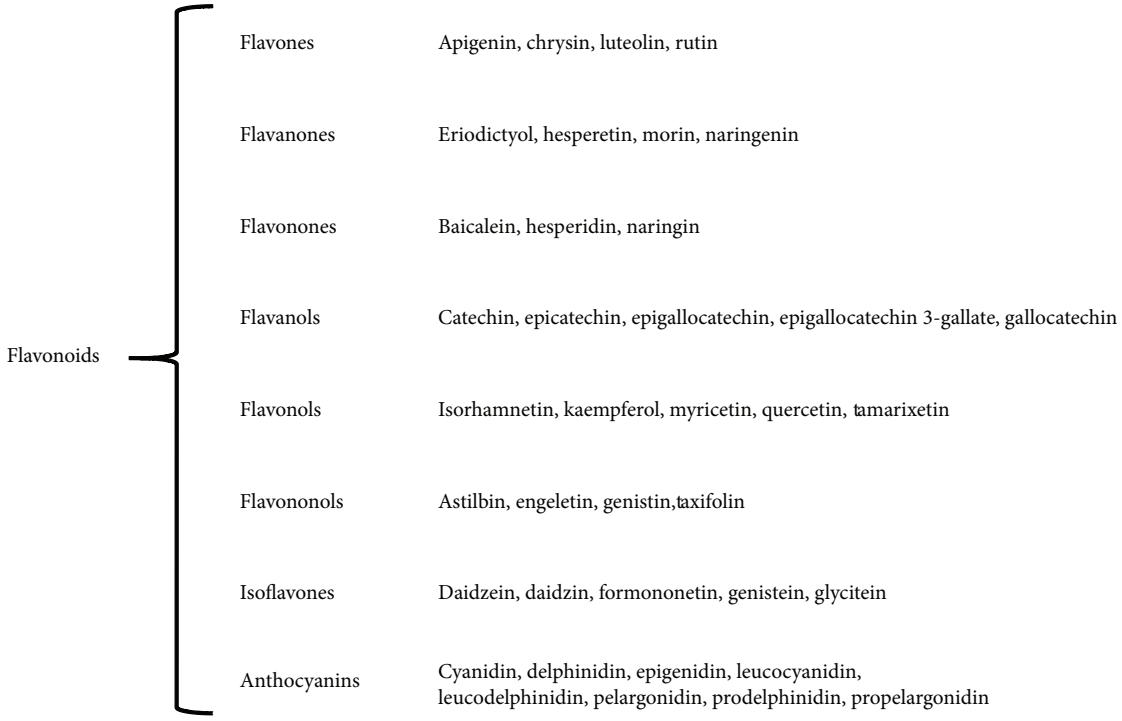

Figure 2: Classification of the eight foremost flavonoid subgroups.

naringin, and baicalein. In fact, the former two can be confused with similarly named compounds from the flavanone subgroup (see above) but constitute different compounds. As for all the polyphenols, the latter is metabolized by the GM [93, 95] and exhibits strong anti-inflammatory properties [79, 96, 97]. Additionally, these compounds also influence lipid metabolism as follows: hesperidin improves lipid metabolism against alcohol injury by reducing endoplasmic reticulum stress and DNA damage [98] and exhibits an antiobesity effect [99]; naringin also influences the lipid profile and ameliorates obesity [100], and finally, baicalein regulates early adipogenesis by inhibiting lipid accumulation and m-TOR signaling [101]. Again, there is a need for studies that take into account the following elements together, that is, GM metabolism of the polyphenols and their specific effect on lipid metabolism, obesity, and inflammation.

3.1.4. Flavanols. This subgroup mainly comprises catechins, which are more abundant in the skin of fruits than in fruit pulp. Catechins found in cranberries may contribute to cancer prevention [102]. Catechins are abundant in green tea, to which has been attributed several beneficial impacts on health. Traditionally, green tea has been used to improve resistance to disease and to eliminate alcohol and toxins by clearing the urine and improve blood flow [103, 104]. Lately, emerging areas of interest have been the effects of green tea for the prevention of cancer and cardiovascular diseases, as well as their effects on angiogenesis, inflammation, and oxidation [105, 106].

This subgroup of flavonoids is one of the few that has been studied to date under the lens of their relationship with GM and their anti-inflammatory actions [107], as well as their role in lipid metabolism and obesity [105, 108]. Among the compounds included in this group, we find the following: catechin, epicatechin, epigallocatechin, epigallocatechin 3-gallate, and gallocatechin. Practically, all of these have already begun to be studied in the light of their relationship between GM and inflammation, as well as that related with lipid metabolism and obesity (see Table 1 for specific examples). However, much work remains to ascertain the mechanisms by which these compounds are able to benefit health.

3.1.5. Flavonols. Compounds in this subgroup have also been studied as related with GM and inflammation or obesity, mainly quercetin and kaempferol, while another three, rutin, myricetin, and isohamnetin, have not to our knowledge been studied within this context. Quercetin protects against high-fat diet-induced fatty liver disease by modulating GM imbalance and attenuating inflammation [109]. Kaempferol also exhibits protective properties, both anti-inflammatory and antioxidant, in adipocytes in response to proinflammatory stimuli [110]. These two works, by Porras et al., and Le Sage et al., respectively, constitute some clear examples of the experimental approximations that need to be done to increase our knowledge on the relationships already mentioned among phytochemicals, GM, inflammation, and obesity. Therefore, this subgroup constitutes that of the leading compounds in the study of the relationship among these three elements (Figure 3).

3.1.6. Flavononols. This is another subgroup with nomenclature problems for the literature search, because the term "flavononols" is usually substituted by "flavonols," which is a different group (see above). For this reason, compounds

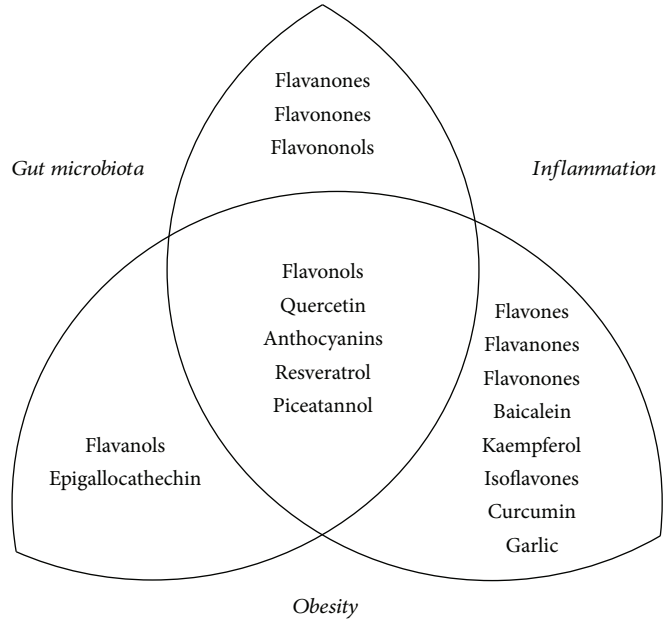

Figure 3: Phytochemicals that affect gut microbiota with anti-inflammatory and/or antiobesity properties.

included in this group were individually searched. This subgroup includes genistein, taxifolin, engeletin, and astilbin. Again, all of these are metabolized by GM and also exhibit potent anti-inflammatory properties [111–114], as well as being able to influence energy metabolism (both lipid and carbohydrate) [115–117]. Despite this, to our knowledge there is a lack of research regarding the possible effects of this subgroup of flavonoids on obesity and/or inflammation through their effect on GM.

3.1.7. Isoflavones. This subgroup has been partially studied with relation to GM and inflammation or obesity. It is made up of phytoestrogens, which are mainly present in soybeans. Isoflavones are metabolized by GM [30, 118, 119]. They also show an anti-inflammatory effect [120], as well as having had a hypocholesterolemic effect attributed to them [121]. The following are found included in this group: daidzein, genistein, glycitein, formononetin, and daidzin. Daidzein is metabolized by GM mainly into equol, which contributes to the beneficial effects of soybeans [122]; thus, it is relevant that dietary fat intake diminishes GM's ability to synthesize equol [123]. In addition, daidzein and genistein reduced lipid peroxidation *in vivo* and increased the resistance of low-density lipoproteins (LDL) to oxidation [124] and both exhibit an anti-inflammatory activity [125]. Glycitein affects gene expression in adipose tissue [126] and demonstrates antiobese and antidiabetic effects [127]. Additionally, together with daidzein and genistein, glycitein exhibits an anti-inflammatory and neuroprotective effect on microglial cells [128]. Finally, formononetin and daidzin have also received attention because of their anti-inflammatory properties [129–131]. Once again, this group would be interesting for further studies regarding their metabolism by GM in relation with inflammation and lipid metabolism for obesity.

3.1.8. Anthocyanins. Anthocyanins are a class of flavonoids that are ubiquitously found in fruits and vegetables and they possess many pharmacological properties, for example, lipid-lowering, antioxidant, antiallergic, anti-inflammatory, antimicrobial, anticarcinogenic, and antidiabetic actions [132–135]. Strawberries constitute a source of anthocyanins and have been recently broadly evaluated for their effect on human health, due to their rich phytochemical content, effectiveness in rodent models, and almost no toxicity observed in pilot studies in humans [136, 137]. In rodent models, for example, strawberries have shown anticancer activity in several tissues [138]. This subgroup includes a long list of compounds, such as cyanidin, delphinidin, epigenidin, leucocyanidin, leucodelphinidin, pelargonidin, prodelphinidin, and propelargonidin. Although there are fewer than 70 papers that correlate at least one of these compounds with anti-inflammatory activity or obesity (or lipid metabolism), there are only a dozen papers, to our knowledge, which correlate any of these compounds with their metabolism by GM, and none of them associate this information among these aspects. Therefore, this constitutes a nearly complete virgin area still to be explored.

3.2. Phenolic Acids

3.2.1. Curcumin. A second subgroup of polyphenols is constituted by phenolic acids, such as curcumin (diferuloylmethane), which is abundantly present in the rhizomes of the *Curcuma longa*, used both in traditional medicine and in cooking. Curcumin has been used for the coadjuvant treatment of a large diversity of diseases, including hepatic disorders, respiratory conditions, and inflammation and also obesity, diabetes, rheumatism, and even certain tumors. One relevant aspect to notice is that even at very high doses, no studies in animals or humans have revealed significant

curcumin toxicity [139]. Curcumin possesses a great protective impact on acute alcoholic liver injury in mice and can improve the antioxidant activity of mice after acute administration of alcohol. It can increase the activity of antioxidant enzymes in liver tissues [140]. Curcumin is also metabolized by GM; the biotransformation of turmeric curcuminoids by human GM is reminiscent of equol production from the soybean isoflavone daidzein [141]. Curcumin modulates GM during colitis and colon cancer [142] and improves intestinal barrier function [141]. In addition, it is largely considered a potent anti-inflammatory and neuroprotective agent [143, 144], as well as a possible factor for the treatment of obesity [145–147]. The research on curcumin is extensive; notwithstanding, there are still very few papers that deal with the relationship of curcumin metabolism by GM, its action over intestinal permeability, and effect on obesity and/or inflammation (Table 1).

3.3. Stilbenes

3.3.1. Resveratrol. The third subgroup of polyphenols comprises stilbenoids, such as resveratrol (3,5,4′-trihydroxystilbene) and piceatannol (3,3′,4,5′-trans-trihydroxystilbene). Resveratrol is a natural, nonflavonoid polyphenolic compound that can be found in grape wines, grape skins (red wine), pines, peanuts, mulberries, cranberries, and legumes, among other plant species, which synthesize it in response to stress or against pathogen invasion [148, 149]. Resveratrol is studied as a potent antioxidant with neuroprotective activity. Several *in vitro* and *in vivo* studies show various properties for resveratrol as a potent antioxidant and antiaging molecule, which also exhibits anti-inflammatory, cardioprotective, and anticancer effects, able to promote vascular endothelial function and enhance lipid metabolism [147, 150]. Principally, it is the anti-inflammatory effect of resveratrol which has been widely reported [151], as well as its antiobesity effect [152]. Regarding the GM effect, resveratrol favored the proliferation of *Bifidobacterium* and *Lactobacillus* and counteracts the virulence factors of *Proteus mirabilis* [29]. In fact, resveratrol exhibits pleiotropic actions, modulates transcription factor NF-κB, and inhibits the cytochrome P450 isoenzyme CYP1 A1, as well as suppressing the expression and activity of cyclooxygenase enzymes, modulating p53, cyclins, and various phosphodiesterases, suppressing proinflammatory molecules, and inhibiting the expression of hypoxia-inducible transcription factor 1 (HIF-1α) and vascular endothelial growth factor (VEGF), among other actions [153]. Some studies analyze the effect of resveratrol on GM combined with their anti-inflammatory and antiobesity actions (Table 1). It constitutes a good example of the potential that the profound study of phytochemicals and their impact on health represents.

3.3.2. Piceatannol. Piceatannol is a hydroxylated analogue of resveratrol found in various plants (mainly grapes and white tea). It is less studied than resveratrol but also exhibits a wide biological activity [154]. It mainly exhibits potent anticancer properties and also antioxidant and anti-inflammatory activities, which make it a potentially useful nutraceutical and possibly an attractive biomolecule for pharmacological use [59]. Recently, Hijona et al. [155] studied its beneficial effects on obesity. Although these are limited, it constitutes a promissory phytochemical molecule.

3.4. Organosulfur Compounds

3.4.1. Garlic. In addition to polyphenols, another group of phytochemicals of relevance for health is the organosulfur compounds. For instance, garlic (*Allium sativum*) is a rich source of organosulfur compounds and exhibits a plethora of beneficial effects against microbial infections as well as cardioprotective, anticarcinogenic, and anti-inflammatory activity [156].

Nearly 80% of garlic's cysteine sulfoxide is constituted by alliin (allylcysteine sulfoxide). When raw or crushed garlic is chopped, the "allinase" enzyme is released which catalyzes sulfonic acid formation from cysteine sulfoxides and when the two react with each other, they produce an unstable compound: thiosulfinate or allicin. The *in vitro* breakdown of allicin produces numerous fat-soluble components: diallyl sulfide; DiAllylDiSulfide (DADS), and DiAllylTriSulfide (DATS). Likewise, vinyldithiins, S-allylcysteine, ajoene, S-1-prpenylcysteine, and S-allylmercaptocysteine are important constituents of garlic powder, oil, and extracts [157, 158].

Naturally occurring products have attracted the attention of researchers as sources of novel drugs and drug leads for the treatment of obesity [159–161]. *Allium* species have been used in herbolary or traditional medicine for the treatment of metabolic diseases, and *Allium*-derived extracts have recently become of interest for their antiobesity effects [162].

The chemical constituents of garlic are enzymes (asalliinase) and organosulfur compounds (such as alliin and its derived agent, allicin). The effect of garlic on different medical conditions (such as hypertension, hyperlipidemia, diabetes mellitus, rheumatic disease, the common cold, arteriosclerosis, and cancer) has been widely investigated. Garlic is known as a hypolipidemic agent because of its role in increasing the hydrolysis of triacylglycerols due to increased lipase activity [163]. Moreover, garlic reduces the biosynthesis of triacylglycerols through its blocking of nicotinamide adenine dinucleotide phosphate. On the other hand, garlic contains abundant antioxidants and can induce antioxidant enzymes [164]. Thus, garlic is a potential hepatoprotective agent against liver disorders [165]. Experimental studies have shown that garlic and its organosulfur compounds might reduce alcohol-related liver enzymes, glutathione reductase, alkaline phosphatase, lactate dehydrogenase, and alcohol dehydrogenase, as well as enhance liver antioxidant enzymes, and alleviate hepatic-fat accumulation [165–172]. However, there has been no clinical trial on patients with liver disorders [164].

4. Concluding Remarks and Perspectives

Several issues need to be solved before natural products can be effectively translated into the clinic. With regard to the best source of bioactive molecules or compounds, the following aspects should be considered: (a) if they are better

acquired directly from food in the diet or from pharmacological sources (purified or through synthetic analogues) and (b) if they should be used alone or as a cotreatment in combination with approved drugs. Therefore, there is a need to develop specific clinical trials. Disadvantages of commercial nutraceutic preparations include the high variability in formulations (preparation methods and chemical composition), as well as the dosage quantification and the different means of administration. Research devoted to the optimization of phytochemical formulation and dosage has become of critical importance. Given the low bioavailability of phytochemicals, the development of more useful synthetic derivatives has become a great concern [173].

Once nutrients and nutraceuticals have been incorporated into the body, the gut environment is essential in maintaining homeostasis; in this sense, like GM, the surface of the intestinal mucous membrane plays a fundamental role in the preservation of homeostasis. Consequently, the correct functioning of its permeability is of great importance [174]. Several pathologies, as well as susceptibility to metabolic diseases, have been linked to alterations in the permeability of the intestinal barrier. Humans possess two interacting genomes: their own and that of their host microbiome, the majority of which resides in the gut, in the layer of mucin glycoproteins (mucus) produced by the cells called goblet cells [168]. The microbiome provides products such as vitamins and nutrients to host cells, thereby establishing a beneficial ecosystem for host physiology and preventing the arrival of pathogens [175]. Thus, a symbiotic relationship is established between both genomes, through the expression of pattern recognition receptors (PRRs) for the sense of the presence of intestinal microbiota, through the microbe-associated molecular patterns (MAMPs). This communication between the two genomes results in the accuracy of the mucosal barrier function, by regulating the production of its components: mucus, antimicrobial peptides, IgA and IL-22, facilitating homeostasis, and immune tolerance [175–177]. Therefore, GM and the human host influence each other by exchanging their metabolic active molecules [178], working together, as a hologenome, to maintain mutual health [179].

Another current challenge is convincing a skeptical health sector of the use of such compounds as medicines, or at least in conjunction with pharmaceutical medicines, which could serve both practitioners and patients better [180]. For instance, research on traditional Chinese medicine has substantially increased recently through the search for its molecular, cellular, and pharmacological bases, with the identification of active substances and the investigation of mechanisms of action [181]. Although the available cumulative data strongly suggest the positive effects of a large variety of phytochemicals in terms of health, it remains insufficient in order to directly extract solid conclusions, due mainly to the lack of confirmation, in human trials, of the results obtained by the animal model studies. Consequently, more research must be focused on the analysis of different phenolic compounds metabolized by GM and their influence on human health [182]. Results are crucial for the precise understanding of the influence of GM on the metabolism of micronutrients and phytochemicals within the human organism, and their metabolism undergone upon ingestion, in order to correctly attribute beneficial health properties to specific polyphenols with a more complete knowledge of their bioavailability, metabolism, and effects on carbohydrate and lipid metabolism, and therefore their use in treating obesity and inflammatory diseases.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors' Contributions

Lucrecia Carrera-Quintanar and Rocío I. López Roa contributed equally to this work.

Acknowledgments

The work was partially supported by Universidad de Guadalajara Grant PRO-SNI 2017 to Daniel Ortuño-Sahagún and SEP-UDG-CA-454 to Barbara Vizmanos and Lucrecia Carrera-Quintanar and CONACyT-México Grant CB-2015-256736 to Rocío I. López Roa. Fellowship support was provided by CONACyT-México Grant 622462 to Marina A. Sánchez-Sánchez.

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Hindawi
Mediators of Inflammation
Volume 2018, Article ID 5201652, 10 pages
https://doi.org/10.1155/2018/5201652

Research Article

Butyrylcholinesterase Levels on Admission Predict Severity and 12-Month Mortality in Hospitalized AIDS Patients

Lijun Xu,[1,2] Biao Zhu,[1,2] Ying Huang,[1] Zongxing Yang,[1,3] Jia Sun,[1] Yan Xu,[1] Jinlei Zheng,[4] Sabine Kinloch,[5] Michael T. Yin,[6] Honglei Weng,[7] and Nanping Wu[1,2]

[1]*The State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, School of Medicine, Zhejiang University, Qingchun Rd, Hangzhou, China*
[2]*Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, School of Medicine, Zhejiang University, Qingchun Rd, Hangzhou, China*
[3]*Department of HIV/AIDS, Xixi Hospital, Hengbu Rd, Hangzhou, China*
[4]*Zhejiang Provincial Center for Disease Control and Prevention, Xincheng Rd, Hangzhou, China*
[5]*Department of HIV Medicine, Royal Free Hospital, Pond Street, London, UK*
[6]*Division of Infectious Diseases, Columbia University Medical Center, New York, NY, USA*
[7]*Department of Medicine II, Section Molecular Hepatology, Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany*

Correspondence should be addressed to Nanping Wu; flwnp2013@163.com

Received 13 August 2017; Accepted 24 January 2018; Published 15 March 2018

Academic Editor: Amedeo Amedei

Background. Butyrylcholinesterase (BChE) is synthesized mainly in the liver and an important marker in many infectious/inflammatory diseases, but its role in acquired immunodeficiency syndrome (AIDS) patients is not clear. We wished to ascertain if BChE level is associated with the progression/prognosis of AIDS patients. *Methods*. BChE levels (in U/L) were measured in 505 patients; <4500 was defined as "low" and ≥4500 as "normal." Associations between BChE level and CD4 count, WHO stage, body mass index (BMI), C-reactive protein (CRP) level, and duration of hospitalization were assessed. Kaplan–Meier curves and Cox proportional hazards model were used to assess associations between low BChE levels and mortality, after adjustment for age, CD4 count, WHO stage, and laboratory parameters. *Results*. A total of 129 patients (25.5%) had a lower BChE level. BChE was closely associated with CD4 count, WHO stage, CRP level, and BMI (all $P < 0.001$). Eighty-four patients (16.6%) died in the first year of follow-up. One-year survival was $64.5 \pm 4.5\%$ for patients with low BChE and $87.6 \pm 1.8\%$ for those with normal BChE (log-rank, $P < 0.001$). After adjustment for sex, age, BMI, WHO stage, and CD4 count, as well as serum levels of hemoglobin, sodium, and albumin, the hazard ratio was 1.8 (95% confidence interval, 1.0–3.2) for patients with a low BChE compared with those with a normal BChE ($P = 0.035$). *Conclusion*. BChE level is associated with HIV/AIDS severity and is an independent risk factor for increased mortality in AIDS patients.

1. Introduction

The digestive system plays an important part in pathogenesis of infection by the human immunodeficiency virus (HIV) [1]. The intestinal mucosa and gut-associated lymph nodes can serve as "HIV reservoirs" [2, 3]. As the largest organ in the digestive system, the liver is associated with HIV infection. For example, dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin is expressed abundantly on liver sinusoidal endothelial cells and promotes HIV infection [4]. Furthermore, the liver can modulate several processes in HIV infection by regulating lymphocyte functions, especially those of liver-associated lymphocytes, which contributes to the pathogenesis of acquired immune deficiency syndrome (AIDS) [5].

On the other hand, HIV infection exerts direct and indirect effects on the liver. Studies have demonstrated that HIV can infect hepatocytes, Kupffer cells [6, 7], and hepatic

stellate cells (HSCs) [8]. HIV infection of these three types of liver cells induces production of inflammatory cytokines, as well as increasing the risk of hepatic steatosis [9, 10]. As a result, circulating levels of several proteins synthesized in the liver (e.g., albumin, prealbumin, and transferrin) decrease in response to injury and inflammation in many critically ill patients, and attenuation of levels of such proteins is associated with an increased risk of mortality in AIDS patients [11].

Previously, we showed that 85% of hospitalized HIV/AIDS patients in China have advanced HIV disease and have nearly 25% mortality within the first year of highly active antiretroviral therapy (HAART), especially within the first 6 months of HAART [12]. Thus, identification of an easily obtainable biomarker that can predict short-term mortality would be an important advancement for care.

Butyrylcholinesterase (BChE) is an important enzyme synthesized in the liver. In China, serum BChE is recognized as a parameter of liver function included in nearly all the patients' routine chemical profile. The normal range of BChE is roughly from 4500 U/L to 15,000 U/L in adult Chinese population, and measurement of serum BChE is very cheap (≈1dollar). It is usually as a prognostic biomarker of liver diseases such as viral hepatitis, cirrhosis, hepatocellular carcinomas, and even liver failure [13–15]. BChE is also an important clinical marker in inflammation [16, 17], severe bacterial infection [18], and fungal infection [19]. Importantly, reduced BChE indicates severe systemic inflammation in critically ill patients [20]. In addition, the BChE level reflected the nutrition state of patients [21]. Interestingly, patients infected with HIV and either HBV or HCV showed a direct correlation between decreased BChE and reduced CD4 counts [22]. However, the relationship between BChE and AIDS is not clear. Since low BChE reflects poor immune status, increased inflammation, infections, and poor nutrition of patients, we hypothesize that BChE may be a potential biomarker for HIV/AIDS patients.

In the present study, BChE was employed to evaluate the progression and prognosis of AIDS patients and compared it against other commonly available biomarkers. We want to know if BChE level was associated with the progression/prognosis of AIDS patients.

2. Materials and Methods

2.1. Ethical Approval of the Study Protocol. The study protocol was in accordance with the 1975 Declaration of Helsinki and approved by the Ethics Committee of the First Affiliated Hospital, School of Medicine, Zhejiang University (Hangzhou, China). Written informed consent was obtained from all patients to participate in the study.

2.2. Study Cohort. Between April 2010 and March 2015, a total of 589 HIV/AIDS patients from the First Affiliated Hospital, School of Medicine, Zhejiang University, were eligible for enrollment in this prospective study. Among them, 84 were excluded for the following reasons: 56 had HIV/hepatitis B virus (HBV) infection, 13 had HIV/HCV infection, 1 patient had HIV/HBV/HCV infection, 4 patients had cancer, and 18 patients were about to undergo surgery. Thus, 505 patients formed the study cohort (Figure 1). Subjects underwent HAART if they had a CD4 cell count ≤ 350 cells/μL or were WHO disease stage III or IV [23, 24]. The basic regimen was zidovudine (AZT) or tenofovir (TDF) plus lamivudine (3TC) combined with either nevirapine (NVP), efavirenz (EFV), or ritonavir-boosted lopinavir (LPV/r).

2.3. Laboratory Tests. Upon hospital admission, blood samples were drawn after a 12-hour fast. BChE was assayed with an Autobiochemical Analyzer (Beckman Coulter, Fullerton, CA, USA) using the choline thiobutyrate method. BChE ≥ 4500 U/L was defined as "normal BChE" and BChE < 4500 U/L as "low BChE." Numbers of CD4+ and CD8+ T cells were measured using a Flow Cytometer (Becton Dickinson, Fullerton, NJ, USA) with fluorescein isothiocyanate-conjugated antihuman CD4, phycoerythrin-conjugated antihuman CD8, and phycoerythrin-Cy5-conjugated antihuman CD3 monoclonal antibodies (Becton Dickinson). HIV-1 RNA was assayed according to a standard protocol of the COBAS® AmpliPrep/TaqMan® 48 Analyzer (Roche, Basel, Switzerland). The lower limit of detection for HIV was 400 copies/mL.

2.4. Follow-Up and Collection of Clinical Data. Follow-up was undertaken at three-month intervals according to a method described previously [12, 25]. The time of follow-up was from the first day of hospital admission. Cases were followed up for 1 year.

2.5. Statistical Analyses. Continuous normal variables are the mean ± standard deviation. Categorical variables are the number of cases (percentage). CD4 count (cells/μL) are expressed as medians (interquartile range, IQR). HIV-RNA levels (copies/mL) were log10-transformed into variables (log copies/mL) to meet the normality criteria for statistical analyses. Continuous variables were compared by one-way ANOVA or Student's t-test. Categorical variables were compared by $\chi 2$ analyses or Fisher's exact test. The effect of BChE on patient survival was analyzed by the Kaplan–Meier method and Cox proportional hazards model. "AIDS-related death" was defined as an "event." Data for patients were censored at the date of the final visit (for those alive at the end of the follow-up period), date last known to be alive (for those with unknown vital signs), or the date of participants for whom the cause of death was not known to be AIDS-related. The clinical laboratory date collected were date only within the first three days of patients' admission. Data not available or beyond the first three days of patients' admission were defined as "missing data." BChE (U/L) had categories of <4500 and ≥4500; age (years) of <30 and ≥30; WHO stage of I, II, III, and IV; CD4 count (cells/μL) of missing data, <50 and ≥50; HIV-RNA (copies/mL) of missing data , $<10^5$ and $\geq 10^5$; hemoglobin (g/L) of missing data, <110 and ≥110; serum concentration of sodium (mmol/L) of missing data, <130 and ≥130; and albumin (g/L) of missing data, <35 and ≥35. These categories were included in the models as time-dependent covariates. These covariates were analyzed first in the univariate Cox model. Then, covariates with $P < 0.2$ in the univariate model were

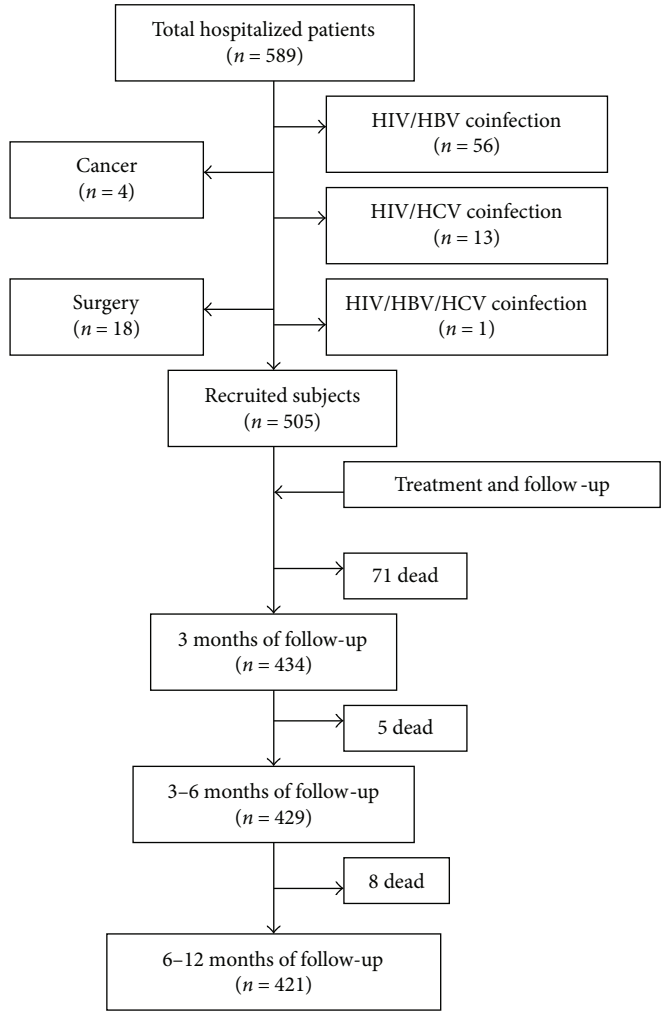

Figure 1: Study flowchart for patient selection.

selected for the multivariate Cox proportional hazards model using the forward stepwise (likelihood ratio) method. The missing date was excluded for multivariate analysis. $P < 0.05$ (two-tailed) was considered significant. Data analyses were undertaken using SPSS v19.0 (IBM, Armonk, NY, USA) and Graphpad Prism version 5.0 (GraphPad Software, La Jolla, California, USA).

3. Results

3.1. Basic Characteristics of Patients. Five hundred and five patients formed the study cohort. Of these, 441 (87.3%) were male and 64 (12.7%) were female. Mean age of patients was 41.8 ± 13.8 years. Prevalence of patients with disease progression of WHO stage III or IV was 80.8%. Patients had advanced immunosuppression status with a median CD4 count of 76 (20–233) cells/μL. Mean level of BChE of patients was 6073.8 ± 2280.8 U/L. Basic characteristics of patients are shown in Table 1.

3.2. BChE Was Closely Associated with HIV/AIDS Progression. In the present study, the CD4 count and WHO stage were used to indicate HIV/AIDS progression. Correlations among serum levels of BChE, CD4 count, and WHO stage were evaluated. A decrease in serum levels of BChE was positively correlated with CD4 depletion ($P < 0.001$) (Figure 2(a)). Serum levels of BChE and WHO stage of HIV/AIDS were also evaluated. The serum level of BChE (in U/L) was 7389.2 ± 2152.2 in patients with WHO stage I/II, 6296.0 ± 2261.4 for stage III, and 5538.2 ± 2138.2 for stage IV. Using ANOVA and LSD post hoc correction, we found that patients at WHO stage I/II had significantly higher BChE levels compared to the levels observed in patients at WHO stage III ($P < 0.001$) and WHO stage IV ($P < 0.001$). The serum level of BChe was negatively correlated with WHO stage ($P < 0.001$) (Figure 2(b)).

Additionally, HIV-RNA was tested in fifty-four patients in our study. No statistically significant correlations were found between BChE and HIV-RNA ($P = 0.132$).

3.3. BChE Was Significantly Correlated with Inflammation and Nutritional Status. White blood count (WBC), neutrophil proportion, and C-reactive protein (CRP) were used as indicators for inflammation/infection in patients. We assessed the relationships between serum levels of BChE and WBC and neutrophil proportion as well as CRP. Pearson

Table 1: Patient basic characteristics ($n = 505$).

Parameter	Value
Age (years) (mean ± SD)	41.8 ± 13.8
Sex [n(%)]	
Male	441 (87.3%)
Female	64 (12.7%)
CD4+ (cells/μL) [median (IRQ)]	76 (20–233)
WHO disease stage [n (%)]	
I/II	97 (19.2)
III	119 (23.6)
Pulmonary tuberculosis	23 (4.6)
Bacterial pneumonia	18 (3.6)
Other diseases	—
IV	289 (57.2)
Pneumocystis pneumonia	106 (21.0)
Extrapulmonary tuberculosis	78 (15.4)
Cryptococcal meningitis	42 (8.3)
Severe bacterial pneumonia	38 (7.5)
Fungal infection in bloodstream	13 (2.6)
Recurrent septicaemia	10 (2.0)
Nontuberculosis mycobacteria	5 (1.0)
Lymphoma	17 (3.4)
Other diseases	—
*HIV-RNA (log_{10} copies/mL)**	4.3 ± 1.4
Serum BChE (U/L)	6073.8 ± 2280.8
Serum albumin (g/L)	35.6 ± 7.5
Body mass index	20.4 ± 3.1
Hemoglobin (g/L)	116.8 ± 26.5
Serum sodium (mmol/L)	137.8 ± 4.6

BChE: butyrylcholinesterase; IQR: interquartile range; WHO: World Health Organization; *HIV-RNA was available in 54 patients.

correlation analyses suggested that a decrease in the serum level of BChE was negatively correlated with the CRP level ($P < 0.001$), WBC ($P = 0.011$), and neutrophil proportion ($P < 0.001$) (Figures 3(a)–3(c)).

We used the body mass index (BMI), serum albumin, and hemoglobin to evaluate the patients' nutrition status. The relationships between the serum level of BChE and BMI and serum albumin and hemoglobin were also studied. Correlation analyses suggested that the serum level of BChE was closely associated with the BMI ($P < 0.001$), serum albumin ($P < 0.001$), and hemoglobin ($P < 0.001$) (Figures 3(d)–3(f)).

3.4. Serum Level of BChE Is Associated with Treatment Outcome. To illustrate further the effects of serum levels of BChE upon hospitalization, the serum level of BChE of patients upon hospital admission and that upon hospital discharge was analyzed among surviving and deceased patients, respectively. Mean serum level of BChE level (in U/L) for surviving patients was 5951.3 ± 2089.7 upon hospital admission and 6399.1 ± 1960.5 upon hospital discharge, respectively ($P < 0.001$). In contrast, mean serum level of BChE level (in U/L) decreased from 4932.7 ± 2118.3 at hospital admission to 4122.9 ± 2009.1 upon transfer to the mortuary among deceased patients, respectively, in patients who died during the hospital admission ($P = 0.004$) (Figure 4(a)). Among surviving patients at WHO stage III, the duration of hospital stay (in days) was 14.5 (range, 7.9–22.8) in patients with a serum level of BChE (in U/L) ≥4500 and 21.5 (14.0–31.8) in patients with a serum level of BChE < 4500 ($P = 0.010$). Among surviving patients at WHO stage IV, the duration of hospital stay (in days) was 20 (range, 13–31) with a serum level of BChE (in U/L) ≥4500 and 21 (14–32) in patients with a serum level of <4500 ($P = 0.597$) (Figure 4(b)).

3.5. Serum Level of BChE Is an Independent Predictor of 1-Year Mortality among Hospitalized HIV/AIDS Patients. Of the 505 patients enrolled in our study, 84 (16.6%) patients had died at 1-year follow-up, including 42 (8.3%) patients with a low serum level of BChE and 42 (8.3%) patients with a normal serum level of BChE ($P < 0.001$). Seventy-one patients (14.1%) had died at 3-month follow-up. Five patients (1.0%) had died at 3–6-month follow-up, and 8 patients (1.6%) had died at 6–12-month follow-up. Kaplan–Meier analyses revealed that 1-year cumulative survivals were 64.5 ± 4.5% for patients with a serum level of BChE < 4500 U/mL and 87.6 ± 1.8% for those with a serum level of BChE > 4500 U/mL (log-rank test, $P < 0.001$) (Figure 5).

3.6. Serum Level of BChE Is a Predictor of Survival in Patients with HIV/AIDS. We stratified patients according to the following criteria: age (years; <30 and ≥30), BMI (kg/m^2; missing data, <18 and ≥18), WHO stage (I, II, III, and IV), CD4 count (cells/μL; missing data, <50 and ≥50), serum level of hemoglobin (g/L; missing data, <110 and ≥110), serum level of albumin (g/L; <35 and ≥35), serum concentration of sodium (mmol/L; missing data, <130 and ≥130), and serum level of BChE (U/L; <4500 and ≥4500). In the unadjusted model, our data suggested that the following factors contributed to mortality: age, BMI, CD4 count, and WHO stage, as well as serum concentrations of sodium, hemoglobin, albumin, and BChE. Hazard ratios (HRs) were 3.5 [95% confidence interval (CI), 2.3–5.3] for patients with a low serum level of BChE compared to those with a normal serum level of BChE. Next, we analyzed 383 patients without data missing. In the multivariate Cox proportional hazards model adjusted for age, CD4 count, and WHO stage, as well as serum levels of sodium, hemoglobin, and albumin, our data suggested that, compared with patients with a normal serum level of BChE, the HR was 1.8 (95% CI, 1.0–3.2) for patients with a low serum level of BChE ($P = 0.035$) (Table 2). These data suggested that the serum level of BChE was a predictor of survival in patients with HIV/AIDS. Patients with a low serum level of BChE had a higher HR for mortality than those with a normal serum level of BChE.

4. Discussion

Measurement of BChE is a very cheap (about one dollar) and routine item in liver function test in nearly all Chinese inpatients. However, the relationships between BChE and HIV

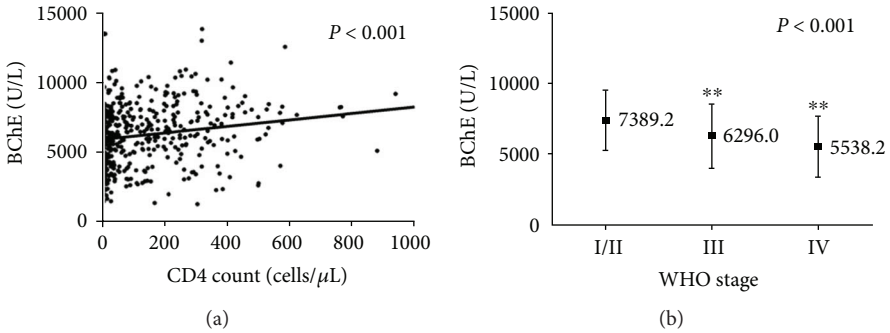

Figure 2: BChE is associated with HIV/AIDS progression. (a). BChE level is correlated with CD4 count ($P < 0.001$). (b). BChE level is negatively associated with HIV/AIDS stage ($P < 0.001$). $^{**}P < 0.01$ using one-way ANOVA test.

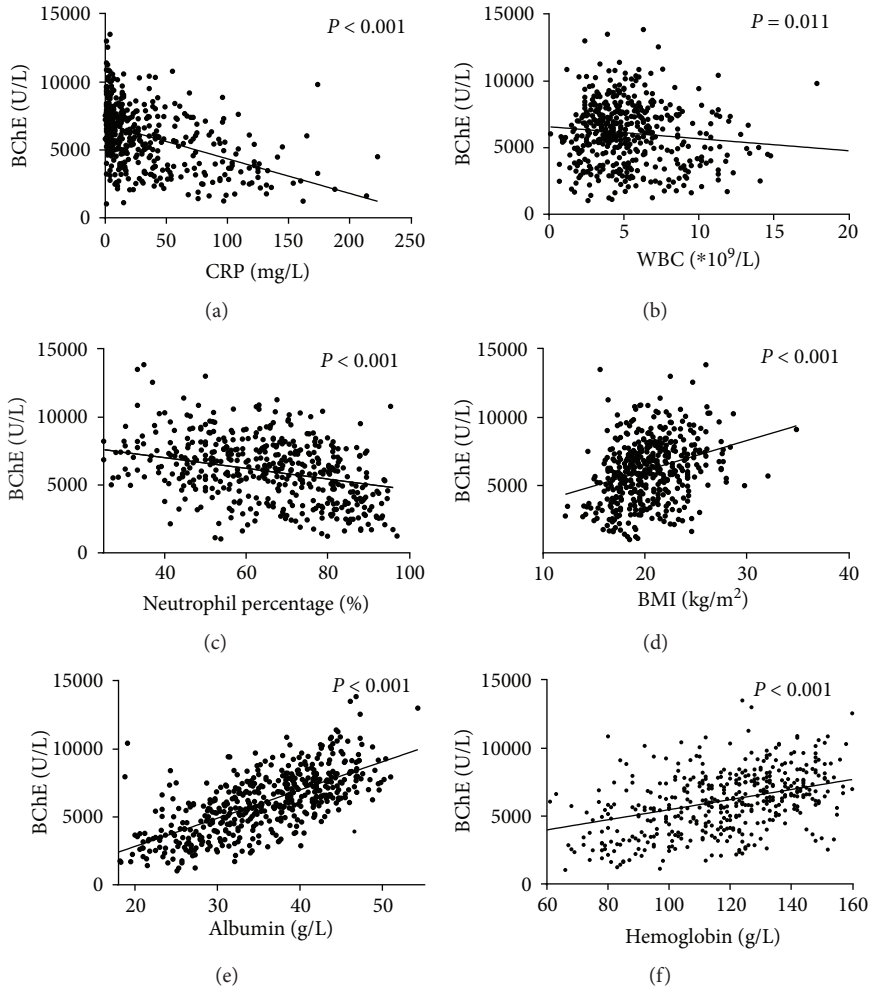

Figure 3: BChE, infection, and nutrition. BChE levels decrease significantly with increasing CRP levels, WBC, and neutrophil proportion ($P < 0.001$, $P = 0.011$, and $P < 0.001$, resp.) (a–c). BChE levels are positively associated with body mass index, albumin, and hemoglobin (all $P < 0.001$) (d–f).

infection remain unclear. In this prospective study of hospitalized patients with HIV/AIDS, we found that a low level of BChE at time of admission was associated with a higher risk of in-hospital death and 1-year mortality. Even after adjustment for sex, age, BMI, WHO stage, and CD4 count, as well as serum levels of hemoglobin, sodium, and albumin in the multivariate model, a low level of BChE was associated with a twofold higher risk of death. BChE may be a useful biomarker to predict clinical outcomes in patients with HIV/AIDS.

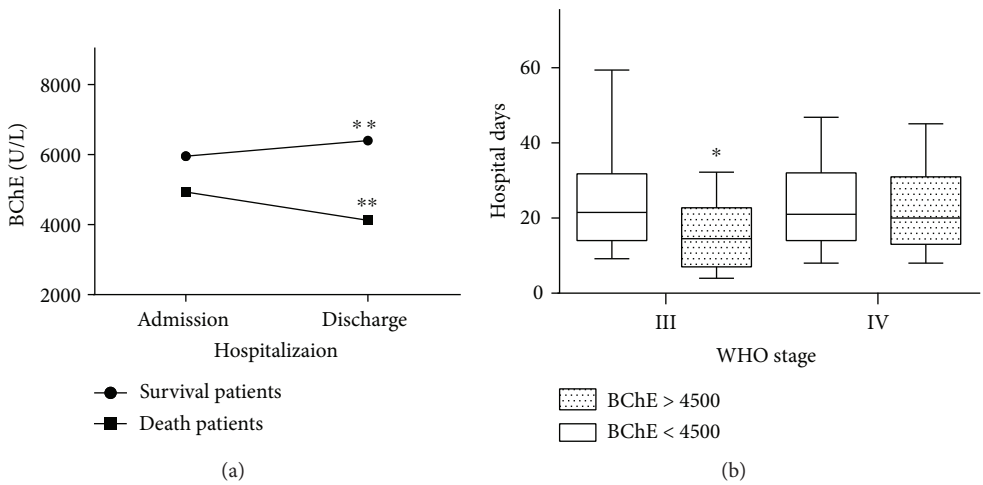

Figure 4: BChE level and hospitalization. (a) Mean BChE level increased from 5951.3 ± 2089.7 U/L to 6399.1 ± 1960.5 U/L ($P < 0.001$) in patients who survived during the hospitalization, whereas, mean BChE level decreased from 4932.7 ± 2118.3 U/L to 4122.9 ± 2009.1 U/L ($P = 0.004$) who died during hospitalization. (b) Patients with a low BChE have a longer duration of hospital stay than patients with a normal level of BChE at WHO stage 3 ($P = 0.010$), but this trend is not obvious among patients at WHO stage IV. $^{*}P < 0.05$, $^{**}P < 0.01$ using t-test.

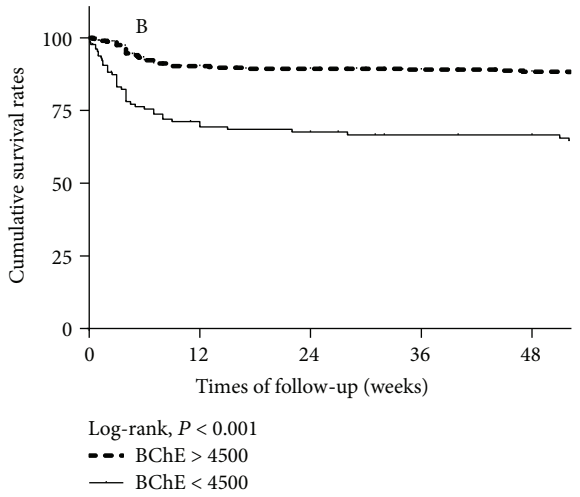

Figure 5: Kaplan–Meier survival curves according to BChE levels. The BChE level affects mainly 1-year mortality. One-year cumulative survival for patients with a low BChE is $64.5 \pm 4.5\%$ and $87.6 \pm 1.8\%$ for patients with a normal BChE level (log-rank, $P < 0.001$).

Cholinesterases include acetylcholinesterase and BChE. Acetylcholinesterase is present mainly at the ends of cholinergic nerves, cholinergic neurons, and red blood cells. BChE is an α-glycoprotein found mainly in the liver and used frequently as a parameter of protein synthesis in this organ [26]. BChE levels are decreased in disorders such as severe liver disease, poisoning with organophosphate compounds, cancer, and cachexia, whereas increased levels of BChE have been reported in obesity, diabetes mellitus, uremia, hyperthyroidism, and hyperlipidemia [26–28].

BChE plays an important role both in acute and chronic inflammatory diseases. Previous study indicated that two kinds of cholinergic status responders, enhancers and suppressors, were found in acute inflammatory phage. Enhancers showed increased BChE levels, complete WBC recovery, and improved cholinergic status modulations to plasma IL-6 levels in terms of acute infection or inflammation, but suppressors did not [29]. Likewise, BChE activities and levels in serum are associated with chronic low-grade and severe inflammatory diseases [16, 17, 20]. Interestingly, BChE is also detectable in human brain, participating in cholinergic status modulation, and nerve-macrophage interaction. Overall, BChE plays an important role in controlling extrasynaptic signalling of the cholinergic anti-inflammatory pathway, parasympathetic dysfunction, and inflammation-related disease [30, 31].

BChE is used as a marker to predict the prognosis of several disorders (inflammatory diseases, infection, malnutrition, malignancy, critical illness, liver disease, and metabolic diseases [32]), but the role of BChE in HIV/AIDS is incompletely understood. Here, we evaluated the association between the serum level of BChE and the prognosis of HIV/AIDS patients. Our data demonstrated that ≈30% of such patients had a reduced serum level of BChE.

Three complex factors predispose to a reduction in serum levels of BChE in AIDS patients. First, malnutrition and wasting syndrome are associated with protein-energy malnutrition (PEM) and accounted for a reduction in the serum level of BChE. Studies have demonstrated that abnormal absorption in the intestine is a common feature in HIV patients with or without diarrhea [1, 33]. Intestinal dysfunction and inadequate intake of nutrients are the main reasons for weight loss and wasting syndrome in HIV/AIDS patients [34]. Serum levels of BChE, total protein, and albumin are lower in malnourished children with marasmus than those measured in normal children; these values tend to increase after 3 weeks of nutritional rehabilitation, and a similar trend in serum levels of BChE has been observed in undernourished adults [35]. Nutritional status may be compromised

Table 2: Risk factors for mortality of patients in univariate/multivariate Cox proportional hazards models.

Factor	Number ($n = 505$)	Deaths ($n = 84$)	Univariate			Multivariate		
			HR	95% CI	P	HR	95% CI	P
Sex								
Male	441 (87.3)	74 (16.8)	1.0	0.9–1.1	0.817			—
Female	64 (12.7)	10 (15.6)	1					
Age (years)								
<30	123 (24.4)	12 (9.8)	1		0.020			—
≥30	382 (75.6)	72 (18.8)	2.1	1.1–3.8				
BMI (kg/m^2)								
Missing data	37 (7.3)	22 (59.5)						
<18	110 (21.8)	21 (19.1)	1.8	1.0–3.0	0.036			—
≥18	358 (70.9)	41 (11.5)	1					
WHO stage								
I/II/III	217 (43.0)	19 (8.8)	1.0		<0.001	1.0		0.008
IV	288 (57.0)	65 (22.6)	5.6	3.0–10.5		2.9	1.3–6.5	
CD4 count (cells/μL)								
Missing data	71 (14.1)	15 (21.1)			<0.001			0.007
<50	181 (35.8)	45 (24.9)	3.0	1.8–4.9		2.4	1.3-4.4	
≥50	253 (50.1)	24 (9.5)	1			1		
Serum sodium (mmol/L)								
Missing data	42 (8.3)	5 (11.9)			0.002			—
<130	28 (5.5)	10 (35.7)	1					
≥130	435 (86.1)	69 (15.9)	2.9	1.5–5.6				
Hemoglobin (g/L)								
Missing data	33 (6.5)	4 (12.1)			0.035			—
<110	166 (32.9)	37 (22.3)	1.6	1.0–2.5				
≥110	306 (60.6)	43 (11.7)	1					
Serum albumin (g/L)								
<35	237 (46.9)	60 (25.3)	3.2	2.0–5.2	<0.001			—
≥35	268 (53.1)	24 (9.0)	1.0					
Serum BChE (U/L)								
≥4500	376 (74.5)	42 (11.2)	1		<0.001	1		0.035
<4500	129 (25.5)	42 (32.6)	3.5	2.3–5.3		1.8	1.0–3.2	

BChE: butyrylcholinesterase; BMI: body mass index; HR: hazard ratio; WHO: World Health Organization.

in PEM and has important adverse effects upon outcome. Some authors have suggested that serum levels of BChE are nutritional and prognostic markers [21]. In our study, most patients (≈80%) were at WHO stage III/IV, so most subjects were underweight or had wasting syndrome. Patients at the latter stages of critical illness lose their appetite, resulting in inadequate intake of nutrients. Also, we found that serum level of BChE was closely associated with the BMI, albumin, and hemoglobin, suggesting that BChE is a potential marker of nutritional status. A lower serum level of BChE reflects the decreased nutrition of patients, which is a predictor of a bad outcome.

Second, infection and inflammation can affect serum levels of BChE. Chronic inflammation plays an important part in the pathogenesis of untreated HIV infection [36]. Such inflammation can result in dysfunction of hepatic protein synthesis, leading to reduction of BChE production [16, 20]. Systemic infection also contributes to BChE attenuation; BChE levels are significantly lower in those with bacteremia and could be useful for early detection of sepsis [18]. Low levels of BChE have also been noted in deep fungal infections, possibly due to enrichment of blood flow through the liver or invasion of fungi (e.g., *Candida albicans*, *Candida tropicalis*) into the liver from the gut by penetration through degenerated barriers of gastrointestinal mucosa [37]. Also, HIV-mediated destruction of the gut mucosa leads to microbial translocation, which induces persistent local and systemic inflammation, thereby promoting HIV/AIDS progression [36]. Translocated microbial products also pass through the liver, contributing to hepatic damage and impaired synthesis of proteins [36]. Earlier studies have suggested two types of cholinergic status responders, named the enhancers and suppressors. In the settings of acute infection or inflammation, the former refers to elevated level of BChE and improved

WBC status as well as ameliorated cholinergic status modulations towards level of plasma IL-6, while the latter didn't show any of the forementioned status. In our study cohort, most patients were immunosuppressed and suffering from severe opportunistic bacterial/fungal infections. Critical illness or infection promotes an inflammatory response that has a rapid, catabolic effect on hepatic protein synthesis. Serum levels of inflammatory cytokines such as interleukin-6, interferon-gamma, and activity of BChE well as BChE activity were not measured in the present study, but we revealed that BChE levels were negatively associated with CRP levels, WBC, and of neutrophils proportion, demonstrating that infection contributes to lower levels of BChE.

Third, HIV infection itself may directly or indirectly affect liver function [38]. HIV-RNA has been detected in sinusoidal cells and hepatocytes in vivo [6]. A high level of HIV-RNA is an independent factor associated with increased levels of alanine aminotransferase in HIV-only-infected patients [39], suggesting that HIV facilitates liver dysfunction. HIV can infect hepatocytes [6], Kupffer cells [7], and HSCs [8, 40]. In our study, HIV-RNA levels were measured only in 54 patients. However, no obvious correlations were found between levels of HIV-RNA and BChE. Studies have suggested that HIV infection can damage liver function by inducing hepatocyte apoptosis [41], thereby impairing the ability of Kupffer cells to clear the products of microbial translocation [7] and promoting HSC production of proinflammatory cytokines [40]. In HIV patients, a systemic increase in lipopolysaccharide levels caused by microbial translocation results in chronic immune activation and contributes to impairment of protein synthesis [42] (including BChE synthesis). Thus, a direct relationship between HIV-RNA and BChE might be hidden by the three factors mentioned above.

Importantly, our research suggests that BChE might be associated with treatment outcomes. Serum levels of BChE increased from hospital admission to hospital discharge among surviving patients. However, serum levels of BChE decreased in patients who died during the hospitalization. These data suggest that effective treatments (including anti-infection agents and nutritional treatment) probably promote BChE synthesis in the liver. BChE was also associated with duration of hospital stay. Median duration of hospital stay among patients with a normal level of BChE was lower than that among cases with a low level of BChE at WHO stage III but not at stage IV. This difference might be because patients at stage IV have more complications and infections (Table 1), which probably conceal the effect of BChE level on median duration of hospital stay.

The main limitation of our study was that first, we did not fully assess the relationship between levels of HIV-RNA and BChE. The HIV-RNA test is relatively expensive in China, and carrying out this test in all patients is difficult. Nevertheless, we and other researchers understand the potential value of HIV-RNA upon liver function. Another limitation was missing data, especially CD4 count which was missing in 14.1% of patients. This was because Chinese government implemented the National Free Antiretroviral Treatment Program (NFATP) in 2003, with a "**Four Free and One Care**" policy (providing free HIV test and CD4+ T cell tests; free antiretroviral treatment for AIDS patients; free drugs to prevent mother-to-child transmission; free education for AIDS orphans; and government care for AIDS patients who live in poverty) [43]. Therefore, some patients had free CD4+ T cell tests performed in local the CDC clinic every three months instead of at our hospital, and we were not able to have access to the CDC data. Other missing data were mainly because the data were not obtained within the first three day of patients' admission. However, our data showed that BChE levels were clearly associated with patients' mortality in the multivariate Cox proportional hazards model, indicating that BChE level is associated with HIV/AIDS severity and is an independent risk factor for increased mortality in Chinese HIV/AIDS patients.

5. Conclusions

Our research suggests that even though it is not a specific marker of AIDS, a lower level of BChE is an independent surrogate marker of disease progression in patients with HIV/AIDS patients. Patients with low serum levels of BChE have a longer duration of hospitalization and higher risk of mortality within 1 year. BChE is a commonly utilized biomarker in China and should be included as a laboratory assessment at time of hospitalization for HIV/AIDS patients. Patients with lower serum levels of BChE may benefit for intensive nutritional support, earlier initiation of antibiotic therapy, and strategies to reduce inflammation.

Additional Points

Article's Main Point. Butyrylcholinesterase (BChE) is associated with HIV/AIDS progression. Patients with low serum levels of BChE have a longer stay in hospital and higher risk of death. Assessment of serum levels of BChE provides a routine biochemical approach for predicting clinical outcome of Chinese HIV/AIDS patients.

Conflicts of Interest

The authors declare that they do not have conflicts of interest.

Authors' Contributions

Lijun Xu and Nanping Wu designed the research. Biao Zhu and Jia Sun collected data. Zongxing Yang was responsible for the data analysis. Ying Huang and Jinlei Zheng followed up the study. Lijun Xu was responsible for the paper writing. Ying Huang , Yan Xu, and Jinlei Zheng followed up the study. Sabine Kinloch, Michael T. Yin, and Honglei Weng revised the paper and rewrote it. Lijun Xu is the first author.

Acknowledgments

The authors thank Hongju Wang and Ruoyu Dong for technical assistance and the staff of the HIV/AIDS ward in the First Affiliated Hospital, School of Medicine, Zhejiang University. This research was supported by the Science and

Technology Department of Zhejiang Province (Contract number LY12H26005).

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Hindawi
Mediators of Inflammation
Volume 2018, Article ID 7141342, 9 pages
https://doi.org/10.1155/2018/7141342

Research Article

Systemic Inflammatory Cytokines Predict the Infectious Complications but Not Prolonged Postoperative Ileus after Colorectal Surgery

G. S. A. Boersema,[1] Z. Wu,[2] A. G. Menon,[3] G. J. Kleinrensink,[4] J. Jeekel,[4] and J. F. Lange[1,3]

[1]*Department of Surgery, Erasmus University Medical Center, Rotterdam, Netherlands*
[2]*Ward I of Gastrointestinal Cancer Center, Key Laboratory of Carcinogenesis and Translational Research, Ministry of Education, Peking University Cancer Hospital & Institute, Beijing 100142, China*
[3]*Department of Surgery, Havenziekenhuis, Rotterdam, Netherlands*
[4]*Department of Neuroscience, Erasmus University Medical Center, Rotterdam, Netherlands*

Correspondence should be addressed to Z. Wu; wuzhouqiao@gmail.com

Received 6 August 2017; Revised 13 December 2017; Accepted 20 December 2017; Published 6 March 2018

Academic Editor: Helieh S. Oz

Aim. Postoperative ileus (POI) is common after surgery. Animal studies indicate that the POI mechanism involves an inflammatory response, which is also activated during postoperative complications. This study aimed to determine whether inflammatory biomarkers might facilitate an early detection of prolonged POI (PPOI) or infectious complications. *Methods*. Forty-seven adult patients who underwent oncological colorectal surgery were included. They filled out a perioperative diary to report their gastrointestinal symptoms. Blood samples were collected preoperatively, and on postoperative day (POD) 1 and 3. Levels of leucocytes, C-reactive protein (CRP), interleukin (IL)-6, TNF-α, and IL-1β were analyzed. *Results*. Patients with PPOI had significantly longer stay in hospital than patients without (13.6 ± 10.5 versus 7.4 ± 3.2 days, $p < 0.001$); they also had higher levels of IL-6 ratios, leucocytes, and CRP levels, but did not reach significance. Higher levels of postoperative IL-6 and CRP levels ($p < 0.05$, resp.) were found in patients with infectious complications. The receiver operating characteristic (ROC) analysis found better diagnostic values of IL-6 ratio on both POD 1 and 3 than that of CRP (POD 1: ROC 0.825, $p < 0.001$). *Conclusion*. Blood levels of inflammatory cytokines cannot predict PPOI after colorectal surgery. Instead, postoperative IL-6 changes may predict the infectious complications with a better diagnostic value than the current leukocytes or CRP tests.

1. Introduction

Surgical resection is still the cornerstone of colorectal cancer treatment. Nevertheless, colorectal surgery is associated with a high morbidity rate of 24–43% [1–3], which significantly compromises a fast recovery after surgery and quality of life after discharge. Infectious complications including surgical site infection and anastomotic leakage are the major causes of postoperative morbidity and mortality [4]. Moreover, many patients also develop postoperative ileus (POI) characterized by a transient impairment of bowel function and reduced motility. In some of them, prolonged POI (PPOI) is diagnosed when POI does not resolve after 5 postoperative days or recurs after an apparent resolution. Such delayed recovery of bowel function leads to other serious outcomes such as longer hospitalization, hospital-acquired infections, pulmonary compromise, and a large increase of medical cost as well [5].

Many studies on animal models have revealed that the mechanism of POI includes an inflammatory response caused by the intestinal manipulation and surgical trauma [6–8]. Therefore, inflammatory markers such as interleukin (IL)-1β, IL-6, TNF-α, and C-reactive protein (CRP) have been suggested to be valuable for the early detection of POI. Previous studies reported that the levels of IL-1β, IL-6, and TNF-α in PPOI patients were significantly higher

Table 1: The variables and definitions of complication and outcome.

Complications/outcome	Definition
PPOI*	Resolution of POI is defined as passage of feces with good toleration of solid food for at least 24 hours. PPOI is diagnosed if POI is not resolved after postoperative day 5; recurrent POI occurring after an apparent resolution of POI was also defined as PPOI [17, 19].
Anastomotic leakage	Defect of the bowel wall integrity at the anastomotic site. A pelvic abscess close to the anastomosis is also considered as anastomotic leakage. The diagnosed leakage were Grade B or C according to classification of Rahbari et al. [18].
Surgical site infection (SSI)	Erythema requiring initiation of antibiotic treatment or a wound requiring partial or complete opening for drainage of a purulent collection.
Pneumonia	Presentation of clinical symptoms including cough, fever, and dyspnoea or consolidation on chest radiography requiring antibiotic treatment with or without a positive sputum culture.
Urinary tract infection	Presents of clinical symptoms, for example, fever, polyuria, and stranguria requiring antibiotic treatment.
Fascia defect	Dehiscence of the abdominal wall with or without the need for reoperation.
Reoperation	During hospital stay, within 30 days postoperative, or during readmission within 30 days after initial discharge.
Length of hospital stay	Day of admission till the day a patient is ready for discharge; this means patient tolerate solid food and had passage of feces, and pain is adequately in control with oral analgesics.
Readmission	Admission within 30 days after discharge for more than 24 hours.
Mortality	Death occurring during hospital stay or within 30 days postoperative.

*Prolonged postoperative ileus.

at postoperative day 5 in abdominal drain fluid than that in normal recoveries [9, 10]. However, due to the wide application of the ERAS (enhanced recovery after surgery) program, peritoneal drainage is no longer routine practice in colorectal patients. In such cases, measuring systematic levels of the inflammatory cytokines seems to be a promising alternative since it can be easily integrated into postoperative blood tests.

This approach is supported by animal studies, which have revealed that elevation of the inflammatory cytokines is also detectable in blood samples in addition to a localized change [6, 7]. Nevertheless, clinical data to support this are still not yet available. Moreover, it is important to note that the classic proinflammatory response is also activated in infectious complications, and increasing levels of the inflammatory cytokines were also reported in these complications [11–16]. To this end, we conducted a prospective cohort study in patients underwent colorectal surgery. In this study, we analyzed the systemic inflammatory markers in perioperative blood samples. The primary goal of this study was to investigate whether the perioperative inflammatory cytokine levels can predict PPOI. Secondarily, we also tried to associate the cytokine levels with the infectious complications.

2. Method

2.1. Study Population and Design. Adult patients admitted to the Academic Colorectal Cancer Center, Havenziekenhuis, Rotterdam, who underwent oncological colorectal surgery, were included after informed consent. In total, 50 patients were planned to be included in this prospective cohort during the period of November 2013 and November 2014. In accordance with the Dutch law on medical research in humans, this study was approved by the Medical Ethical Committee of the Erasmus University Medical Center, Rotterdam, Netherlands (permit number: MEC-2013-246, NL43053.078.13) and patients gave their written consent after receiving oral and written information.

All patients were asked to fill out a questionnaire before surgery and every day after surgery until postoperative day (POD) 7. The questions refer to their food and fluid intake, bowel movements and defecation, gastrointestinal symptoms, and visual analogue scale (VAS) pain score. Data collection included age, gender, body mass index (BMI), American Society of Anesthesiologists (ASA) score, medication use, smoking, operative procedure, and postoperative complications including anastomotic leakage, fascia dehiscence, surgical site infection (SSI), urinary tract infection, pneumonia, and postoperative course.

2.2. Selection of Variables and Definitions. To ensure the objectivity of the primary endpoint, PPOI was not diagnosed by the participating surgeons but via the retrospective review of the patient diary and medical record. The participating doctors diagnosed the other complications based on the criteria referred from the literature [17–19] (see Table 1).

2.3. Blood Sample Analysis. Peripheral blood was drawn from each patient before surgery (baseline) and on the first and third postoperative days in the morning, together with the routine blood tests. Leucocytes and CRP measurements were part of the standardized care and the outcomes were retrieved from the medical chart. Blood samples were centrifuged and plasma was stored at −80°C into two aliquots for each sample. Enzyme-linked immunosorbent assays (ELISAs) were performed according to manufacturer's instructions to quantify the concentrations of systematic inflammatory markers IL-6 and TNF-α (PeproTech Inc., Rocky Hill, USA) and IL-1β (R&D Systems, Minneapolis, MN, USA) in blood plasma.

2.4. ERAS Protocol. All patients were treated according to the ERAS protocol. Two hours before surgery patients preoperatively received a carbohydrate-loaded drink. In some cases of low anterior resection, an enema was given under prescription of the surgeon. In general, left-sided colectomy and (low) anterior resections received bowel preparation with 2 liters of Macrogol 3350 (Klean-Prep 69 gr, Norgine Ltd., Harefield, United Kingdom). Immediately after surgery, nasogastric tubes were removed and patients are allowed to move or drink fluid food. Normal diet was offered from the first postoperative day and so on.

2.5. Statistical Analysis. The statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, USA, version 21.0 for Windows). Demographic data were presented in n (%), mean (SD), or median (95% confidence interval). Mann–Whitney U test, chi-square test, Pearson correlation test, and receiver operating characteristic (ROC) analysis were employed according to a proper indication. A 2-tailed p value < 0.05 was considered to indicate statistical significance.

3. Results

Between October 2013 and November 2014, 54 patients were included. Among them, three patients were excluded because of protocol violations of the inclusion criteria, and four patients retracted the informed consent. In total, 47 patients were included for analysis.

3.1. PPOI versus Non-PPOI. In total, 72% (34/47) of the patients recovered from POI within five postoperative days (POD 5) and were assigned to the non-PPOI group; 28% (13/47) patients recovered on or after 6 days postoperatively (8/13) or had recurrence of POI (5/13) and were therefore defined as PPOI. Univariate analysis showed a similar baseline and operative characteristics in the patients with or without PPOI (Table 2).

IL-6 levels were detectable and measurable in all the samples. However, TNF-α and IL-1β were not detectable in the majority of samples. The detailed proportions of detectable samples are listed in Supplementary Table S1.

The absolute median values of cytokines of positive samples are presented in Supplementary Table S2. In the detected samples, we found several samples with substantially higher levels of cytokines compared with other samples, resulting in a large variation in results. We also found that cytokine levels of these patients remained high after surgery. Therefore, a cytokine ratio was calculated with the following equation: ratio POD1 (or 3) = cytokine level on POD1 (or 3)/cytokine level before surgery for further analysis. Cytokine levels and ratios describe the ratio of cytokine levels at postoperative days 1 and 3 divided by the preoperative cytokine level.

The PPOI group showed higher IL-6 ratios on POD 3: 5.90 ± 9.11 than in the non-PPOI group: 2.44 ± 3.84 (Figure 1). Due to a low number of valid values, we found no differences in IL-1β ratio and TNF-α ratio between the two groups.

Table 2: Baseline and surgical characteristic comparison between the PPOI and non-PPOI patients.

	Non-PPOI (%) $n = 34$	PPOI (%) $n = 13$
Patient characteristics		
Age (yrs.)	67.6 ± 10.4	71.2 ± 11.2
Gender		
Male	21 (62)	6 (46)
Female	13 (39)	7 (54)
BMI (kg/m^2)	27.2 ± 4.7	24.7 ± 4.2
ASA score		
I	6 (18)	4 (31)
II	14 (41)	4 (31)
III	9 (26)	1 (8)
IV	0	0
Missing	5 (15)	4 (31)
Cardiac comorbidity	11 (32)	3 (23)
Diabetes mellitus	6 (18)	1 (8)
Smoker	6 (18)	1 (8)
COPD	7 (21)	1 (8)
Use of statins	12 (36)	3 (23)
Use of antihypertensive	12 (36)	7 (54)
Neoadjuvant radiotherapy	2 (6)	0
Chemoradiation	4 (12)	1 (8)
Abdominal surgery in history	12 (35)	3 (23)
Operation characteristics		
Type of operation		
Low anterior resection	10 (29)	2 (15)
Sigmoid resection	6 (18)	2 (15)
Right hemicolectomy	9 (26)	8 (62)
Left hemicolectomy	5 (15)	0
Colon transversum resection	1 (3)	1 (8)
Abdominal perineal resection	3 (9)	0
Approach		
Laparotomy	13 (38)	5 (38)
Laparoscopy	20 (59)	7 (54)
Conversion	1 (3)	1 (8)
Stapled versus hand sutured[#]		
Sutured	19 (58)	9 (69)
Stapled	14 (42)	4 (31)
Anastomotic configuration[*]		
Side-end	10 (29)	5 (42)
Side-side	14 (41)	7 (58)
End-end	6 (18)	0
Stoma construction	11 (32)	2 (13)
Prophylactic drainage	4 (12)	1 (8)
Nasogastric tube[**]	10 (29)	6 (50)

PPOI = prolonged postoperative ileus; non-PPOI = patients without PPOI. Data are n (%), mean (SD). BMI = body mass index; ASA = American Society of Anesthesiologists classification; COPD = chronic obstructive pulmonary disease; [#]$n = 33$ in non-PPOI, $n = 13$ in PPOI group; [*]$n = 30$ in non-PPOI group, $n = 12$ in PPOI group; [**]$n = 12$ in PPOI group.

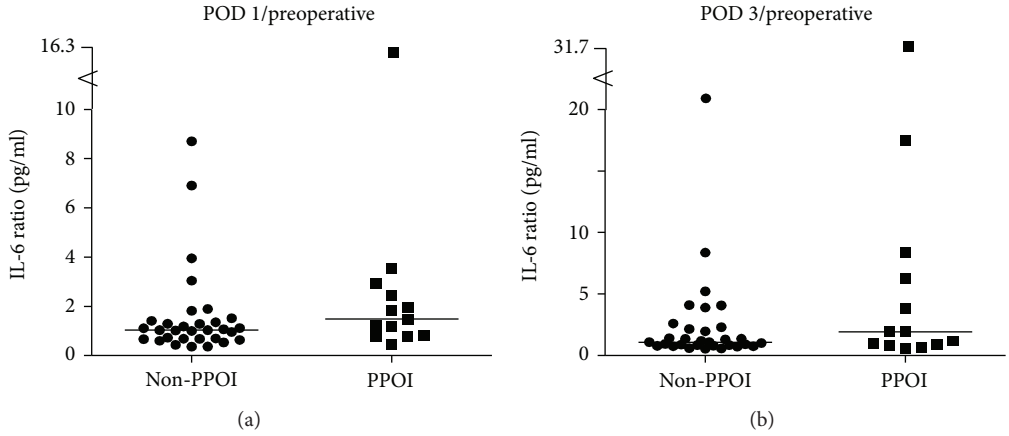


Figure 1: (a) and (b). IL-6 ratio in normal recoveries (non-PPOI) versus PPOI patients, every single dot represents a patient, the line indicates the median, and there are no significant differences.

Both leucocytes and CRP were higher in the PPOI group, but there were no significantly differences between non-PPOI and PPOI groups at any time point (Figure 2). Also, a higher postoperative VAS score was seen in the PPOI group, though no statistical difference was observed.

In total, 13.0% (6/46) were diagnosed with colorectal anastomotic leakage. In the PPOI group, a significantly higher percentage of anastomotic leakage was seen, 38.5% (5/13) versus 3.0% (1/33) in the non-PPOI group, $p = 0.005$. The hospital stay duration was significant longer in PPOI patients 13.6 ± 10.5 versus 7.4 ± 3.2 in the non-PPOI cases, $p < 0.001$ (Figure 2(d)).

3.2. Infectious versus Non-Infectious Complication. There were no significant differences between the baseline and surgical characteristic comparison of patients with and without infectious complications (Supplementary Table S3). Different from the PPOI patients, patients with the infectious complications had significantly higher IL-6 ratios and CRP levels on POD 1 and POD 3 ($p < 0.05$, respectively, Figure 3(a)), while the leucocyte count, though also higher, was not significantly different from patients without infectious complications, Figure 3(c). Further detailed analysis showed that significantly higher levels of IL-6 ratios on POD 1 and POD 3 were found in SSI and colorectal anastomotic leakage (CAL) patients as illustrated in Figure 3(a), while the differences in CRP were not significant (Figure 3(b)). No differences were observed in IL-1β and TNF-α ratios between the groups.

We performed the ROC analysis to determine the diagnostic value of CRP and IL-6 ratio in detection of infectious complications. Both on POD 1 and POD 3, IL-6 ratio had a larger area under curve (AUC) than that of CRP (Figure 4). Further analysis showed that the diagnostic value was achieved on POD 1 with a cutting-off point of 1.21 of IL-6 ratio, which yielded a sensitivity of 76% and a specificity of 86%. Although the sensitivity was relatively low (43%), a cutting-off point of 1.93 on POD 1 reached a specificity of 100%, meaning all patients with an IL-6 ratio higher than 1.93 on POD 1 were diagnosed with infectious complications later on.

4. Discussion

The importance of developing effective strategies to predict and eventually to treat the postoperative complications cannot be overemphasized [20]. In this study, we investigated the association between the inflammatory cytokines and the postoperative complications. We found that systematic changes of IL-6 predicted the infectious complications but not prolonged POI after colorectal surgery.

To develop the effective strategies, researchers depend on the translational knowledge from animal studies, which have been continuously contributing to the understanding of POI mechanism. Several experimental studies have reported the important role of IL-6 in the development of POI. Even little manipulation of the bowel induces activity of IL-6, which results in activation of nitric oxide and prostaglandins and causes migration of leucocytes into the circular muscle of the bowel and ends up with PPOI eventually [21–24]. However, with fruitful data obtained from animal studies, clinical attempts to predict POI by determining inflammatory mediator levels, the important mechanism in PPOI pathophysiology, remain limited. Zhu et al. found that peritoneal levels of IL-1, IL-6, and procalcitonin were higher in PPOI patients [10], indicating localized parameters are sensitive for PPOI prediction. Clinical data also found that IL-6 levels are higher in patients undergoing open surgery when compared with patients undergoing laparoscopic surgery [21], while open procedures had been demonstrated to delay recovery of POI [8].

In this study, we report a prospective cohort investigating the association between the inflammatory cytokines and the postoperative complications. We found that systematic changes of IL-6 predicted infectious complications but did not predict PPOI after colorectal surgery. In contrast to many previous animal studies, our results indicate that systematic cytokine levels yield poor predictive value in PPOI diagnosis. This can be partly explained by inevitable confounding factors (e.g., sex, age, type of surgery, and preoperative risk factors) in patient subjects which dilute the influence of POI on systematic inflammatory response [25], while those factors are usually controlled in animal

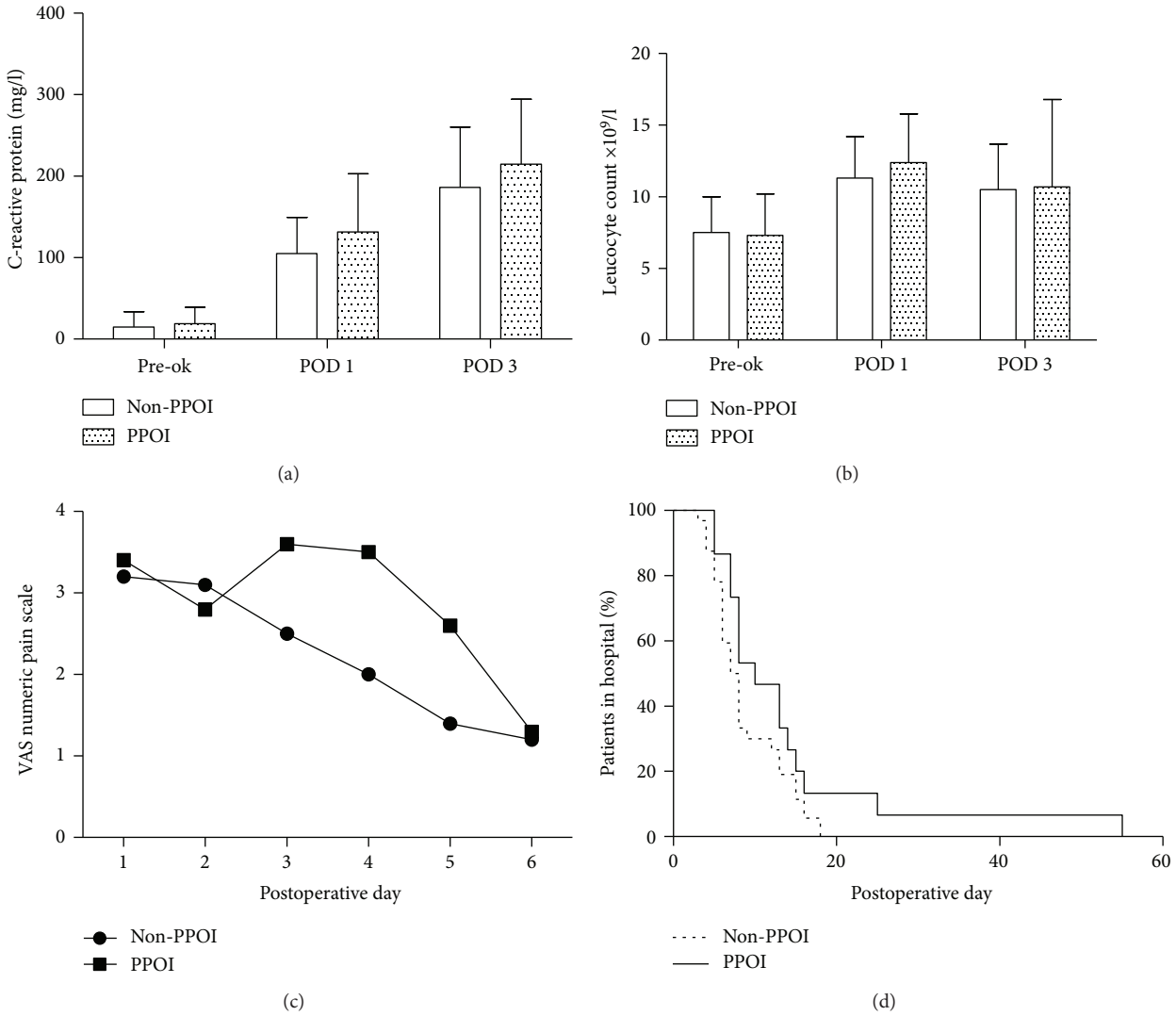


Figure 2: The leucocyte count and CRP and VAS scores in non-PPOI patients versus PPOI. In panels (a) and (b), bars represent the mean and error bars the SD. There are no significant differences. Panel (c) presents the VAS (visual analogue scale for pain) score, from postoperative day 1 up to postoperative day 6. Panel (d) presents patients with or without PPOI and the time in days of being ready for discharge. Patients with PPOI had a significantly longer hospital stay $p < 0.001$.

studies. Nevertheless, we believe that our included patients properly represent the common colorectal patient population. An ideal parameter should be able to identify the high-risk patients. In addition, many animal models used in POI research have very different inflammatory response compared with human [26, 27]. For instance, different from the animal data, our study found that systematic TNF-α and IL-1β levels were extremely low, which was also reported by Ellebæk et al. [28]. In addition, our previous meta-analysis also found that in peritoneal samples, IL-6 is already significantly higher in CAL patients on POD 1, while elevation of TNF-α and IL-1β, both at much lower concentration, was not observed in the first 3 postoperative days [29].

As is shown in our results, cytokine levels are individually dependent. This has not yet been previously investigated in surgical patients. Picotte et al. also reported great variation in systematic IL-6 levels [30]; therefore, we chose ratio instead of absolute levels of cytokines to rule out the individual baseline variations, which resulted in a higher diagnostic value of the infectious complications than CRP in the ROC analysis.

Based on our results, it seems that only in severe complications but not PPOI can the overwhelming inflammatory response be detected in serum in clinical settings. For those complications, leukocyte count and CRP are commonly used to assist an early diagnosis [31, 32]; thus, we also included them into our analysis. In accordance to the previous studies, our data also support the value of CRP in the diagnosis of infectious complications. Nevertheless, the ROC analysis further demonstrates that IL-6 yielded better diagnostic value than CRP in predicting the infectious complications. It is important to note that the diagnostic value of IL-6 became evident very early on POD 1. All patients had a ratio higher

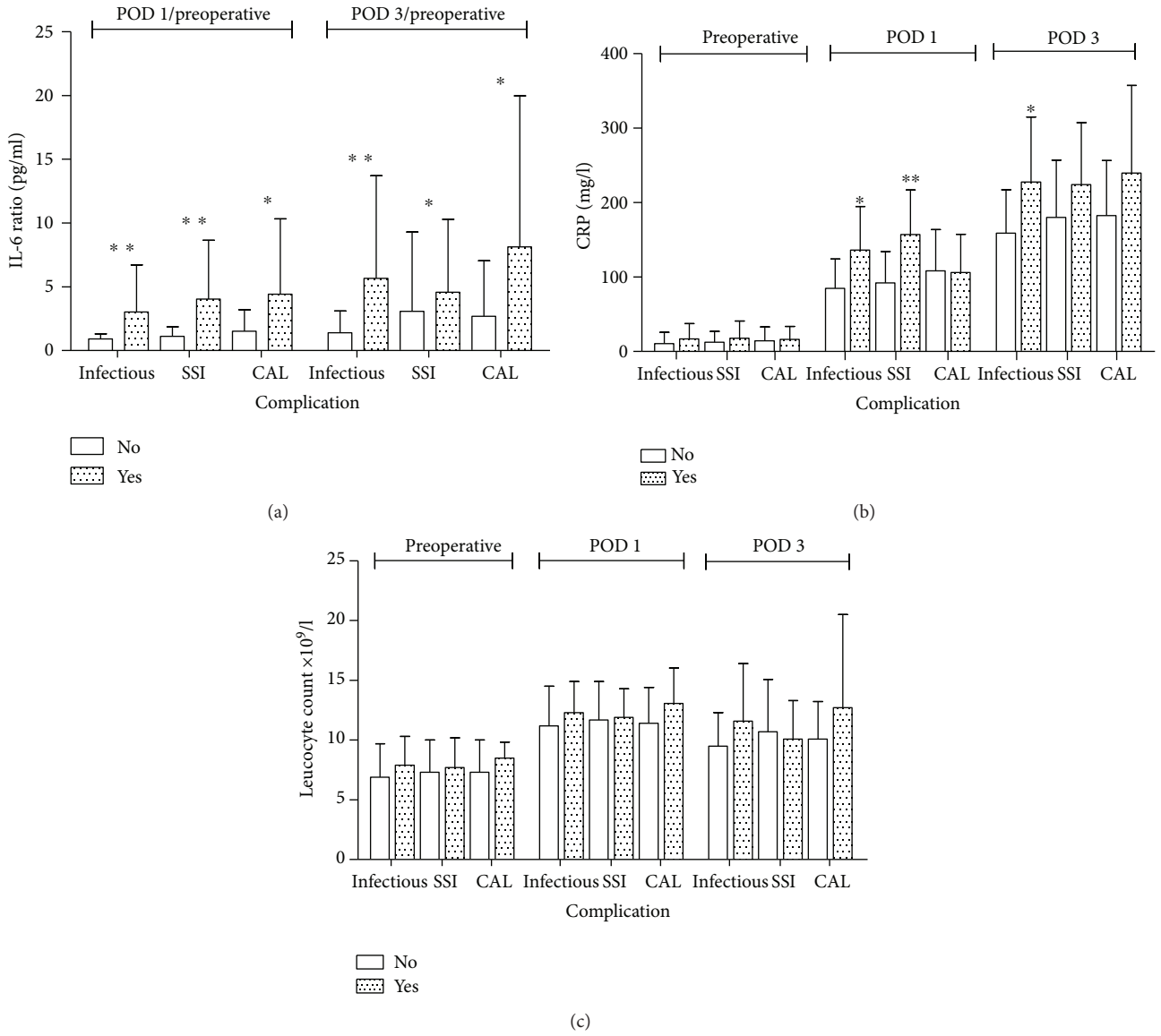


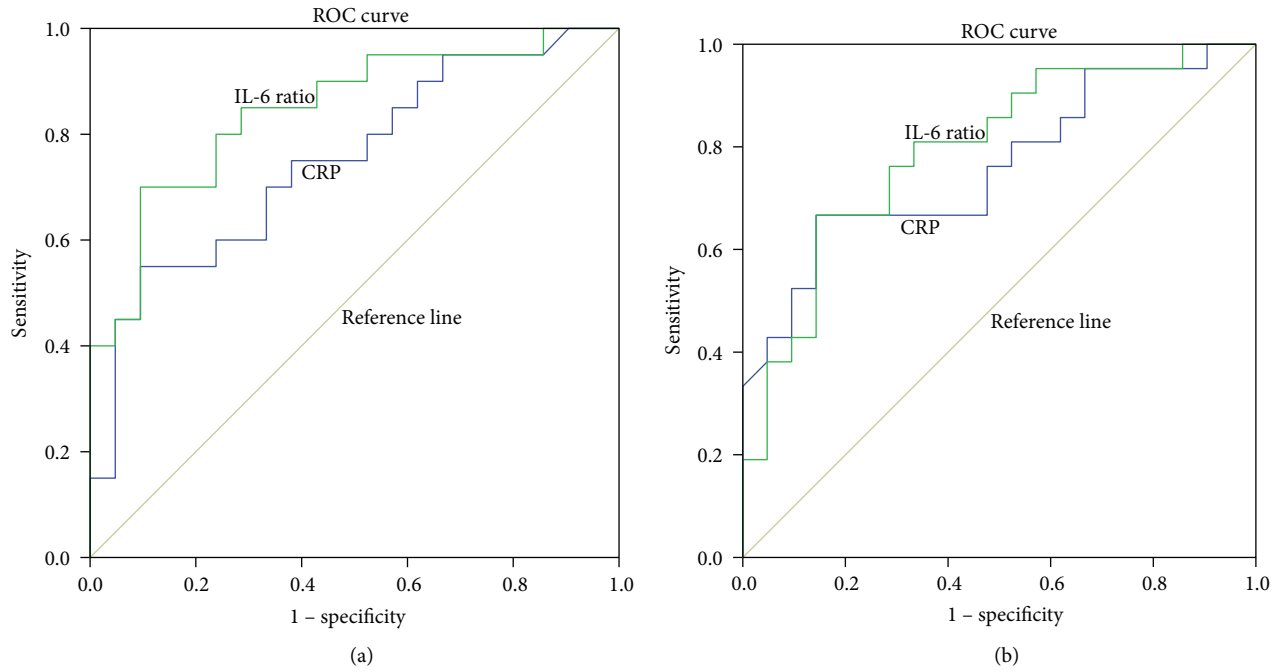
Figure 3: Comparison of IL-6, leucocyte count, and CRP between the patient group with infectious complication(s) (SSI, AL, pneumonia, UWI) and without infectious complication or with or without SSI (surgical site infection) or with or without CAL (colorectal anastomotic leakage). Panel (a) shows that all IL6 ratios are significant higher on both time points between all three groups; the infectious group POD 1 $p < 0.001$ and POD 3 $p = 0.001$, SSI; POD 1 $p = 0.001$ and POD 3 $p = 0.017$, CAL; POD 1 $p = 0.027$ and POD 3 $p = 0.050$. (b) On POD 1 and POD 3, the CRP levels were significantly higher in the infectious complication groups (POD 1 $p = 0.009$, POD 3 $p = 0.008$). In the SSI groups, CRP levels were significantly higher in patients with SSI compared to patients without SSI on POD 1, $p < 0.001$. Also in the groups with CAL had higher numbers of CRP though not significant. (c) Although the leucocyte count is higher in the infectious and CAL groups, there were no significant differences. Bars represent the mean, error bars, and the SD; p values are indicated with an asterisk; $^{*}p$ value ≤ 0.05, $^{**}p$ value ≤ 0.001.

than 1.93 developed infectious complications, indicating the importance of IL-6 evaluation as a promising innovative biomarker for clinical practice.

Although many previous studies exclude patients with other complications from the PPOI group, we still included them to represent a common patient population. This is because it is possible to exclude those patients with complications (e.g., anastomotic leakage) from the POI or PPOI group in a retrospective database. But in a prospective cohort or clinical practice, a surgeon has to differentiate POI or PPOI from other severe complications that require more invasive interventions because many infectious complications first manifest abdominal symptoms before systematic manifestations. This may explain the significantly higher rate of the complications in the PPOI patients in our data.

5. Conclusion

POI remains the most common complication after gastrointestinal surgery, without a satisfactory parameter for its early detection or prediction. In this study, we report a prospective cohort study investigating the association between



POD	Biomarker	AUC	SE	p	95% Cl	
POD 1	CRP	0.732	0.078	0.010	0.579	0.884
	IL-6	0.825	0.067	<0.001	0.693	0.956
POD 3	CRP	0.731	0.077	0.008	0.580	0.882
	IL-6	0.801	0.068	0.001	0.668	0.934

Figure 4: ROC analysis showed CRP and IL-6 ratio on POD 1 (a) and POD 3 (b), on both days; the AUC was higher in IL-6 ratio.

inflammatory cytokines and postoperative complications. We found that serum IL-6 changes predict the infectious complications but not PPOI after colorectal surgery. How to translate knowledge from rodent POI studies to clinical practice is evidently an urgent issue to be addressed. Further exploration of IL-6 seems promising and may assist an early detection of the infectious complications after surgery.

Disclosure

G. S. A. Boersema and Z. Wu should both be considered the first authors. This study was presented to the European Colorectal Congress, St. Gallen, Switzerland, December, 2016.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

J. F. Lange, J. Jeekel, G. J. Kleinrensink, A. G. Menon, Z. Wu, and G. S. A. Boersema designed the research; G. S. A. Boersema and Z. Wu performed the research; G. S. A. Boersema and Z. Wu analyzed the data; G. S. A. Boersema, Z. Wu, and J. F. Lange wrote the article, and all authors drafted the article or revised it critically for important intellectual content and approved the current version. G. S. A. Boersema and Z. Wu contributed equally to designing and performing the trial, data interpretation, and writing of the manuscript.

Acknowledgments

The authors would like to thank all medical assistance from the Academic Colorectal Cancer Center, Havenziekenhuis, Rotterdam, for their help. This study was funded by "Stichting Coolsingel", Rotterdam; Beijing Municipal Science and Technology Project, Beijing Municipal Science and Technology Commission (D141100000414004); and Program for Clinical Application and Development, Beijing Municipal Science and Technology Commission (Z151100004015070).

Supplementary Materials

Table S1: percentage of positive samples in the non-PPOI and PPOI group. Table S2: absolute level of cytokine in plasma of the positive samples. Table S3: baseline and surgical characteristic comparison between patients with infectious complications and normal recovery. *(Supplementary Materials)*

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Hindawi
Mediators of Inflammation
Volume 2018, Article ID 2380319, 8 pages
https://doi.org/10.1155/2018/2380319

Review Article

Maresins: Specialized Proresolving Lipid Mediators and Their Potential Role in Inflammatory-Related Diseases

Shi Tang,[1,2] **Ming Wan**,[2] **Wei Huang**,[1] **R. C. Stanton**,[3,4,5] **and Yong Xu**[1,6]

[1]*Endocrinology Department, The Affiliated Hospital of Southwest Medical University, Luzhou, Sichuan 646000, China*
[2]*Endocrinology Department, The Affiliated Hospital of Nuclear Industry 416 Hospital, Chengdu, Sichuan 610000, China*
[3]*Joslin Diabetes Center, Boston, MA, USA*
[4]*Beth Israel Deaconess Medical Center, Boston, MA, USA*
[5]*Harvard Medical School, Boston, MA, USA*
[6]*Collaborative Innovation Center for Prevention and Treatment of Cardiovascular Disease of Sichuan Province, Southwest Medical University, Luzhou, Sichuan 646000, China*

Correspondence should be addressed to Yong Xu; xywyll@aliyun.com

Received 7 September 2017; Revised 21 December 2017; Accepted 25 December 2017; Published 20 February 2018

Academic Editor: Sung-Ling Yeh

Acute inflammatory responses are host-protective and normally self-limited; these responses can maintain cell homeostasis and promote defense against various infections and damage factors. However, when improperly managed or inappropriately activated, acute inflammation can lead to persistent and uncontrolled chronic inflammation, which is associated with many other chronic diseases including cardiovascular disease and metabolic disease. Recently, studies have shown that resolution of acute inflammation is a biosynthetically active process. Specialized proresolving lipid mediators (SPMs) known as resolvins and protectins are autacoids that resolve inflammation. A new family of anti-inflammatory and proresolving lipid mediators have recently been reported, known as maresins, which are biosynthesized from docosahexaenoic acid (DHA) by macrophages, have a conjugated double-bond system, and display strong anti-inflammatory and proresolving activity. Here, we review the biological actions, pathways, and mechanisms of maresins, which may play pivotal roles in the resolution of inflammation.

1. Introduction

Acute inflammatory responses are defined as the activation of the innate immune system when the body is damaged or invaded by pathogens; leukocytes migrate from the circulation to the site of trauma or microbial invasion, forming inflammatory exudates and the release of inflammatory mediators of interleukin (interleukin, IL-1β, IL-6), tumor necrosis factor-α (TNF-α), high mobility group box-1 protein (HMGB1), prostaglandins, and so forth. This is followed by local vascular expansion, increase in permeability, leukocyte exudation, and, consequently, removal of pathogens. Inflammation is often accompanied by local painful swelling that is red and hot, along with other symptoms [1].

Proinflammatory cytokine production is a major feature of the inflammatory response. Often positive, the inflammatory response is temporary, only occurring locally, and is activated to fight invasion of pathogens and to promote repair of damaged tissue. However, when uncontrolled or inappropriately activated, acute inflammation can lead to persistent chronic inflammation, causing asthma and neurological degenerative disorders, as well as metabolic diseases, including diabetes, obesity, cardiovascular disease, and even cancer; if the inflammatory response is left unchecked, many inflammatory mediators are released into the blood, causing sepsis, which can lead to death [2]. Therefore, it is very important to regulate the inflammatory response at a clinical level.

Inflammation is an important defense mechanism of the host, which is driven not only by a series of proinflammatory mediators but also by a set of inflammatory self-limited mechanisms to regulate the development and resolution. Due to the these self-limited mechanisms, when inflammation has developed to an appropriate stage, the body

produces endogenous proresolving lipid mediators, which remove inflammatory cells and proinflammatory mediators, repair damaged tissue, and terminate inflammatory responses in time [3, 4]. Therefore, insufficient secretion and/or dysfunction of proresolving lipid mediators do not allow the timely resolution of inflammation, which then progresses to chronic inflammation [5].

Resolution of inflammation is an active and highly regulated cellular and biochemical process [6]. Timely resolution of inflammation is crucial for preventing severe and chronic inflammation. Recently, several endogenous proresolving lipid mediators have been discovered, including lipoxins, resolvins, protectins, and maresins, which are heavily involved in driving inflammatory resolution and successfully terminating inflammation [7, 8]. Hence, specialized proresolving lipid mediators are a new focus for research. Many studies have shown the benefits of these lipid mediators that can limit tissue infiltration of polymorphonuclear leukocytes (PMNs), reduce collateral tissue damage by phagocytes, shorten the resolution interval (Ri), enhance macrophage phagocytosis and efferocytosis, and counterregulate proinflammatory chemical mediators [9].

2. Synthesis and Classification of Maresins

The omega-3 fatty acids eicosapntemacnioc acid (EPA) and DHA, which are found in fish oils, have long been known to be important for maintaining organ function and health, as well as reducing the incidence of inflammation [10, 11]. Maresins (*ma*crophage mediators in *re*solving *in*flammation) are derived from the omega-3 fatty acid DHA [12]. Maresins are produced by macrophages via initial lipoxygenation at the carbon-14 position by the insertion of molecular oxygen, producing a 13S,14S-epoxide-maresin intermediate that is enzymatically converted to maresin family members maresin 1, maresin 2, and maresin conjugate in tissue regeneration (MCTR) [9] (Table 1).

Maresin 1 was the first maresin to be identified [12]. Biosynthesis of maresin 1 in macrophages involves initial oxygenation of DHA with molecular oxygen, followed by epoxidation of the 14-hydroperoxy-intermediate that is subsequently converted to 13S,14S-epoxy-maresin. The complete stereochemistry of this epoxide intermediate is 13S,14S-epoxy-docosa-4Z,7Z,9E,11E,16Z,19Z-hexaenoic acid [13]. This epoxide intermediate is then proposed to be enzymatically hydrolyzed via an acid-catalyzed nucleophilic attack by water at carbon-7, resulting in the introduction of a hydroxyl group at that position and double-bond rearrangement to form the stereochemistry of bioactive maresin 1 [14].

However, when the 13S,14S-epoxy-maresin intermediate is followed by conversion via soluble epoxide hydrolase (sEH), it is then converted to additional bioactive products by human macrophages. Here, we nominated the new bioactive macrophage product as maresin 2 [15].

Recently, a new series of bioactive peptide-lipid-conjugated mediators that are produced during the later stages of self-resolving infections have been uncovered [16]. Researchers identified these mediators from human milk, mouse exudates, and human macrophages [17], and they cause lipoxygenation of DHA, producing a maresin-epoxide intermediate that is converted to sulfido-conjugate (SC) with triene double bonds, which belongs to the maresin family. Given that their production was initiated by oxygenation at carbon-14, these mediators were named maresin conjugates in tissue regeneration (MCTRs) [18].

3. Key Biosynthesis Enzymes of Maresins

Human macrophage 12-lipoxygenase (12-LOX) initiates biosynthesis of maresins and, more importantly, is responsible for producing 13S,14S-epoxy-maresin [15] (Figure 1). Activation of 12-LOX in macrophages oxidizes DHA at carbon-14 sites in the major S-configuration and is also involved in the conversion of the 14-hydroperoxy group of 4Z,7Z,10Z,12E,16Z,19Z-docosahexaenoic acid to the 13S,14S-epoxide intermediate process, showing cyclooxygenase activity, manifested as alcohol capture [19]. 12-LOX also catalyzes the formation of lipoxins by leukotriene A4 (LTA4), which is susceptible to epoxide inhibition, for example, LTA4 or 13S,14S-epoxide intermediates [20]. Interestingly, the 13S, 14S-epoxide intermediates only inhibit the conversion of 12-LOX to arachidonic (eicosatetraenoic) acid and do not play a role in DHA conversion, suggesting that 13S,14S-epoxide intermediates can exert a positive feedback on the maresin synthesis pathway and enhance resolution of the inflammation [19]. In addition, the level of messenger RNA expression of 12-LOX was shown to remain unchanged during differentiation of human monocytes to macrophages (M0, M1, and M2) [15].

Studies have shown that the biosynthesis of maresin 2 relates to the mammalian sEH protein (Figure 1); mammalian sEH enzymes catalyze the hydrolysis of a broad category of epoxides, including epoxyeicosatrienoic acids, LTA4, and even hepoxilins [15, 21]. sEH enzymes are active in mononuclear cells and macrophages [22, 23].

In the proposed MCTR biosynthetic pathway, human macrophage 12-LOX is the initiating enzyme, converting docosahexaenoic acid to 13S,14S-epoxide intermediates, which is converted to MCTR1 by leukotriene C4 synthase (LTC4S) and catalyzed glutathione S-transferase MU 4 (GSTM4). Both of these enzymes expressed in human macrophages and catalyze the conversion of LTA4 to leukotriene C4 (LTC4), which displays potent vasoactive and smooth muscle constricting actions. What is interesting is that GSTM4 gave higher affinity to 13S,14S-eMaR, whereas LTC4S has a higher affinity to LTA4. This quality may determine the balance between the LTC4 and the MCTR1. MCTR1 is the proposed precursor to MCTR2 and MCTR3, and gamma-glutamyltransferase (GGT) converts MCTR1 to MCTR2, which is then converted to MCTR3 by a dipeptidase (DPEP) enzyme (Figure 1). Both of the enzymes participate in the cysteinyl leukotriene pathway, and the GGT enzyme gave higher affinity to MCTR1 than LTC4. Their relative expression at sites of inflammation may lead to different disease processes; they also provide targeted therapeutic strategies to upregulate SPM formation [24]. However, the mechanism of maresins and their receptors is not clear, and thus, additional experiments are needed to investigate further.

Table 1: Classification and structure of maresins.

Designation	Chemical structures	Key enzyme	Bioactions and function
Maresin 1	7R,14S-Dihydroxy-docosa-4Z,8E,10E,12Z,16Z,19Z-hexaenoic acid [18]	12-Lipoxygenase, epoxide hydrolysis [49]	Limits PMN infiltration [50]; enhances macrophage phagocytosis and efferocytosis [51]; shortens resolution interval and suppresses oxidative stress [52]; counterregulates proinflammatory chemical mediators [53]; controls pain and enhances tissue regeneration [47]
Maresin 2	13R,14S-Dihydroxy-4Z,7Z,9E,11E,16Z,19Z-hexaenoic acid [54]	12-Lipoxygenase, soluble epoxide hydrolase [54]	Limits PMN infiltration; enhances macrophage phagocytosis [54, 55]
MCTR1	13R-Glutathionyl,14S-hydroxy-4Z,7Z,9E,11E,13R,14S,16Z, 19Z-docosahexaenoic acid [16]	12-Lipoxygenase, leukotriene C4 synthase, and glutathione S-transferase MU 4 [53, 56]	Stimulates tissue regeneration and reduces neutrophil infiltration: MCTR3 ≈ MCTR2 > MCTR1
MCTR2	13R-Cysteinylglycinyl, 14S-hydroxy-4Z,7Z,9E,11E,13R,14S,16Z,19Z-docosahexaenoic acid [56]	12-Lipoxygenase, gamma-glutamyltransferase [53, 56]	Shortens resolution interval (Ri) : MCTR2 > MCTR3 > MCTR1 Regulates local eicosanoids during infections: MCTR1 > MCTR3 > MCTR2
MCTR3	13R-Cysteinyl,14S-hydroxy-4Z,7Z,9E,11E,13R,14S,16Z, 19Z-docosahexaenoic acid [17]	12-Lipoxygenase, dipeptidase [53, 56]	Enhances macrophage phagocytosis: MCTR3 > MCTR1 > MCTR2 [9, 17, 56]

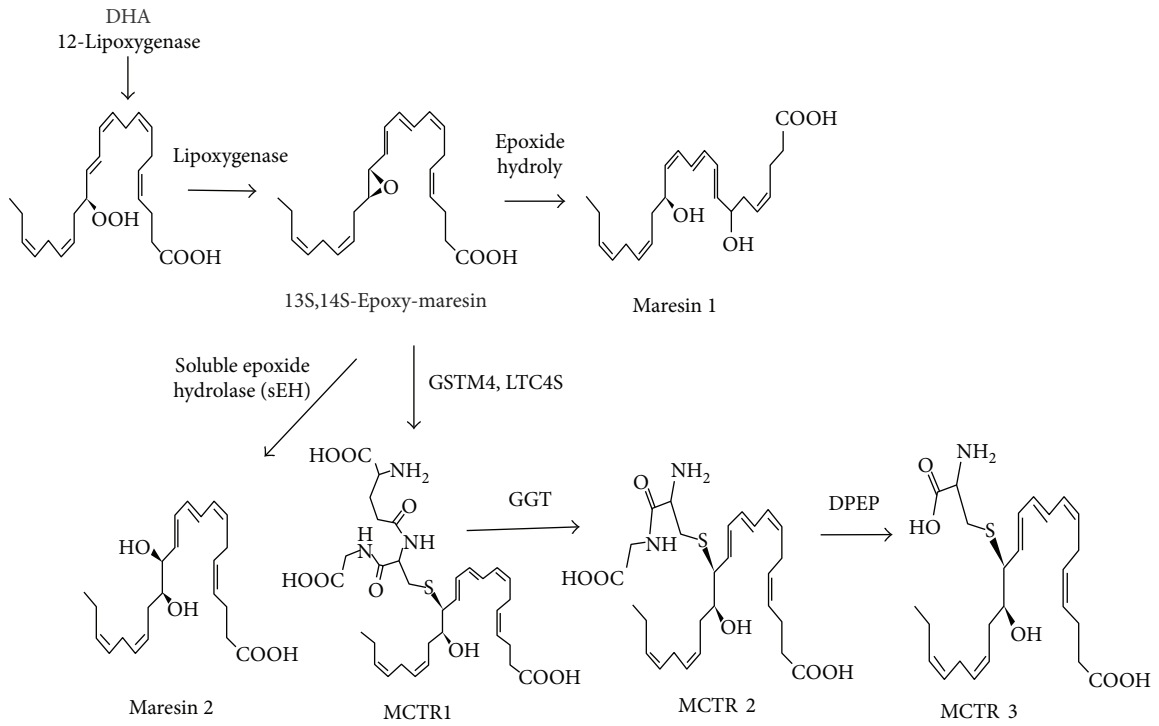

Figure 1: Maresin biosynthetic pathway [48].The pathway is initiated by the lipoxygenation of DHA to yield 13S,14S-epoxy-maresin. This intermediate is then enzymatically hydrolyzed to maresin 1 or via a soluble epoxide hydrolase (sEH) to maresin 2. 13S,14S-epoxy-maresin is also a substrate for glutathione S-transferase MU 4 (GSTM4) and leukotriene C4 synthase (LTC4S) yielding MCTR1, which is then converted to MCTR2 by gamma-glutamyl transferase (GGT) and to MCTR3 by dipeptidase (DPEP).

4. Biological Actions of Maresins

Acute inflammation can lead to persistent and uncontrolled chronic inflammation, which can lead to severe diseases such as lung disease, vascular disease, and metabolic disease [25, 26].Currently, antibiotics are still the main treatment of acute infection following clinical diagnosis. However, with the serious threat of emerging pathogens, especially

antibiotic-resistant ones, it is imperative to research and develop new therapeutic interventions of increasing the host anti-infective mechanisms [27].

Inflammatory resolution has become a new focus of inflammation research, and specialized proresolving lipid mediators have become a new strategy for inflammatory therapy [9]. The synthesis of anti-inflammatory drugs with endogenous anti-inflammatory mediators has important clinical significance. Studies have shown that targeted intervention with specialized proresolving lipid mediators can reduce the use of antibiotics for treating infection in the host reaction process, thus providing a new way to seek and develop more effective antimicrobial therapies [28].

There is an increasing understanding of the roles of proresolving lipid mediators in treating infection. As a new family of anti-inflammatory and proresolving lipid mediators, it has been previously confirmed that maresins limit the further recruitment of PMNs and inhibit neutrophil infiltration in vivo yet stimulate the nonphlogistic recruitment of mononuclear cells. When macrophages encounter maresins, they increase phagocytosis and efferocytosis, resulting in the removal of microbes. Biosynthesized maresins counterregulate the proinflammatory cytokines such as IL-1β, IL-6, and TNF-α. They also regulate nuclear factor kappa B (NF-κB) gene products and increase the regulation of T cell de novo synthesis and intracellular levels of cyclic adenosine monophosphate, regenerate tissue, and play a role in antinociceptive action [9, 29] (Figure 2).

5. Maresins in Lung Disease

Acute inflammation is a form of innate immune defense and is the primary response to injury and infection. In the lungs, dysregulated acute inflammation and failure to resolve inflammation are the major contributors of numerous lung diseases, which can result in lung injury, contributing to pulmonary fibrosis that severely impairs essential gas exchange processes [26].

IL-6 is a pleiotropic cytokine best recognized as a primary mediator of the acute phase response [30]. IL-6 not only activates neutrophils but also delays the phagocytosis of macrophages in acute inflammation, which can promote a "cytokine storm." A number of stimuli, including inflammatory cytokines and growth factors, such as TNF-α, IL-1, and platelet-derived growth factor (PDGF), are associated with increases in vascular cell-derived IL-6 [31, 32]. IL-6, IL-1, and TNF-α are all sensitive indicators of inflammatory reaction, which can reflect the condition of patients and evaluate the severity of inflammatory reaction. By early monitoring of these important indicators, we can take appropriate measures to stop the further development of the inflammatory response. IL-6 can play a positive role in some specific aspects of lung disease. Inhibition of IL-6 (or IL-6R) may be a therapy for asthma, chronic obstructive pulmonary diseases (COPD), and other lung diseases.

Maresin 1 as a specialized proresolving lipid mediator has been shown to reduce airway inflammation associated with acute and repetitive exposure to organic dust by activating protein kinase C (PKC) isoforms α and ε [33], limiting neutrophil infiltration, and decreasing IL-6, TNF-α, and chemokine C-X-C motif ligand 1 levels, which suggests that maresin 1 could contribute to an effective strategy for reducing airway inflammatory diseases induced by agricultural-related organic dust environmental exposure [34]. 100 nmol/L maresin 1 can attenuate the proinflammatory cytokines (TNF-α, IL-1β, and IL-6), chemokines, pulmonary myeloperoxidase activity, and neutrophil infiltration in an LPS-induced acute lung injury (ALI) mouse and can significantly inhibit LPS-induced ALI by restoring oxygenation, attenuating pulmonary edema, and mitigating pathohistological changes [35]. This study also shows that maresin 1 exhibits novel mechanisms in the resolution of inflammation in that it can inhibit proinflammatory mediator production by LTA4 hydrolase and can block arachidonate conversion by human 12-LOX, rather than merely terminating phagocyte involvement [20]. Furthermore, maresin 1 can also maintain the permeability of lung epithelial cells by upregulating the expression of claudin-1 and Zonula occludens protein 1 (ZO-1) in ALI [36].

Recently, metabololipidomics of murine lungs identified temporal changes in endogenous maresin 1 during self-limited allergic inflammation. Exogenous maresin 1 augmented de novo generation of regulatory T cells (Tregs), which interacted with innate lymphoid cells (ILC2s) to markedly suppress cytokine production in a transforming growth factor β1- (TGF-β1)-dependent manner, suggesting the use of maresin 1 as the basis for a new proresolving therapeutic approach in asthma and other chronic inflammatory diseases [37]. In addition, the study also found that treating mouse type II alveolar epithelial cells with maresin 1 significantly prevented TGF-β1-induced fibronectin and alpha-smooth muscle actin (α-SMA) expression and restored E-cadherin levels in vitro, as well as attenuating bleomycin-induced lung fibrosis in vivo [38]. These studies suggest that maresin 1 can be used as a promising new strategy for treating lung inflammation-related diseases.

6. Maresins in Vascular Disease

Vascular injury induces a potent inflammatory response that influences vessel remodeling and patency, limiting the long-term benefits of cardiovascular interventions such as angioplasty. Inflammatory resolution is central to vascular repair. Chatterjee et al. [14] confirmed that maresin 1 imparted a strong anti-inflammatory phenotype in human vascular smooth muscle cells and endothelial cells, associated with reduced monocyte adhesion and TNF-α-induced production of reactive oxygen species (ROS) and NF-κB activation by inhibiting IκB kinase (IKK) phosphorylation, NF-kappa-B inhibitor alpha (IκB-α) degradation, and nuclear translocation of the NF-κB p65 subunit. Maresin 1 also inhibited mouse aortic smooth muscle cell migration, relative to a PDGF gradient, and reduced TNF-α-stimulated p65 translocation, superoxide production, and proinflammatory gene expression. In vivo, maresin 1 reduced neutrophil and macrophage recruitment and increased polarization of M2 macrophages in the arterial wall [39]. These results offer new opportunities to regulate the vascular injury response and

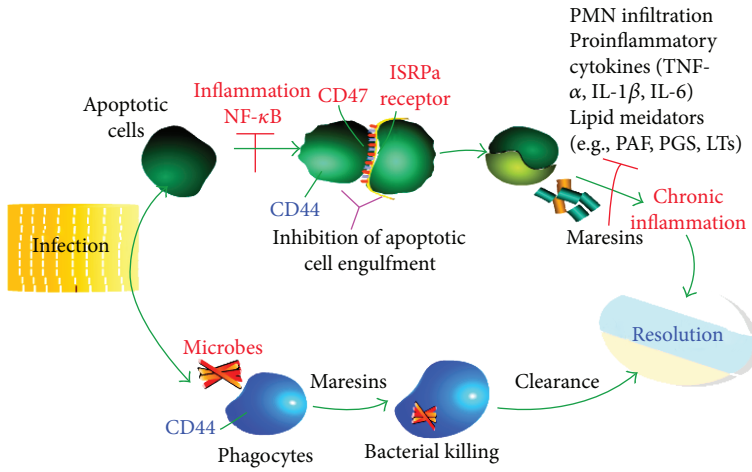

Figure 2: Maresins in the resolution pathway. Maresins stimulate efferocytosis and the uptake of debris for successful clearance from tissues and resolution. Maresins block NF-κB and counterregulate proinflammatory mediators and lipid mediators; inhibition of containment of apoptotic cells leads to chronic inflammation.

promote vascular homeostasis. In addition, research has shown, for the first time, that human platelets express the SPM receptors G-protein-coupled receptor 32 (GPR32) and ALX, and maresin 1 regulates platelet hemostatic function by enhancing platelet aggregation and spreading, while suppressing the release of proinflammatory and prothrombotic mediators, indicating maresin 1 could be a novel class of antiplatelet agents that play an important role in the resolution of inflammation in cardiovascular diseases [40].

7. Maresins in Metabolic Disease

Chronic low-grade inflammation associated with metabolic diseases is sustained and detrimental. SPMs can stop and limit further PMN entry and stimulate macrophage intake and clearance of apoptotic cells, debris, and bacteria; treatment with specific SPMs improves metabolism and immunity [28]. Viola et al. [41] found that maresin 1 prevented atheroprogression by inducing a shift in macrophage profile toward a reparative phenotype and stimulated collagen synthesis in smooth muscle cells. Recently, a study has confirmed that maresin 1 reduced the expression of MCP-1 (monocyte chemotactic protein 1), TNF-α, IL-1β, and the proinflammatory M1 macrophage phenotype marker Cd11c, while it upregulated adiponectin and glucose transporter-4 protein (Glut-4) and increased protein kinase B (Akt) phosphorylation in white adipose tissue (WAT) in diet-induced obese (DIO) mice; maresin 1 also improved the insulin tolerance test and increased adiponectin gene expression, Akt and adenosine monophosphate-activated protein kinase (AMPK) phosphorylation, and the expression of M2 macrophage markers Cd163 and IL-10 in genetic (ob/ob) obese mice [42]. Our previous research showed that maresin 1 may have a protective effect on diabetic nephropathy by mitigating the expression of the NLRP3 inflammasome, TGF-β1, and fibronectin (FN) in mouse glomerular mesangial cells [43]. Furthermore, Hong et al. [44] found that maresin-like mediators (14,22-dihydroxy-docosa-4Z,7Z,10Z,12E,16Z,19Z-hexaenoic acids) were produced by leukocytes and blood platelet (PLT) and were involved in wound healing by restoring reparative functions to diabetic macrophages; in addition, these mediators could ameliorate the inflammatory activation of macrophages and had the potential to suppress chronic inflammation in diabetic wounds caused by the activation of macrophages. Resolution of inflammation may be an essential criterion in developing future therapeutic interventions aimed at counteracting inappropriate inflammation in metabolic disease.

8. Maresins in Inflammatory Bowel Disease

The gut is regarded as being in a state of controlled inflammation; resolution of inflammation is thus critical to avoid excessive damage to host tissue. It has been previously reported that maresin 1 consistently protects mice in models of experimental colitis by inhibiting the NF-κB pathway and consequently multiple inflammatory mediators, such as IL-1β, TNF-α, IL-6, and porcine interferon γ (IFN-γ), while enhancing the macrophage M2 phenotype [45]. Recently, Wang et al. [46] found that maresin 1 treatment ameliorated iron-deficient anemia by reducing colonic inflammation and inhibiting hepcidin expression though the IL-6/STAT3 pathway. In addition, maresin 2 showed the potential anti-inflammatory action in mouse peritonitis initiated by intraperitoneal injection of zymosan. This study found that maresin 2 is equivalent to maresin 1 in limiting neutrophil infiltration, whereas maresin 1 is more effective in enhancing macrophage phagocytosis than maresin 2 [15]. Current studies on maresin 2 are still limited and require additional experiments to explore its biological effects and mechanisms.

9. Maresins Stimulate Tissue Regeneration and Control Pain

Acute inflammatory responses are protective, and the cardinal signs of inflammation are heat, redness, swelling, and eventual loss of function. Proresolving mediators have been shown to be the stop signals of inflammation and act in the

host defense mechanism to reduce pain and enhance wound healing and tissue regeneration [39]. Transient receptor potential V1 (TRPV1) was found to be expressed in primary sensory neurons and plays an important role in mediating heat pain and heat hyperalgesia after injury [47]. Serhan et al. [47] have confirmed that maresin 1 dose-dependently inhibited TRPV1 currents in neurons, blocked capsaicin-induced inward currents, and reduced both inflammation-induced and chemotherapy-induced neuropathic pain in mice. Meanwhile, maresin 1 markedly reduced vincristine-induced mechanical allodynia and accelerated surgical regeneration in planaria, increasing the rate of head reappearance. Recently it was reported that macrophages produce a family of bioactive peptide-conjugated mediators known as maresin conjugates in tissue regeneration (MCTR) [16]. These mediators have been found to rescue *Escherichia coli* infection-mediated delay in tissue regeneration in planaria and were shown to protect mice from second-organ reflow injury, promoting repair by limiting neutrophil infiltration, upregulating nuclear antigen KI-67, and roof plate-specific spondin 3 [16]. To assess the ability of each synthetic MCTR to promote tissue regeneration in planaria, one study found that each of the three synthetic MCTRs dose-dependently (1–100 nM) accelerated tissue regeneration in planaria by 0.6–0.9 days; MCTR3 and MCTR2 were more potent than MCTR1. In mice, MCTRs were also found to regulate tissue repair and regeneration in lung tissue where administration of their key enzymes during ischemia-reperfusion-mediated injury protected the lung from leukocyte-mediated damage and upregulated the expression of molecules that are associated with cell proliferation and tissue repair in the lung [16]. Furthermore, each MCTR promoted resolution of *E. coli* infections in mice by increasing bacterial phagocytosis, limiting neutrophil infiltration, and promoting efferocytosis [48]. Therefore, these results demonstrate the potent actions of maresins in regulating inflammation resolution, tissue regeneration, and pain resolution.

10. Conclusion and Prospects

Maresins are part of the latest families of anti-inflammatory lipid mediators, which display both anti-inflammatory and proresolving activities in acute or chronic inflammatory-related diseases. Maresins are synthesized by the lipoxygenase enzyme oxidation pathway during the inflammation-subsiding period and conjugate triene double bonds. Studies have confirmed that maresins protect the body by limiting neutrophil infiltration, enhancing macrophage phagocytosis, reducing the production of proinflammatory factors, inhibiting NF-κB activation, stimulating tissue regeneration, and controlling pain. Therefore, maresins as potent inflammatory self-limiting factors are expected to become highly promising anti-inflammatory intervention drug targets. And as inflammation is closely related to fibrosis, studying maresin may also provide new directions for the prevention and treatment of viscera fibrosis. In addition, further investigations are required to understand the relationship between novel endogenous pathways to control pathogens and microbial pathogenesis diversity. We envisage more basic research and clinical research on maresins. We also expect to discover maresin-related stable analogues or new family members of specialized proresolving lipid mediators as potential reserve molecules for exploiting endogenous anti-inflammatory mechanisms to limit excessive pathogen-mediated inflammatory responses in future therapeutic strategies.

Abbreviations

SPMs: Specialized proresolving lipid mediators
DHA: Docosahexaenoic acid
PMNs: Polymorphonuclear leukocytes
Ri: Resolution interval
EPA: Eicosapntemacnioc acid
MCTR: Maresin conjugate in tissue regeneration
sEH: Soluble epoxide hydrolase
12-LOX: 12-Lipoxygenase
LTC4S: Leukotriene C4 synthase
GSTM4: Glutathione S-transferase MU 4
GGT: Gamma-glutamyltransferase
DPEP: Dipeptidase
NF-κB: Nuclear factor kappa B
PDGF: Platelet-derived growth factor
TGF-β1: Transforming growth factor β1
TRPV1: Transient receptor potential V1.

Conflicts of Interest

There is no conflict of interest.

Acknowledgments

The authors thank Stanton RC and BioMed Proofreading LLC for the English copyediting.

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Hindawi
Mediators of Inflammation
Volume 2018, Article ID 8430614, 10 pages
https://doi.org/10.1155/2018/8430614

Research Article

Dietary n-3 PUFA May Attenuate Experimental Colitis

Cloé Charpentier,[1,2] Ronald Chan,[3] Emmeline Salameh,[1] Khaly Mbodji,[1] Aito Ueno,[3,4] Moïse Coëffier,[1,5] Charlène Guérin,[1] Subrata Ghosh,[3,6] Guillaume Savoye,[1,2] and Rachel Marion-Letellier[1]

[1]*INSERM UMR 1073, UFR de Médecine-Pharmacie, 22 boulevard Gambetta, 76183 Rouen Cedex, France*
[2]*Department of Gastroenterology, Rouen University Hospital, 1 rue de Germont, 76031 Rouen Cedex, France*
[3]*University of Calgary, Gastrointestinal Research Group, Snyder Institute for Chronic Diseases, Calgary, AB, Canada*
[4]*Center for Advanced IBD Research and Treatment, Kitasato Institute Hospital, Tokyo, Japan*
[5]*Nutrition Unit, Rouen University Hospital, 1 rue de Germont, 76031 Rouen Cedex, France*
[6]*Institute of Translational Medicine, University of Birmingham, Birmingham, UK*

Correspondence should be addressed to Subrata Ghosh; sughosh@ymail.com

Received 28 July 2017; Revised 13 October 2017; Accepted 31 October 2017; Published 15 February 2018

Academic Editor: Sung-Ling Yeh

Background. Inflammatory bowel diseases (IBD) occurred in genetically predisposed people exposed to environmental triggers. Diet has long been suspected to contribute to the development of IBD. Supplementation with n-3 polyunsaturated fatty acids (PUFA) protects against intestinal inflammation in rodent models while clinical trials showed no benefits. We hypothesized that intervention timing is crucial and dietary fatty acid pattern may influence intestinal environment to modify inflammation genesis. The aim of this study was to evaluate the dietary effect of PUFA composition on intestinal inflammation. *Methods*. Animals received diet varying in their PUFA composition for four weeks before TNBS-induced colitis. Colon inflammatory markers and gut barrier function parameters were assessed. Inflammatory pathway PCR arrays were determined. *Results*. n-3 diet significantly decreased colon iNOS, COX-2 expression, IL-6 production, and LTB4 production but tended to decrease colon TNFα production ($P = 0.0617$) compared to control diet. Tight junction protein (claudin-1, occludin) expressions and MUC2 and TFF3 mRNA levels were not different among groups. n-9 diet also decreased colon IL-6 production ($P < 0.05$). *Conclusions*. Dietary n-3 PUFA influence colitis development by attenuating inflammatory markers. Further research is required to better define dietary advice with a scientific rationale.

1. Introduction

Inflammatory bowel diseases (IBD) affects genetically predisposed people exposed to environmental triggers [1]. Amongst environmental factors, dietary habits have long been suspected to contribute to the development of IBD [2]. IBD patients often considered diet as a potential trigger for initiating the disease or causing a relapse [3], and this concept led to exclusion diets especially in children [4].

An increased incidence of IBD has been associated with diets high in animal protein. Indeed, association between dietary pattern (fat/protein) and Crohn's disease (CD) risk was found in a study from Japan [5] while increased consumption of animal protein has been associated with higher IBD risk in a study from France [6]. A systematic review demonstrated that this Western dietary pattern (high fat, high n-6 polyunsaturated fatty acids (PUFA), and high meat) is associated with an increased IBD risk [7]. More recently, a study was conducted in 103 IBD patients using food frequency questionnaire over 1 year and the authors found a positive association between meat intake and disease relapse [8]. Similarly, Western diet had a deleterious impact on gut barrier function and dysbiosis in IBD murine models [9].

While n-3 and n-6 PUFA are essential in human nutrition, a Western diet is characterized by an unbalanced ratio

of both types of PUFA (n-3/n-6 ratio). Indeed, linoleic acid (LA, n-6 PUFA) consumption has markedly increased (3-fold throughout the 20th century) [10]. Numerous epidemiological studies highlighted the role of dietary intake of monounsaturated fatty acids (MUFA) or PUFA in ulcerative colitis (UC) development. Higher intake of LA is associated with an increased risk of UC [11], while docosahexaenoic acid (DHA) (n-3 PUFA) [12] or oleic acid (n-9 MUFA) [13, 14] consumption is beneficial. Ananthakrishnan et al. found that greater fish intake was associated with lower risk of CD [15].

We and others demonstrated an anti-inflammatory effect of n-3 polyunsaturated fatty acids in rodent IBD models [16–21] while clinical trials failed [22]. We hypothesized that intervention timing is crucial and dietary fatty acid pattern may influence intestinal environment to modify inflammation genesis [23].

The aim of the study was to investigate the dietary influence of fatty acid composition before the onset of intestinal inflammation by administration of 2,4,6-trinitrobenzene sulfonic acid (TNBS). For this purpose, rats were fed with diets varying in n-3/n-6/n-9 ratio to reproduce dietary pattern from a pragmatic to a Western diet.

2. Materials and Methods

2.1. Animals and Study Design. Young Sprague-Dawley male rats weighing 75–100 g were purchased from Janvier (Le Genest St. Isle, France) and allowed to access food and water ad libitum. After 1 week acclimatization, 50 rats were randomly divided into 5 experimental groups; the control (CTRL) group was fed with control diet and received the vehicle, while colitic groups including TNBS, n-3, n-6, and n-9 groups were fed with control diet, n-3 diet, n-6 diet, and n-9 diet, respectively, and received TNBS for the colitis induction. Weight changes throughout the study were monitored every day. After 4 weeks of experimental diets (day 28 to day 1), the rats underwent 24 hours food deprivation prior to the TNBS or vehicle administration. During the colitis induction (day 0 to 2), rats were provided control diet. The overview of experimental design is illustrated in Figure 1(a).

All animal handling and treatment procedures were performed in accordance with both French national regulations and European Union regulations (Official Journal of the European Community L 358, 18/12/1986) and RML is authorized to use this animal protocol by the French government (Authorization n°76-106).

2.2. Diets. Four types of isocaloric and isolipidic experimental diets were prepared with several fatty acid proportions:

(i) The normal diet matched a balanced diet with a n-3/n-6/n-9 ratio equal to 1 : 4 : 16 as a fat ratio recommended by dietary guidelines and described as a well-balanced diet in the literature [24]. The control diet was given to CTRL and TNBS groups. The recommended dietary n-6/n-3 ratio is about 4 in human nutrition which is comparable to the control diet in this study.

(ii) n-3 diet had a n-3/n-6/n-9 ratio equal to 1 : 1 : 4. We chose a n-3/n-6 ratio equal to 1 : 1 because this ratio was a target by dietary advice in a Japanese clinical trial for IBD patients [25]. In addition, a n-3/n-6 ratio is commonly used in experimental studies investigating the effect of n-3 therapy [24].

(iii) n-6 diet fitted the Western diet with a n-3/n-6/n-9 ratio at 1 : 16 : 16. The dietary n-6/n-3 ratio is about 15 in human Western diets [26], and this ratio is useful to underline the imbalance that characterizes Western diets.

(iv) n-9 diet had a similar n-3/n-6 ratio to CTRL diet but is enriched in n-9 MUFA. This n-3/n-9 ratio equal to 1 : 24 is comparable to the ratio observed in people following the Mediterranean diet [26].

Detailed diet composition is shown in Table 1.

2.3. Induction of Colitis. Administration of TNBS (Sigma-Aldrich Company, Saint-Quentin-Fallavier, France) was employed for colitis induction as previously described [16] in TNBS, n-3, n-6, and n-9 groups (colitic groups). The rats were sacrificed using anesthetic reagents (ketamine and xylazine) at day 2 for further analyses.

2.4. Western Blot. PBS, protease inhibitor cocktail, and phosphatase inhibitor cocktail were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). The 4–12% NuPAGE gels and SeeBlue multicolored standard were obtained from Invitrogen (Cergy-Pontoise, France). Frozen colon samples were homogenized in PBS with 0.1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail. Homogenates were centrifuged (12,000g, 15 min, 4°C) and the supernatants were collected. Protein concentration was determined following Bradford's colorimetric method. Aliquots of supernatants containing equal amounts of protein (30 μg) were separated on 4–12% NuPAGE and then transferred to a nitrocellulose membrane (Hybond, GE Healthcare, UK). The mouse monoclonal antibody anti-PPARγ (sc-7273), the goat polyclonal anti-COX-2 (sc-1747), the mouse anti-iNOS (sc-7271), the rabbit polyclonal anti-HNF-4 (sc-8987), and the HRP-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Tebu, Le Perray-en-Yvelines, France). The rabbit anti-claudin-1 and the mouse anti-occludin were, respectively, obtained from Life technology and Invitrogen. After blocking, membranes were incubated with specific primary antibodies at the dilution of 1 : 100 (iNOS), 1 : 500 (COX-2, HNF-4, and PPARγ), and 1 : 1000 (claudin-1, occludin. After three washes, membranes were then incubated with the secondary HRP-linked anti-goat IgG (for COX-2), anti-rabbit IgG (for HNF-4, claudin-1), and anti-mouse IgG (for iNOS, PPARγ, and occludin) antibodies. The enhanced chemiluminescence light-detecting kit (GE Healthcare, USA) was utilized for immunodetection. Densitometric data were measured following normalization to the housekeeping protein (β-actin) by a Scientific Imaging Systems (Image QuantTL, GE Healthcare).

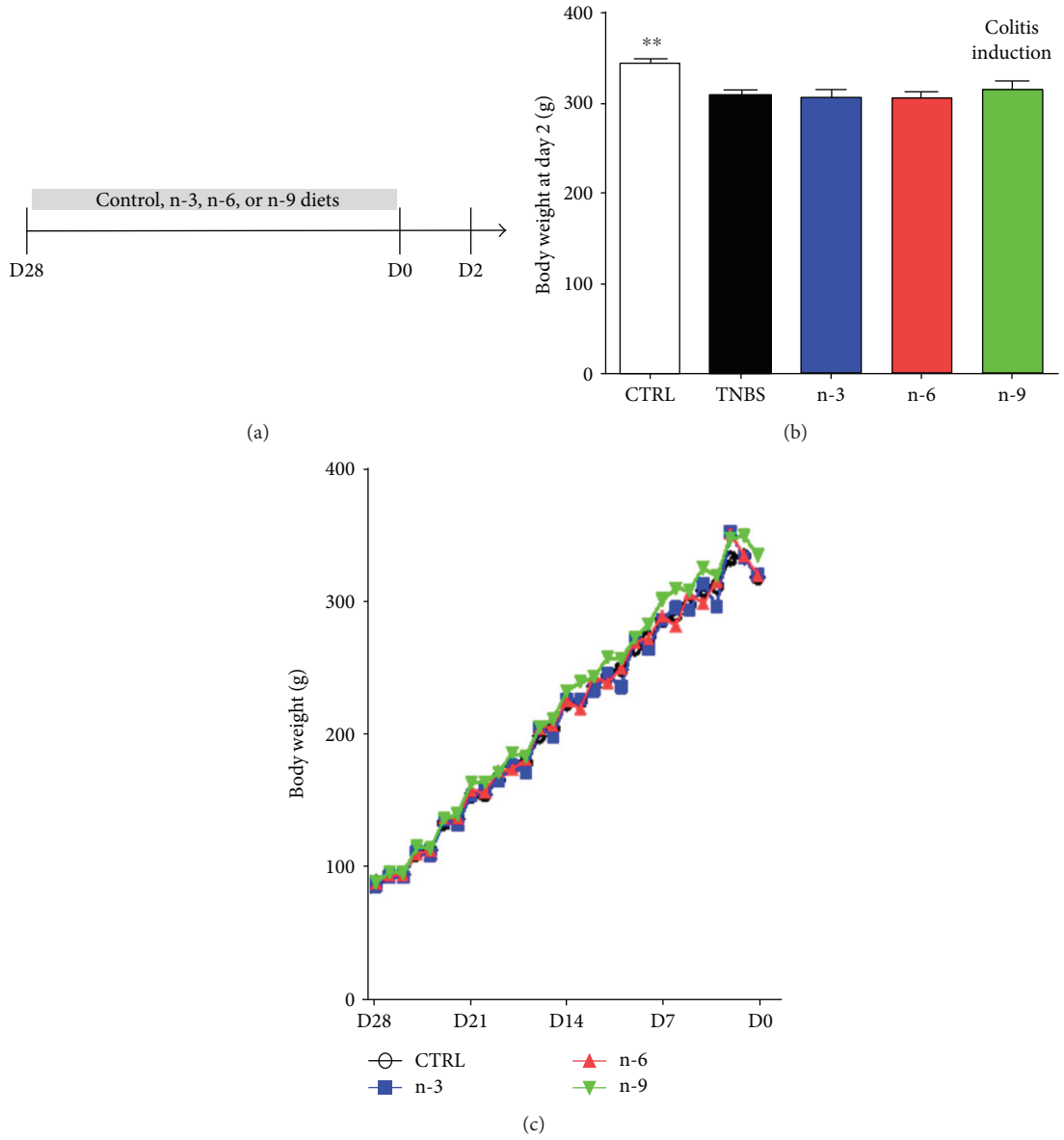

Figure 1: Experimental design and clinical parameters in rats receiving diets varying in their unsaturated fatty acid composition followed by TNBS-induced colitis. (a) Experimental design. Rats received diets varying in their PUFA composition for four weeks before colitis induction at day 0. Rats were killed at day 2. (b) Body weight at day 2. (c) Body weight follow-up from day 28 to day 2. ∗∗ means $P < 0.01$ versus all colitis groups (TNBS, n-3, n-6, and n-9).

Table 1: Fatty acid composition of the experimental diets.

	CTRL	n-3 diet	n-6 diet	n-9 diet
Total fat (g/1000 g of diet)	49.7	49.4	49.7	49.8
Saturated fat (g)	10.2	9.9	9.3	9.6
MUFA (g)	29.8	26.2	20.1	32.8
PUFA (g)	9.8	13.3	20.3	7.4
n-6 fatty acids (g)	7.9	7.3	19.1	6.1
n-3 fatty acids (g)	1.8	6.1	1.2	1.3
n-9 fatty acids (g)	29.3	25.8	19.8	32.2
n-3/n-6/n-9 ratio	1 : 4:16	1:1 : 4	1 : 16:16	1:4:24

2.5. RNA Isolation and Gene Expression Analyses. Colon samples were frozen in liquid nitrogen and stored at −80°C before RNA preparation. Total RNA was isolated from rat colon specimens using a commercial RNA purification kit (SV total RNA isolation kit, Promega, Madison, WI) and mRNA expression of Muc2 (primer sequences F: CCTTGC TCTGCCATACCCGT, R: ACACTGGTCCTCTCCTCCCT) and TFF-3 (F: TAACCCTGCTGCTGGTCCTG, R: GTTT GAAGCACCAGGGCACA), and the internal control (GAPDH) was measured by qRT-PCR. Furthermore, gene expressions in Toll-like receptor signaling pathway were determined by real-time PCR array according to the manufacturer's protocol (PAMM-0018ZD, SA Biosciences, Frederick, MD) on CFX96 thermocycler (Bio-Rad, Hercules, CA). Data

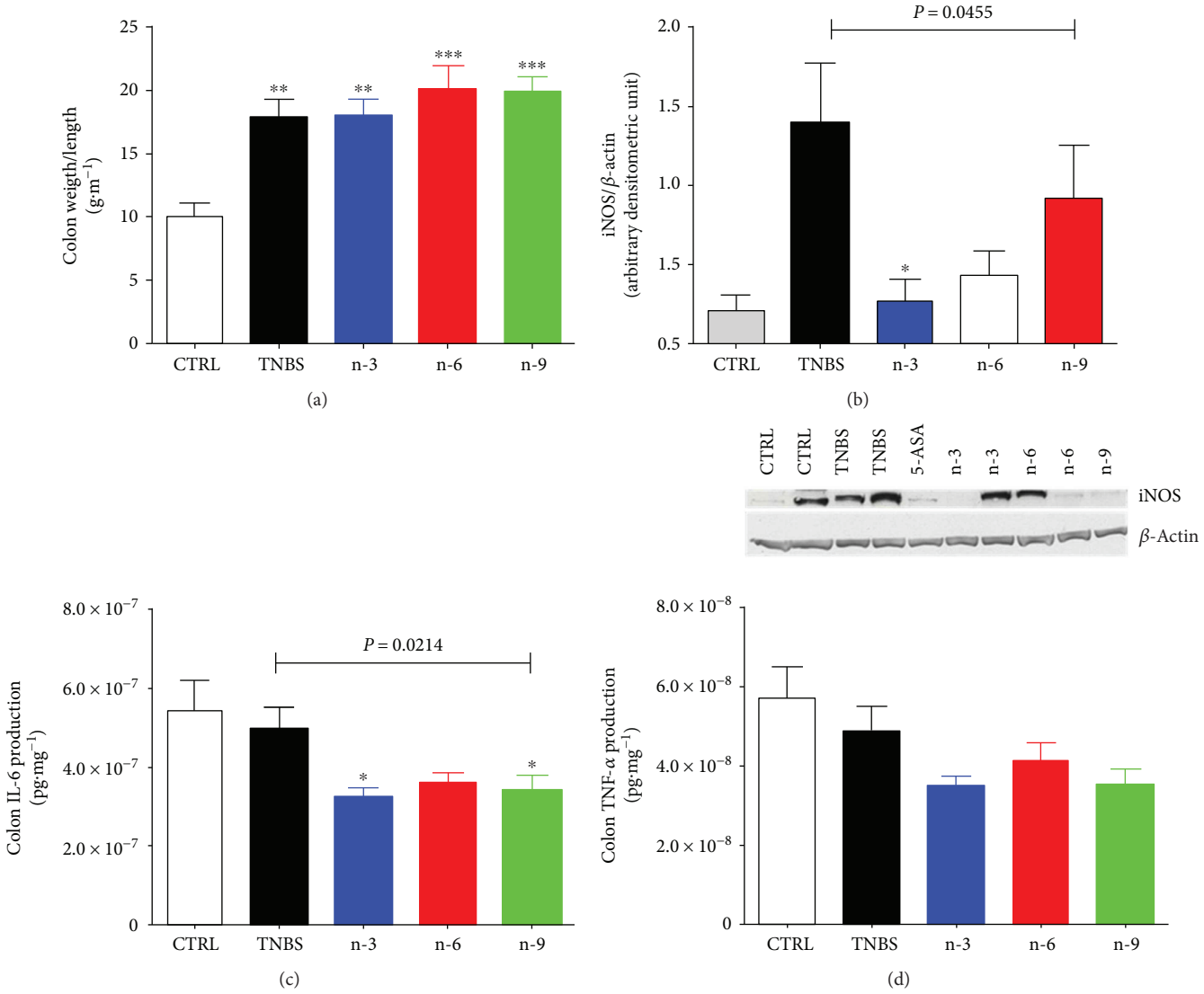

Figure 2: Inflammatory markers in rats receiving diets varying in their unsaturated fatty acid composition for 4 weeks followed by TNBS-induced colitis. (a) Colon/weight length at day 2. (b) Colon iNOS expression with a representative gel at day 2. (c) Colon IL-6 and (d) TNFα production. 5-Aminosalicylic acid (5-ASA) is used a positive anti-inflammatory control. Data from colitic rats were compared by 1-way ANOVA followed by Tukey posttests. ** means $P < 0.01$ versus CTRL, *** means $P < 0.001$ versus CTRL, and * means $P < 0.05$ versus TNBS.

are expressed in fold regulation. The fold change (fold difference) is calculated by the equation $2(-\Delta\Delta CT)$. For the fold regulation, the software transforms fold change values less than 1 (meaning that the gene is downregulated) by returning the negative inverse.

2.6. Colon Cytokines and LTB4 Production. Concentrations of TNFα, IL-1β, and LTB4 in the colon homogenates were detected by ELISA (R&D Systems, Lille, France) following the manufacturer's instructions.

2.7. Proteolytic Pathway Activities. The evaluation of proteolytic activities (caspase-like and chymotrypsin-like) was performed by spectrofluorometric microtiter plate fluorometer (Mithras LB 940, Berthold Technologies) using fluorogenic proteasome substrate in the presence or absence of specific proteasome inhibitors as previously described [27].

2.8. Statistical Analysis. Statistical comparisons were performed using GraphPadPrism 5. Data are expressed as mean ± SEM. Body weight changes and food intake were analyzed with 2-way ANOVA for repeated measures with Tukey's post hoc tests. All the other variables were analyzed by one-way ANOVA with Bonferroni post hoc test or Kruskal-Wallis test as appropriate. Differences were considered significant at $P < 0.05$.

3. Results

3.1. TNBS-Induced Colitis Decreased Body Weight. Colitic groups had a lower body weight compared to control rats

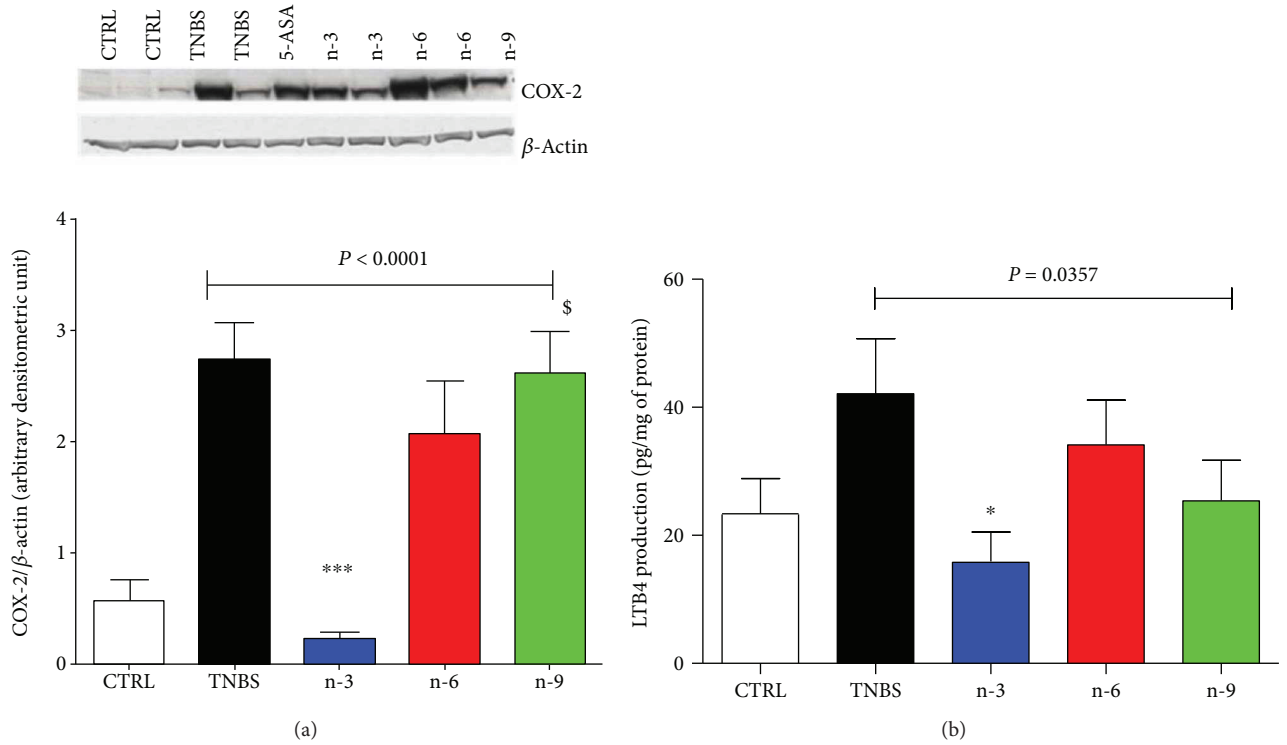

Figure 3: Eicosanoid pathway in rats varying in their unsaturated fatty acid composition for 4 weeks followed by TNBS-induced colitis. (a) Colon cyclooxygenase-2 (COX-2) expression with a representative gel and (b) colon LTB4 production at day 2. 5-Aminosalicylic acid (5-ASA) is used a positive anti-inflammatory control. Data from colitic rats were compared by 1-way ANOVA followed by Tukey posttests. $*$ means $P < 0.05$ versus TNBS, $***$ means $P < 0.001$ versus TNBS, and \$ means $P < 0.05$ versus n-3.

($P < 0.01$) at day 2 but there is no difference among colitic groups (Figure 1(b)).

3.2. TNBS-Induced Colitis Increased Inflammatory Markers. Colon weight/length ratio was increased in colitic rats compared to control group ($P < 0.01$ for TNBS and n-3, $P < 0.001$ for n-6 and n-9, Figure 2(a)) without significant differences among colitic groups (Figure 2(a)). Colon iNOS was significantly higher in colitic groups compared to control group ($P = 0.0141$, Figure 2(b)).

3.3. n-3 Diet Decreased Colon Inflammatory Markers. Among colitis groups, n-3 group had a lower colon iNOS compared to TNBS group ($P < 0.05$, Figure 2(b)). Colon IL-6 production was significantly lower in n-3 and n-6 groups compared to TNBS group ($P < 0.05$ for both, Figure 2(c)) while colon TNFα production did not significantly differ among colitis groups (Figure 2(d)) but tend to decrease in n-3 group compared to TNBS group ($P = 0.0617$). Transcription factors HNF-4α and PPARγ expressions were not different among groups (data not shown).

3.4. n-3 Diet Decreased COX-2 Expression and LTB4 Production in the Colon. Among colitis groups, n-3 group had a lower colon COX-2 expression compared to TNBS group ($P < 0.001$, Figure 3(a)). In addition, colon LTB4 production was lower in the n-3 group compared to TNBS group ($P < 0.05$, Figure 3(b)).

3.4.1. Gut Barrier Function Was Not Affected by Dietary Treatments. Tight junction proteins claudin-1 and occludin were not different among groups ($P = 0.4750$ and $P = 0.8553$, resp., Figures 4(a) and 4(b)). TFF3 mRNA levels were not different among groups ($P = 0.3729$, Figure 4(c)). MUC2 mRNA levels were not different among groups (1-way ANOVA, $P = 0.0381$, posttests $P > 0.05$, Figure 4(d)).

3.5. Colitis or Dietary PUFA Did Not Modify Proteasome Activity. Chymotrypsin and trypsin-like activities were not different among colitic groups ($P = 0.3510$ and $P = 0.0651$, resp., data not shown).

3.6. Dietary Modulation of Inflammatory Gene Expression. In colitis groups, n-3 diet upregulated IL-1A, TLR-2, and MA2K3 genes while n-9 diet upregulated TLR-4 genes ($P = 0.044$, $P = 0.013$, and $P = 0.021$, resp., Table 2). n-6 upregulated HMGB1 ($P = 0.042$) without affecting TLR pathways ($P > 0.05$, Table 2).

4. Discussion

Numerous experimental studies found anti-inflammatory effects of n-3 PUFA in intestinal inflammation while randomized clinical trials failed to demonstrate efficacy [2, 23]. We previously hypothesized that the discrepancy between clinical trials and experimental studies could result from the timing of the intervention [23]. In our previous studies in colitis models [16–19], we tested nutritional intervention

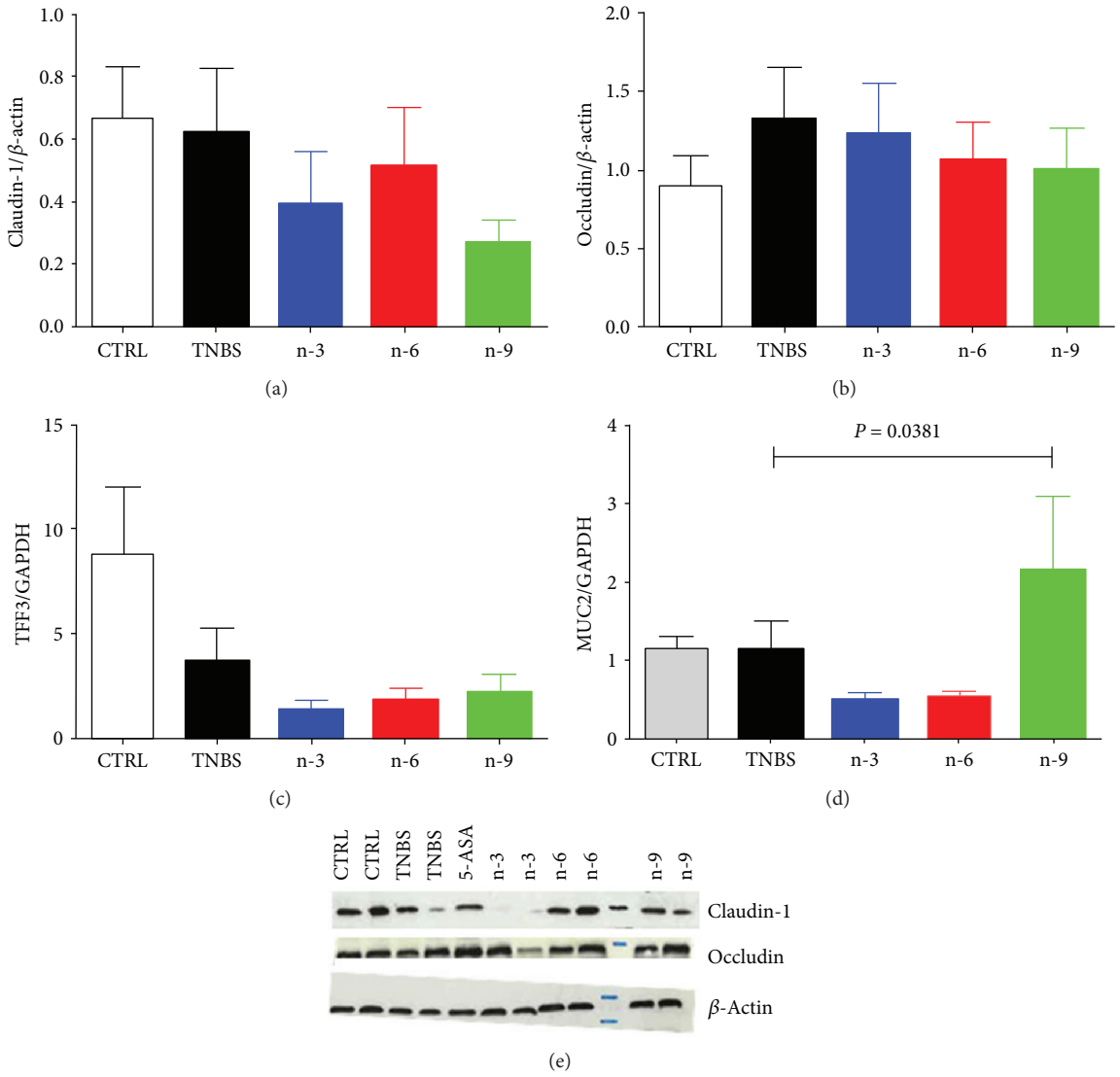

Figure 4: Gut barrier parameters in rats receiving diets varying in their unsaturated fatty acid composition for 4 weeks followed by TNBS-induced colitis. (a) Colon claudin-1 expression, (b) occludin expression, (c) trefoil factor 3 (TFF3) mRNA level, and (d) MUC2 mRNA level at day 2. (e) Representative gel of colon claudin-1 and occludin expression. 5-Aminosalicylic acid (5-ASA) is used a positive anti-inflammatory control. Data from colitic rats were compared by 1-way ANOVA followed by Tukey posttests.

with n-3 PUFA in a curative manner. We now speculated that nutritional intervention with fatty acid should be preventive as reflected in the epidemiological studies. In epidemiological studies, dietary intake of PUFA modifies IBD risk, and identification of their potential mechanisms is now required. To this purpose, we fed rats for four weeks with diets differing in their PUFA composition before the onset of colitis.

In the present study, n-3 diet downregulated colon iNOS expression (Figure 2(a)) in rats with TNBS-induced colitis similar to previous studies performed by us [16, 19] or others [21]. Indeed, n-3 PUFA can regulate oxidative stress. Camuesco et al. found that olive oil enriched with fish oil decreased oxidative activity by restoring glutathione concentration and reducing iNOS expression in the colon of rats [21]. Dietary n-3 PUFA exerted anti-inflammatory properties. Indeed, n-3 diet decreased colon COX-2 and colon LTB4 production (Figure 3). This result is in accordance with our previous studies showing an inhibitory effect of nutritional intervention with n-3 PUFA on COX-2 and LTB4 [16, 19]. Similarly, it has been shown that antagonizing arachidonic acid-derived eicosanoids reduced inflammation and colitis severity in mice [28]. In addition, alteration of eicosanoids is one of the PUFA main mechanisms [29]. In the present study, n-3 diet also downregulated colon proinflammatory cytokines such as IL-6 (Figure 2(c)) and tend to decrease TNFα production.

In the present study, IL-1A gene expression was upregulated by n-3 diet (Table 2). This result is in accordance with an *in vitro* study showing that EPA treatment increased IL-1A secretion in human keratinocytes [30]. In our study, we observed a significant decreased IL-6 production (Figure 2(c)) while IL-6 gene expression did not differ (Table 2). The discrepancy between gene expression and

Table 2: Inflammation pathway PCR array in rats receiving diets varying in their unsaturated fatty acid composition followed by TNBS-induced colitis. Colon RNA from rats receiving diets varying in their unsaturated fatty acid composition before the onset of TNBS-induced colitis. The results were compared to rats receiving TNBS with a control diet. Data in bold are significantly different from TNBS rats.

	n-3 diet		n-6 diet		n-9 diet	
	Fold regulation	*P* value	Fold regulation	*P* value	Fold regulation	*P* value
Il1a	**2.29**	**0.044**	1.33	0.224	1.50	0.157
Il1b	1.60	0.125	1.09	0.776	−1.03	0.879
Il12a	−1.64	0.113	−1.12	0.532	−1.41	0.226
Il6	1.54	0.363	−2.76	0.219	−1.10	0.656
TNF	1.40	0.138	1.32	0.260	1.24	0.393
Ifng	1.06	0.417	2.30	0.124	1.78	0.183
Il10	1.49	0.378	−1.36	0.793	1.16	0.776
Il1r1	1.39	0.205	1.26	0.446	1.11	0.864
Hmgb1	1.09	0.492	**1.52**	**0.042**	1.02	0.851
Map2k3	**1.65**	**0.021**	1.36	0.117	1.2	0.278
Il2	−1.27	0.904	4.23	0.258	3.13	0.275
Clec4e	1.46	0.372	−2.13	0.184	−1.37	0.724
Lta	−1.04	0.572	−2.44	0.351	−1.09	0.952
Cd86	−1.11	0.569	−1.12	0.778	−1.52	0.163
Fos	−1.05	0.345	−1.3	0.379	−1.32	0.214
Irf1	−1.32	0.451	1.08	0.988	1.02	0.693
Jun	−1.37	0.340	1.09	0.762	−1.34	0.158
Tlr1	1.23	0.419	−1.05	0.803	1.06	0.654
Tlr2	**1.73**	**0.013**	1.18	0.320	1.16	0.371
Tlr3	−1.38	0.320	1.25	0.659	1.11	0.846
Tlr4	1.49	0.140	1.34	0.251	**2.01**	**0.005**
Tlr5	−1.09	0.805	1.18	0.815	1.49	0.758
Tlr6	1.26	0.381	1.02	0.855	1.05	0.960
Tlr7	−1.37	0.397	−1.44	0.207	−1.29	0.299
Tlr9	−1.24	0.802	−1.18	0.877	−1.47	0.285

protein concentration is a frequent finding in the literature. In a previous study, we observed that TNBS administration led to a 60% increase of TNFα production, while a 12-fold increase of gene expression was observed [16]. Studies that have tested correlations between gene expression and protein levels have found that mRNA and protein abundances are differentially expressed, suggesting a frequent posttranscriptional regulation of gene expression [31].

Dietary n-3 PUFA increased TLR2 gene compared to control diet while n-9 diet increased TLR4 gene (Table 2). In the literature, the inhibitory effect of n-3 PUFA on TLR2 is controversial. TLR2 protein expression was downregulated by EPA in mouse adipose stem cells [32] while a study investigating the effect of a range of saturated and unsaturated fats on TLR2 and TLR4 activation found no effect [33]. The investigators of this study did not find any effect on DHA, EPA, or oleic acid to activate TLR2 and TLR4 in HEK-Blue cells [33]. Nevertheless, these fatty acids were able to downregulate cytokine production such as TNFα, IL-6, and MCP-1 secretion in human adipose tissue and adipocyte cultures [33]. We studied dietary effects on TLR expression but we did not explore their effects on the intestinal microbiota. It has been demonstrated that fish oil is able to attenuate n-6 PUFA-induced dysbiosis in a colitis model [34].

Dietary n-6 increased gene expression of high-mobility group box 1 (HMGB1, Table 2). An increased of colon HMGB1 by dietary n-6 PUFA was observed in rats with colon cancer [35]. HMGB1 can activate multiple signaling pathways such as TLR but we did not observe any increase in TLR signaling by n-6 diet. Other signaling pathways such as receptor for advanced glycation end products (RAGE) signaling may be involved [36]. Indeed, increased RAGE via dietary n-6 has been reported in experimental colon cancer models [37].

Except for il-1a, MAP2k3, and TLR genes, we observed only modest effect of n-3 diet in inflammatory gene expression. Contrary to other studies, we aimed to evaluate a dietary effect on n-3 PUFA before inflammation genesis while numerous studies are interested in a pharmacological effect of n-3 PUFA in a curative manner [21, 38]. Numerous studies have used long chain n-3 PUFA [39] while the experimental diets used in the present study did not contain any long chain PUFA; these diets cannot directly reproduce a typical omnivore human diet. Route of administration is also a crucial point, and we used diets varying in their unsaturated fatty acid composition while n-3 PUFA are often administered by

gavage. These experimental design discrepancies may explain our effects on inflammatory gene expression.

Fatty acids are endogenous ligands for HNF-4α [40], and the role of HNF-4α in the intestinal inflammatory homeostasis has been demonstrated in mice with the intestinal epithelial deleted HNF-4α [41]. We hypothesized that dietary PUFA can regulate HNF-4 but we did not observe any modifications of colon HNF-4α expression among groups. Similarly, nuclear receptor PPARγ can be activated by PUFA and is a regulator of intestinal inflammation [42, 43], but its expression is not different among groups.

Dietary PUFA did not affect barrier function in our study. We investigated tight junction proteins, MUC2 and TFF3 mRNA levels, and we did not find any significant effect among groups (Figure 4). Some studies found a protective effect of n-3 PUFA on barrier function. Hudert et al. have used transgenic mice carrying the *C. elegans fat-1* gene encoding an n-3 fatty acid desaturase that converts n-6 to n-3 fatty acids and they induced DSS colitis in these mice [44]. They found that *fat-1* mice were protected from colitis induction compared to wild-type mice with decreased inflammatory markers [44]. They also found that fat-1 mice exhibited an increased production of protective markers such as TFF3 [44]. Fish oil supplementation in rats with TNBS-induced colitis also increased the number of goblet cell with mature mucin granules [38]. Nevertheless, our experimental design is different from these studies. Indeed, we investigated the effect of PUFA at a dietary dose while the previous studies investigated PUFA as immunonutrients.

In the present study, n-3 diet group which showed n-3/n-6 ratio equals to 1 attenuated inflammatory markers in the colon. This preventive approach has been already tested in small clinical trials. In a Japanese study, the efficacy of n-3 diet therapy in IBD patients has been already evaluated [25]. The authors of this study combined a double nutritional approach to achieve a n-3/n-6 ratio of 1 for their patients by dietary advice and nutritional supplementation [25]. Their patients were prohibited from consuming the main source of n-6 PUFA consumption such as vegetable oils or dressings. They also provided a n-3 PUFA food exchange table to privilege and n-3 supplementation [25]. The authors of this study found a higher n-3/n-6 ratio in the remission group [25]. In a Norwegian study, they evaluated the effect of 600 g of salmon consumption per week for 8 weeks in 12 active UC patients and they found decreased clinical inflammatory index [45]. A proof of concept study is now required to evaluate n-3 PUFA in a preventive manner. As we cannot directly target IBD physiopathology with a nutritional therapy before the IBD diagnosis, we may first evaluate n-3 therapy in CD postoperative patients. Indeed, postoperative phase is considered as a perfect window to evaluate predisposing factors to IBD recurrence.

Similarly, in a recent epidemiological study, women with a prudent diet (characterized by greater intake of fruits, vegetables, and fish) had a lower CD risk [15]. In addition, greater intake of fish (P trend = 0.01) has been specifically associated with lower risk of CD [15].

In conclusion, prudent diet with a high n-3/n-6 ratio may contribute to partially limit colitis genesis. Further research will be mandatory to determine mechanisms underlying dietary effects to better define dietetic advice with a scientific rationale.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Hindawi
Mediators of Inflammation
Volume 2018, Article ID 3048532, 12 pages
https://doi.org/10.1155/2018/3048532

Research Article

Formononetin Administration Ameliorates Dextran Sulfate Sodium-Induced Acute Colitis by Inhibiting NLRP3 Inflammasome Signaling Pathway

Dacheng Wu,[1,2] Keyan Wu,[1,2] Qingtian Zhu,[2] Weiming Xiao,[1,2] Qing Shan,[2] Zhigang Yan,[1,2] Jian Wu,[1,2] Bin Deng,[1,2] Yan Xue,[1,2] Weijuan Gong,[1,2,3] Guotao Lu,[1,2] and Yanbing Ding[1,2]

[1]*Department of Gastroenterology, Affiliated Hospital of Yangzhou University, Yangzhou University, Yangzhou, China*
[2]*Laboratory of Gastroenterology, Affiliated Hospital of Yangzhou University, Yangzhou University, Yangzhou, China*
[3]*Department of Immunology, School of Medicine, Yangzhou University, Yangzhou, China*

Correspondence should be addressed to Guotao Lu; gtlu@yzu.edu.cn and Yanbing Ding; ybding@yzu.edu.cn

Received 12 July 2017; Revised 29 September 2017; Accepted 22 October 2017; Published 8 January 2018

Academic Editor: Sung-Ling Yeh

Formononetin is a kind of isoflavone compound and has been reported to possess anti-inflammatory properties. In this present study, we aimed to explore the protective effects of formononetin on dextran sulfate sodium- (DSS-) induced acute colitis. By intraperitoneal injection of formononetin in mice, the disease severity of colitis was attenuated in a dose-dependent manner, mainly manifesting as relieved clinical symptoms of colitis, mitigated colonic epithelial cell injury, and upregulations of colonic tight junction proteins levels (ZO-1, claudin-1, and occludin). Meanwhile, our study found that formononetin significantly prevented acute injury of colonic cells induced by TNF-α in vitro, specifically manifesting as the increased expressions of colonic tight junction proteins (ZO-1, claudin-1, and occludin). In addition, the result showed that formononetin could reduce the NLRP3 pathway protein levels (NLRP3, ASC, IL-1β) in vivo and vitro, and MCC950, the NLRP3 specific inhibitor, could alleviate the DSS-induced mice acute colitis. Furthermore, in the foundation of administrating MCC950 to inhibit activation of NLRP3 inflammasome, we failed to observe the protective effects of formononetin on acute colitis in mice. Collectively, our study for the first time confirmed the protective effects of formononetin on DSS-induced acute colitis via inhibiting the NLRP3 inflammasome pathway activation.

1. Introduction

Inflammatory bowel disease (IBD) is a kind of noninfectious inflammatory disease, mainly consisting of ulcerative colitis (UC) and Crohn's disease (CD). Epidemiological data showed that IBD has become a global problem, the incidence of IBD in high-risk areas remained stable in these years, while in low-risk areas such as southern Europe, Asia, and most developing countries, the incidence is increasing [1].

The specific mechanism of IBD is still unclear, while there is no doubt that intestinal mucosal immune dysfunction plays an important role in the pathogenesis of IBD [2, 3]. Immunosuppressors, such as 6-mercaptopurine, azathioprine, methotrexate, and cyclosporin A, which could block the abnormal immune activation, are often recommended to treat IBD patients in the clinical setting [4, 5]. Nevertheless, this kind of immunosuppressors could affect the balance of normal immune system and even cause severe adverse effects, including myelosuppression, infection, and liver damage. Hence, seeking for novel immunomodulatory drugs that could effectively alleviate mucosal inflammation with minimum or no side effects seems to be vital in the clinical prevention and treatment of IBD.

Formononetin is a kind of isoflavone compound and widely exists in the natural plants. It is one of the major biologically active compounds in a variety of Chinese medicinal herbs, such as astragalus, which has been traditionally used for treatment of diabetes over 2000 years in China. Current

research evidences indicated that formononetin played several different effects, such as, anti-inflammatory [6, 7], antioxidative [8], antitumor [9], and promoting apoptosis [10]. Lima Cavendish et al. confirmed that formononetin has an anti-inflammatory effect on rats with peritonitis [6] and could protect mice from lipopolysaccharide-induced acute lung injury [7]. However, the specific effects of formononetin on acute colitis have not been well defined and documented.

Inflammasome, a key component in host inflammation regulation, was put forward by Tschopp research team for the first time in 2002 [11]. The inflammasome is a cytosolic multimeric protein complex composed of nucleotide-binding domain and leucine-rich repeat-containing proteins (NLRs) or AIM2, adaptor protein apoptosis-associated speck-like protein containing CARD (ASC), and cysteine-aspartic acid protease- (caspase-) 1. There has been found four kinds of inflammasome: NLRP1, NLRP3, NLRC4, and AIM2 [12–14], among which NLRP3 inflammasome is the most thoroughly studied. Previous study had shown that NLRP3 inflammasome may play a critical role in the pathogenesis of IBD. Clinical research indicated that the susceptibility to CD may be related to NLRP3 gene polymorphism [15], in a study of Chinese population (232 CD cases, 56 UC cases, and 247 healthy control cases), the results indicated that NLRP3 gene expression was not significantly different between IBD group and control group; There were no significant variations in NLRP3 gene expression in the CD subgroup analysis; While in the UC subgroup analysis, the two mutations of SNPS-rs10754558 and rsl0925019 on NLRP3 gene were associated with the incidence of UC, demonstrating that NLRP3 gene played an important role in the pathogenesis of UC in Chinese patients [16]. In an acute colitis model in mice induced by dextran sulfate sodium (DSS), NLRP3 gene knockout or medical inhibition of the NLRP3 inflammasome activation both exerted protective effects on mice [17, 18]. However, whether formononetin had a regulatory effect on NLRP3 inflammasome pathway remained still unclear. In this study, we aimed to explore the protective effects of formononetin on DSS-induced acute colitis and investigate the potential underlying mechanism.

2. Materials and Methods

2.1. Mice and Reagents. Eight- to ten-week-old male C57BL/6 mice were purchased from Comparative Medicine Centre of Yangzhou University (Yangzhou, Jiangsu Province, China). All mice were housed in a specific pathogen-free (SPF) standard room under 12/12 h light-dark cycle at 26°C, relative humidity 45%, given water ad libitum, fed standard laboratory chow, and were allowed to acclimatize to the new environment for at least 1 week. All methods were carried out in accordance with the principles of laboratory animal care (NIH publication Number 85Y23, revised 1996), and all experimental protocols were approved by the experimental animal ethics committee of Affiliated Hospital of Yangzhou University.

Formononetin (F141481) was purchased from Aladdin Bio-Chem Technology company (Aladdin, Shanghai, China), and DSS was purchased from MP Biomedical company (MP Biomedical, Santa Ana, CA, USA). The primary antibodies: myeloperoxidase (MPO, ab9535), cluster of differentiation 68 (CD68, ab955), claudin-1 (ab15098), occludin (ab31721), zonula occludens-1 (ZO-1, ab59720), interleukin-1β (IL-1β, ab9722), and GAPDH (ab8245) were purchased from Abcam company (Abcam, Cambridge, UK), anti-NLRP3 (15101) antibody was purchased from CST company (Cell Signaling Technology, Boston, USA), and anti-ASC (sc-22514-R) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Goat anti-rabbit and rabbit anti-mouse secondary antibodies were purchased from Abcam company (Abcam, Cambridge, UK). Recombinant human tumor necrosis factor-alpha (TNF-α) was purchased from Cayman company (Cayman, Michigan, USA). MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay was purchased from Promega company (Promega, Madison, WI, USA).

2.2. Experimental Design and Procedures In Vivo. Experimental mice were randomly assigned to five groups ($n = 8$ per group): vehicle, DSS, low-dose formononetin (25 mg/kg, L-dose), middle-dose formononetin (50 mg/kg, M-dose), and high-dose formononetin (100 mg/kg, H-dose) group. Except the vehicle group, mice in the other four groups were given DSS solution (2.5%, dissolved in distilled water) ad libitum to induce the acute colitis model; meanwhile, the vehicle group was administered distilled water equivalently. Mice in the drug intervention groups (L-dose, M-dose, and H-dose group) were daily injected with formononetin (dissolved in 5% DMSO) intraperitoneally, while the vehicle group and DSS model group were administered 5% DMSO in the same way.

To inhibit the NLRP3 inflammasome in vivo, mice were treated with MCC950 (50 mg/kg, $n = 8$) intraperitoneally (the mice in the control group were treated with PBS, $n = 8$) every other day from one day before to day 7 of DSS administration [18]. Meanwhile, high-dose formononetin (100 mg/kg) was treated daily on day 1–8 of DSS administration ($n = 8$) to identify the underlying molecular mechanisms of formononetin on acute colitis.

The severity of acute colitis was judged by measuring body weight, stool consistency, and the occurrence of gross blood in the stool in mice every day, and the disease activity index (DAI) score was also used to assess the disease severity of colitis. DAI score was calculated by grading on a scale of 0 to 4 using the following parameters [19]: loss of body weight (0, normal; 1, 0–5%; 2, 5–10%; 3, 10–15%; and 4, >15%), stool consistency (0, normal; 2, loose stool; and 4, watery diarrhea), and the occurrence of gross blood in the stool (0, negative; 2, slight bleeding; and 4, gross bleeding). The combined DAI was scored by two independent investigators.

Nine days after DSS administration, animals were anaesthetized with the intraperitoneal administration of sodium pentobarbital (50 mg/kg) then sacrificed; their distal colonic tissues were dissected immediately. A portion of the colonic tissues was fixed for histological analysis and the rest were stored at −80°C for further investigation. Blood samples were obtained from the tail veins and stored at −80°C for analysis.

2.3. Histological Examination. Distal colonic tissues were fixed in 4% paraformaldehyde (dissolved in PBS) and embedded in paraffin and stained with hematoxylin and eosin. Two investigators who were blind to the experimental treatment scored the degree of colonic injury by light microscopy. The severity of colonic injury was evaluated by the following parameters [20]: epithelial damage (0, normal; 1, minimal loss of goblet cells; 2, extensive loss of goblet cells; 3, minimal loss of crypts and extensive loss of goblet cells; and 4, extensive loss of crypts); and infiltration (0, normal; 1, infiltrate around crypt bases; 2, infiltrate in muscularis mucosa; 3, extensive infiltrate in muscularis mucosa with edema; and 4, infiltration of submucosa).

2.4. Cell Culture and Treatment In Vitro. Human colon carcinoma cell line HCT-116 was purchased from Shanghai cell bank of Chinese Academy of Sciences (Shanghai, China) and used for in vitro experiment. HCT-116 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum at 37°C with a humidified atmosphere containing 5% CO_2. TNF-α (100 ng/ml) was used to establish a cell injury model [21], and the HCT-116 cells were incubated with different concentrations of formononetin (25 μM, 50 μM) or 0.1% DMSO (vehicle) to verify the protective effect of formononetin. Twelve hours after formononetin intervention, cellular protein was extracted and stored for further experiments.

Cell viability was evaluated by using the MTS assay. After 1 h of adhesion of HCT-116 cells, different doses of formononetin (dissolved in DMSO) or TNF-α (dissolved in PBS) were plated in 96-well plates and wells containing only HCT-116 (0.1% DMSO or PBS in complete medium) were used as control groups. Twelve hours later, cells were treated with MTS in accordance with the manufacturer's instructions.

2.5. Immunofluorescence (IF) and Western Blot (WB). IF and WB analyses were carried out as previously described [22, 23]. Briefly, for IF analysis, slides were incubated overnight at 4°C in a humid chamber with an antibody against MPO (1 : 500 dilution), CD68 (1 : 200 dilution), claudin-1 (1 : 200 dilution), occludin (1 : 200 dilution), ZO-1 (1 : 200 dilution), and NLRP3 (1 : 200 dilution) and then incubated by biotinylated secondary antibody (1 : 500 dilution) for 60 minutes. For WB analysis, the polyvinylidene difluoride (PVDF) membranes were blocked by 5% (*w*/*v*) bovine serum albumin in Tris-buffered saline/0.05% tween-20 (TBST) at room temperature for 2 h in a covered container and incubated overnight at 4°C with primary antibodies against NLRP3 (1 : 1000 dilution), ASC (1 : 1000 dilution), IL-1β (1 : 1000 dilution), claudin-1 (1 : 1000 dilution), occludin (1 : 1000 dilution), ZO-1 (1 : 1000 dilution), and GAPDH (1 : 2000 dilution) in blocking buffer. On the next day, membranes were washed with TBST (3 ∗ 10 min) and incubated with a secondary goat anti-mouse or goat anti-rabbit IgG horseradish peroxidase (HRP) antibody (1 : 10,000 dilution) diluted in 5% (*w*/*v*) dry nonfat milk in TBST for 1 h at room temperature. Finally, membranes were washed with TBST (3 ∗ 10 minutes), developed by using the ECL detection system (Santa Cruz Biotechnology), quickly dried, and exposed to ECL film.

2.6. Enzyme-Linked Immunosorbent Assay (ELISA). ELISA analyses were carried out as previously described [24]. Briefly, the colon tissues were homogenated in PBS and then carried out by centrifugation (12000 rpm, 4°C, 30 min) to obtain supernatant. The TNF-α and IL-1β levels were measured with the commercial kits (Affymetrix ebioscience, Santiago, USA).

2.7. Statistical Analysis. Statistical analysis was performed by SPSS 22.0 software. Results are presented as mean ± standard deviation (SD). The Kruskal–Wallis test followed by the Mann–Whitney *U* test was used to evaluate the differences in histopathological scores. Statistical analysis was performed using one-way ANOVA followed by the Student–Newman–Keuls test as a post hoc test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Formononetin Protected against DSS-Induced Acute Colitis in Mice. As expected, colonic injury, weight loss, bloody stool, and watery diarrhea were observed in mice after DSS feeding. After formononetin administration, we observed that formononetin could alleviate the clinical symptoms of acute colitis mice in a dose-dependent manner. The body weight of mice in DSS group decreased by 15%, in contrast, the body weight of mice in H-dose administration group decreased by 6.7% after nine days DSS feeding (Figure 1(a)); meanwhile, the DAI scores were 11 and 4.5 in two groups, respectively. (Figure 1(b)). Then, we further examined the degrees of colonic histopathological injury to assess the disease severity of colitis and found that the histopathological manifestations in vehicle group presented as a normal colonic appearance, while in DSS group, the histopathological characteristics were displayed as colonic epithelial cell injury and a great number of inflammatory cells infiltrating into the mucosa and the submucosa of colon; in contrast, H-dose formononetin (100 mg/kg) markedly alleviated the histological features of colonic mucosa damage, characterized as lower degree of epithelial cell injury and inflammatory infiltration. Additionally, the protective effects of formononetin on acute colitis appeared to be dose-dependent and we failed to confirm the protective effects of formononetin in L-dose group (25 mg/kg) (Figures 1(e) and 1(f)). Furthermore, as for the changes in the average length for colon after formononetin treatment (Figures 1(c) and 1(d)), the changing trend was consistent with the results of histopathological manifestations.

3.2. Formononetin Reduced Colonic Inflammation in Mice. Neutrophil and macrophage infiltrations could be viewed as evaluative parameters for disease severity in acute colitis mice. As Figure 2(a) showed, the MPO and CD68 staining results were positive in mice with colitis, indicating a great number of neutrophils and macrophage infiltrating into the colonic tissues. After formononetin administration, there were fewer infiltrating neutrophils and macrophages in injured colonic tissue. In addition, the level of inflammatory

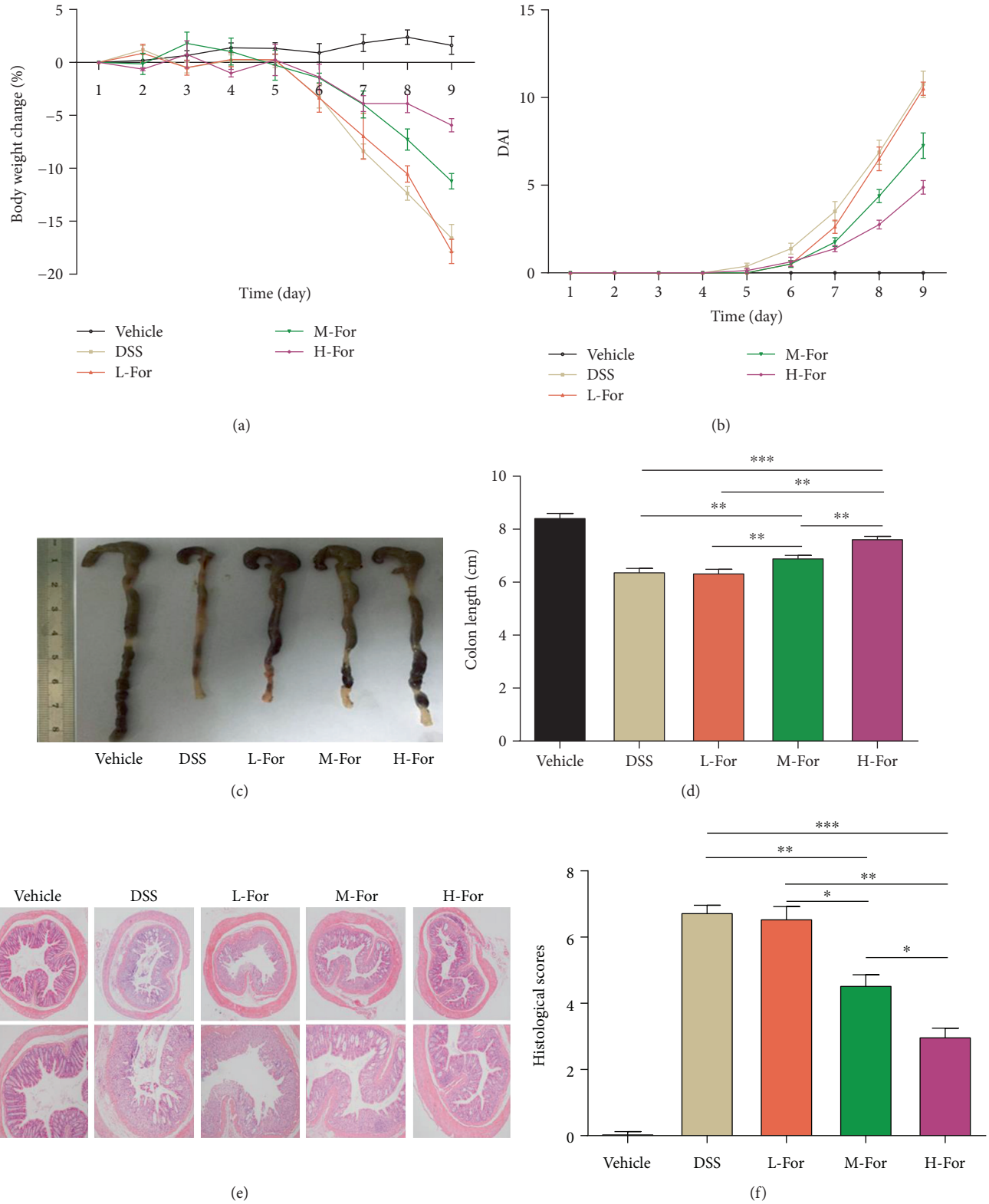

Figure 1: Formononetin (For) attenuates dextran sulfate sodium- (DSS-) induced acute colitis in mice. (a) Body weights of mice and (b) disease activity index (DAI) during the disease process, (c) morphological changes in the mice colons, (d) variations of colon length of mice, (e) representative HE staining, and (f) histological scores of colonic tissue. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

cytokines in the colon of mice was detected by ELISA method to assess the severity of acute colitis; as expected, the levels of TNF-α and IL-1β were significantly decreased after formononetin treatment (Figure 2(b)). The results of inflammatory infiltration and inflammatory cytokine expression turned out to be consistent with the pathological results.

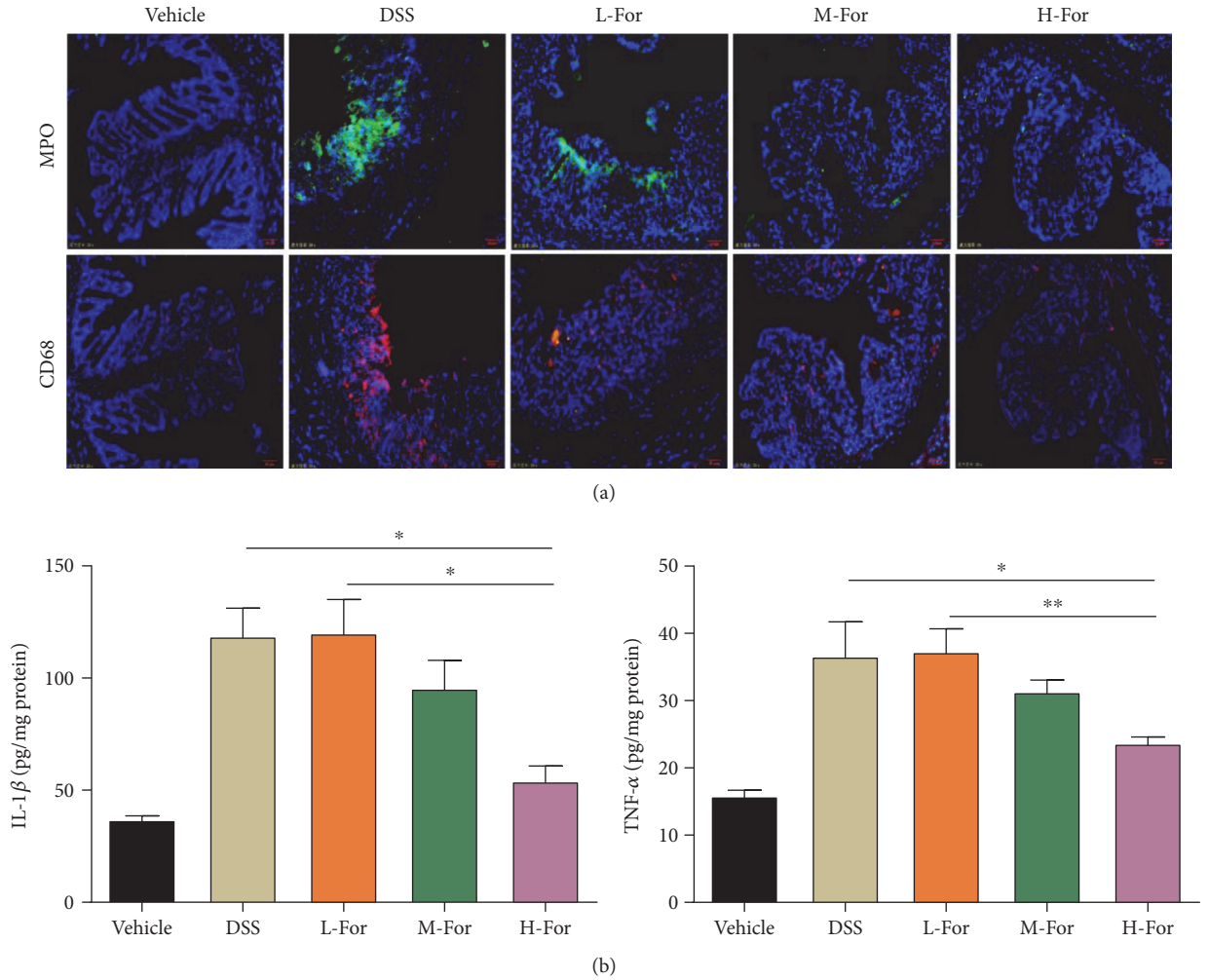

Figure 2: Effect of For on the colonic inflammation in mice. (a) Representative immunofluorescence images for MPO (Green) and CD68 (Red) in the colonic tissue. (b) The level of inflammatory cytokines in the colonic tissue. $^{*}p < 0.05$ and $^{**}p < 0.01$.

3.3. Formononetin Relieved DSS-Induced Colonic Epithelial Tight Junction Disruption in Mice. Tight junction (TJ) is an important structure foundation that maintains the mechanical barrier functions between intestinal mucosa epithelial cells and plays a critical role in the intestinal epithelial barrier integrity [25, 26]. We observed that the expressions of tight junction proteins, such as claudin-1, occludin, and ZO-1 remarkably reduced in the colonic tissues of DSS-induced colitis mice by adopting WB and immunofluorescence methods, and as we expected, the reduced expressions of tight junction proteins were in positive correlation with the integrity of intestinal epithelial barrier structure. Unsurprisingly, after formononetin administration, the reduced expressions of claudin-1, occludin, and ZO-1 proteins were counteracted (Figures 3(a)–3(c)), which provided another powerful evidence for the protective effects of formononetin on mice colitis.

3.4. Formononetin Inhibited NLRP3 Pathway in Mice Colonic Epithelial Cells. Previous studies suggested that NLRP3 inflammasome pathway played an important role in colonic tissue injury in acute colitis in mice [17, 18, 27]. In this study, by using immunohistochemical staining, we firstly detected that the expression of NLRP3 in colonic tissue was elevated in DSS-induced colitis and formononetin reduced the activation of NLRP3 pathway (Figure 4(a)). Next, we observed that the expressions of NLRP3, IL-1β, and ASC proteins in colonic tissue were increased, which suggested that the NLRP3 inflammasome pathway was activated in the pathogenesis of colitis in mice. As we expected, formononetin administration reduced the expressions of NLRP3, IL-1β, and ASC significantly (Figures 4(b) and 4(c)), indicating that formononetin could exert the protective effect on colonic tissue injury by inhibiting the activation of NLRP3 pathway.

3.5. Formononetin Protected against Colonic Epithelial Tight Junction Disruption and Inhibited NLRP3 Pathway In Vitro. In order to further explicit the protective effects of formononetin on acute colitis, the colonic cell line HCT-116 was employed to carry out the in vitro experiment. Firstly, we tested the cell viability of HCT-116 cells with TNF-α and

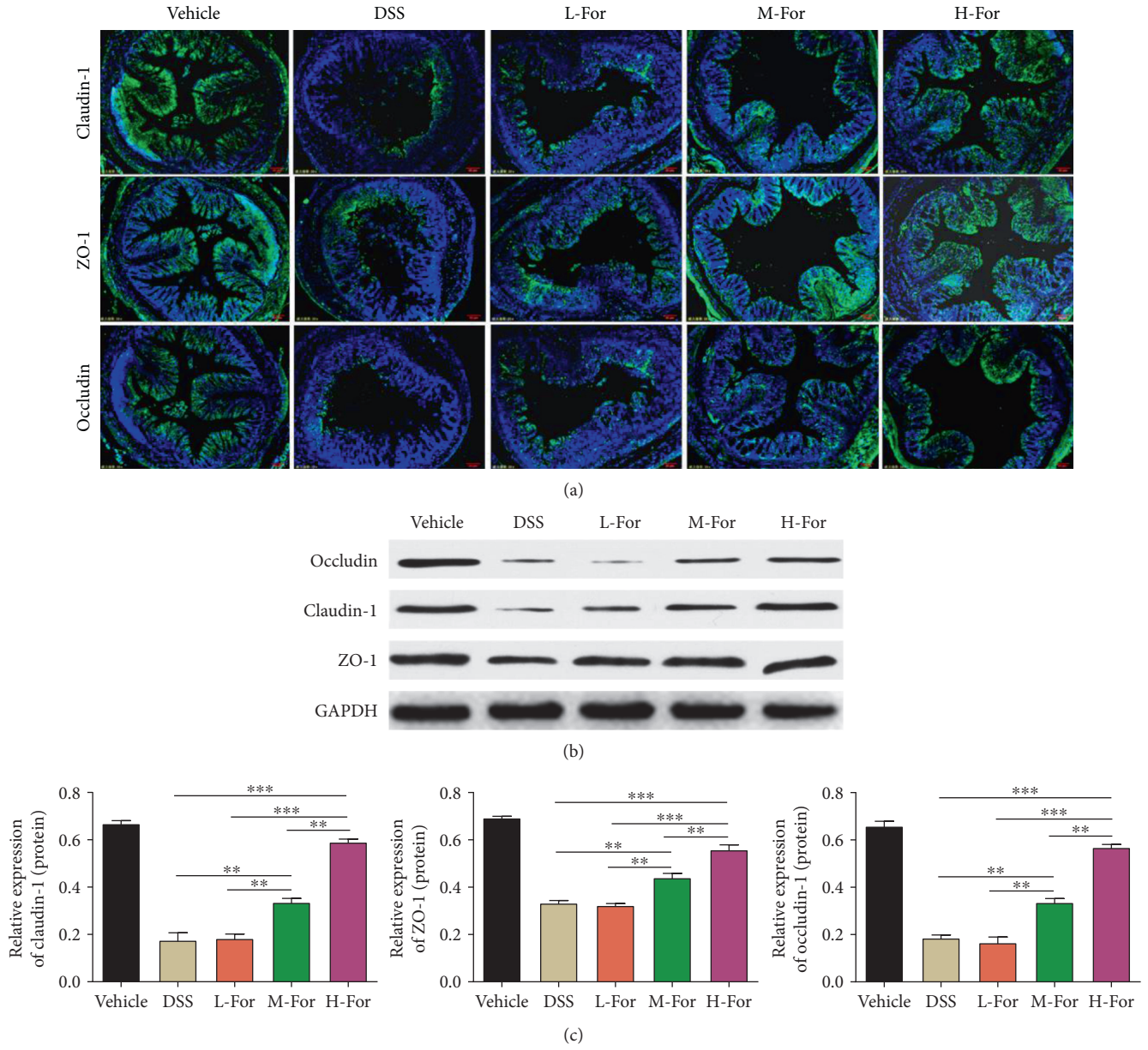

Figure 3: For relieved DSS-induced colonic epithelial tight junction destruction in mice. (a) Representative immunofluorescence images for claudin-1 (Green), occludin (Green), and ZO-1 (Green) in the colonic tissue. (b, c) Protein levels of claudin-1, occludin, and ZO-1 in the colon tissues were analyzed by western blotting. $^{**}p < 0.01$, and $^{***}p < 0.001$.

formononetin treatment (Supplementary Figure S1, A-B), and the results showed that formononetin exerted no toxicity to HCT-116 while TNF-α dose-dependently reduced the cell viability of HCT-116 cells. According to previous literature [21], TNF-α (100 ng/ml) was used to establish the colonic cell injury model in vitro experiment. Next, after treatment with TNF-α and formononetin (25 μM, 50 μM), the expressions of tight junction proteins (claudin-1, occludin, and ZO-1) and NLRP3 inflammasome pathway (NLRP3, IL-1β, and ASC) were detected by WB methods. Unsurprisingly, formononetin protected the colonic cells from injury by the increased expressions of claudin-1, occludin, and ZO-1 (Figures 5(a) and 5(b)) together with the decreased expressions of NLRP3, IL-1β, and ASC (Figures 5(c) and 5(d)).

3.6. NLRP3 Inhibitor MCC950 Could Eliminate the Protective Effect of H-Dose Formononetin on Acute Colitis in Mice. To further identify the underlying mechanisms of formononetin on acute colitis in mice, according to the above results, MCC950, the NLRP3-specific inhibitor [28], and high-dose formononetin (100 mg/kg) were adopted to carry out the following experiment. The experimental protocol with formononetin and MCC950 in acute colitis model was shown in Figure 6(a); MCC950 (50 mg/kg) was injected intraperitoneally every other day to inhibit the NLRP3 activity of mice. Similar to the previous literature results [18], MCC950 could significantly alleviate the loss of body weight, reduce the DAI score, and relieve the pathological injury of colon in mice, while administration of H-dose formononetin and MCC950

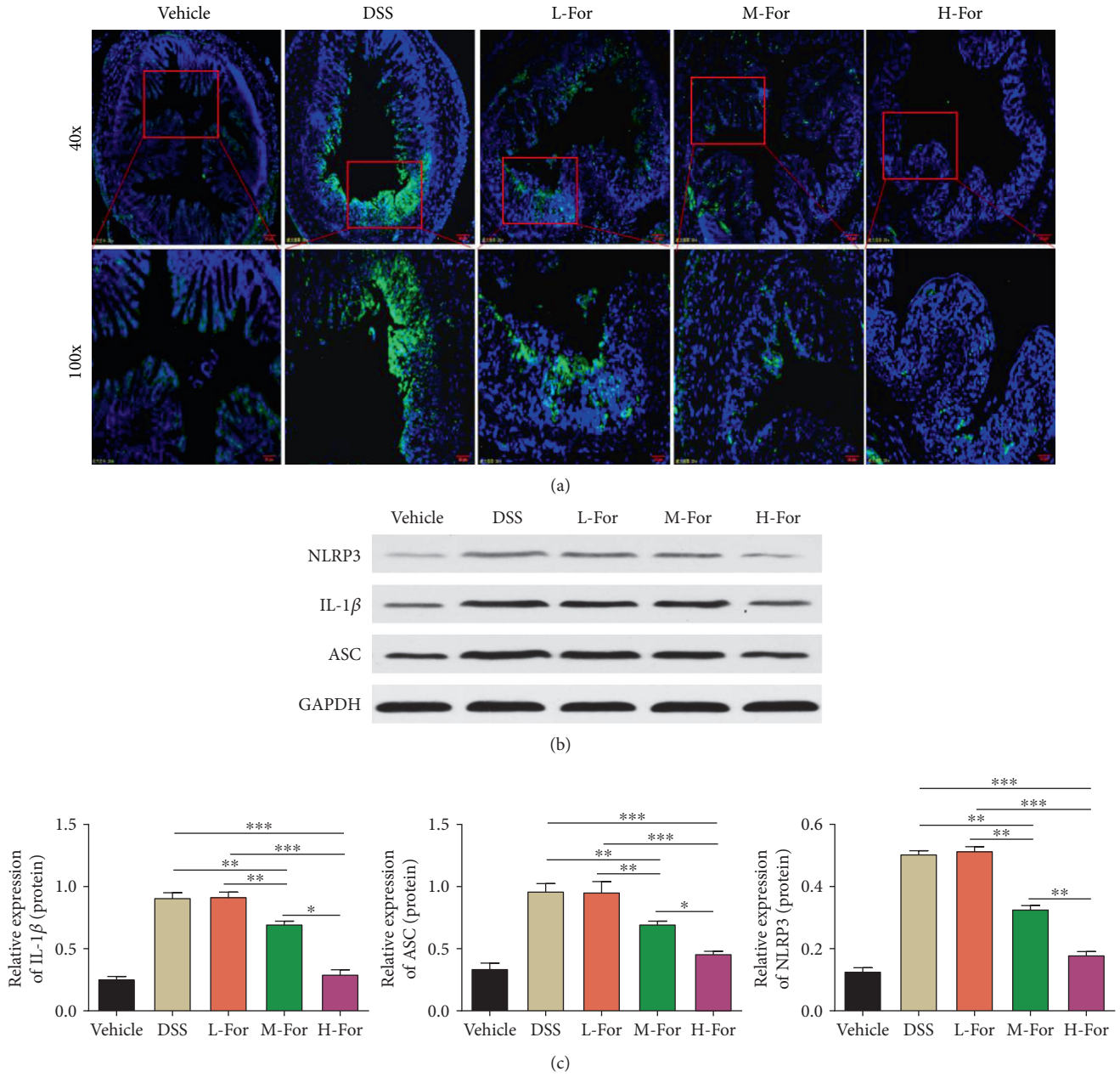

Figure 4: For inhibited NLRP3 pathway in mice colonic epithelial cells. (a) Representative immunofluorescence images for NLRP3 (Green) in the colonic tissue. (b, c) Protein levels of NLRP3, ASC, and IL-1β in the colon tissues were analyzed by western blotting. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

together failed to show synergetic effects, which meant that the protective effects of alleviating the loss of body weight, reducing the DAI score, and relieving the pathological injury of colon in mice showed no significant difference between H-dose formononetin + MCC95 group and MCC950 group (Figures 6(b)–6(g)). In addition, our results showed that the expressions of NLRP3, IL-1β, and ASC were reduced while the expressions of claudin-1, occludin, and ZO-1 were enhanced in mice colonic tissues after MCC950 administration, but the expressions of above proteins failed to show significant difference between H-dose formononetin + MCC95 group and MCC95 group (Figures 6(h) and 6(i), Supplementary Figure S3). All these findings indicated that formononetin exerted protective effects on DSS-induced acute colitis in mice via inhibiting NLRP3 inflammasome pathway.

4. Discussion

In this study, we for the first time verified that formononetin could alleviate the DSS-induced acute colitis in mice by inhibiting NLRP3 pathway. Ulcerative colitis (UC) is a kind of nonspecific inflammatory bowel disease, of which the

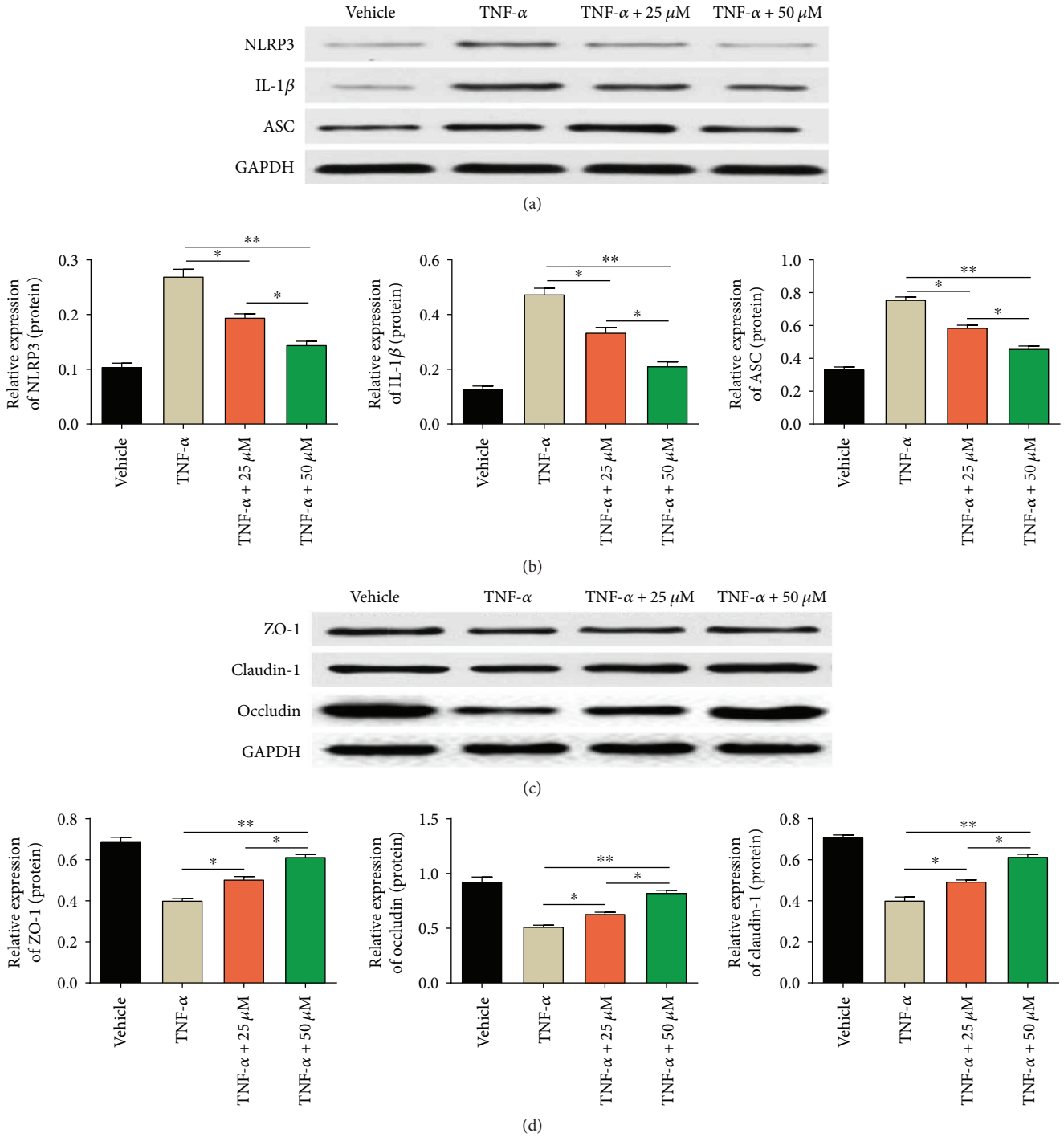

Figure 5: For protected against colonic epithelial tight junction injury and inhibited NLRP3 pathway in vitro. (a, b) Protein levels of NLRP3, ASC, and IL-1β and (c, d) claudin-1, occludin, and ZO-1 were analyzed by western blotting. $^{*}p < 0.05$ and $^{**}p < 0.01$.

main pathological features present as the inflammatory responses and ulceration formations in the mucosa and submucosa of the rectum or colon. Patients usually present with abdominal pain, bloody stool, and diarrhea as the main symptoms, in whom the active and remission stages often emerged in turn and eventually form a recurrent chronic disease course. DSS was used for the first time to establish the classical acute colitis model in rodents by Ohkusa in 1985 [29]. This novel DSS-induced colitis model was easy to prepare, economical, and could replicate the pathogenesis of human colitis suitably and accurately, so DSS-induced model was one of the most commonly used animal model for colitis studies [30, 31]. In this study, we adopted 2.5% DSS to induce a stable acute colitis mice model.

This present study focused on the protective effects of formononetin on DSS-induced colitis in mice. Previous studies have shown that 20 mg/kg formononetin could effectively protect against lipopolysaccharide-induced acute lung injury

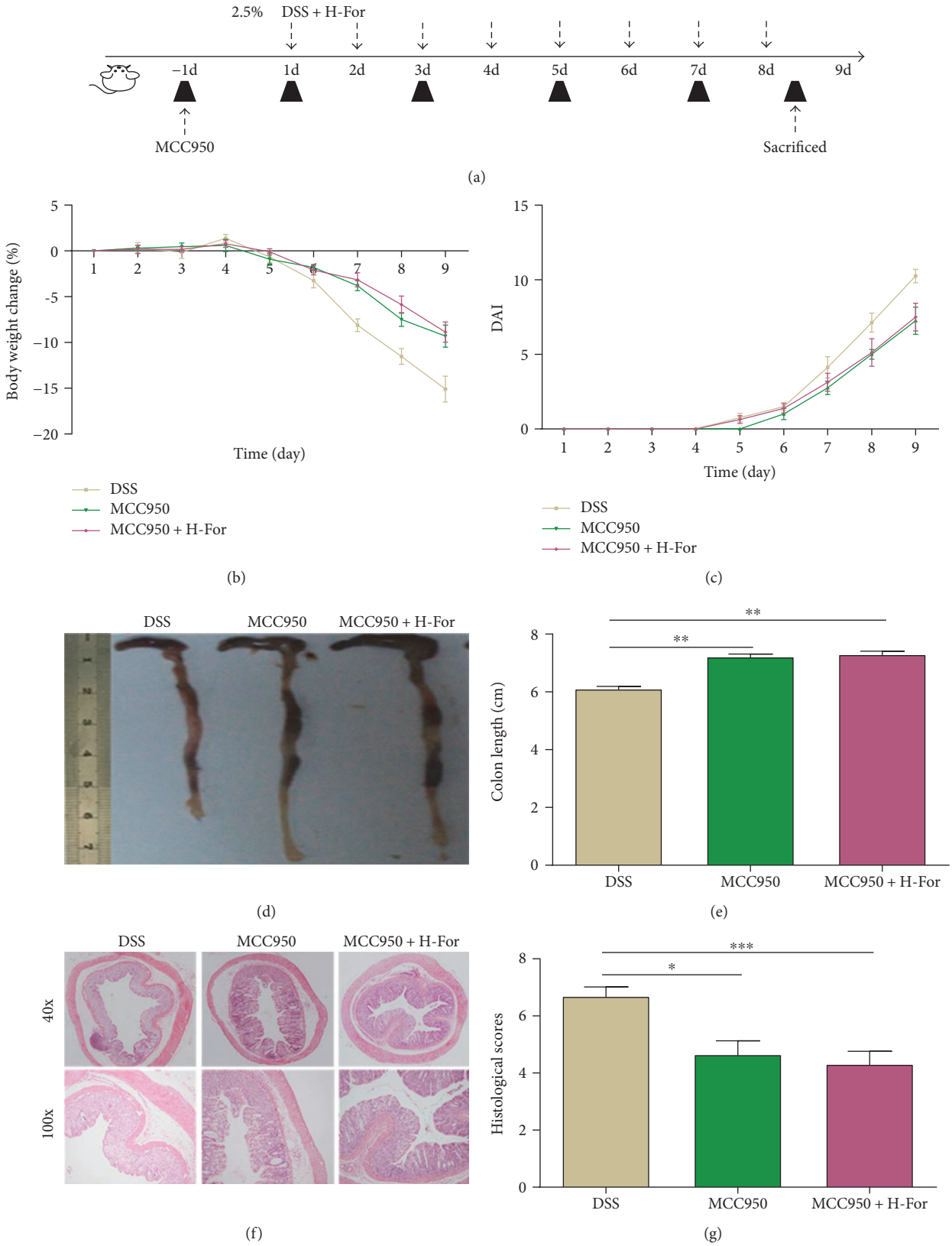

Figure 6: Continued.

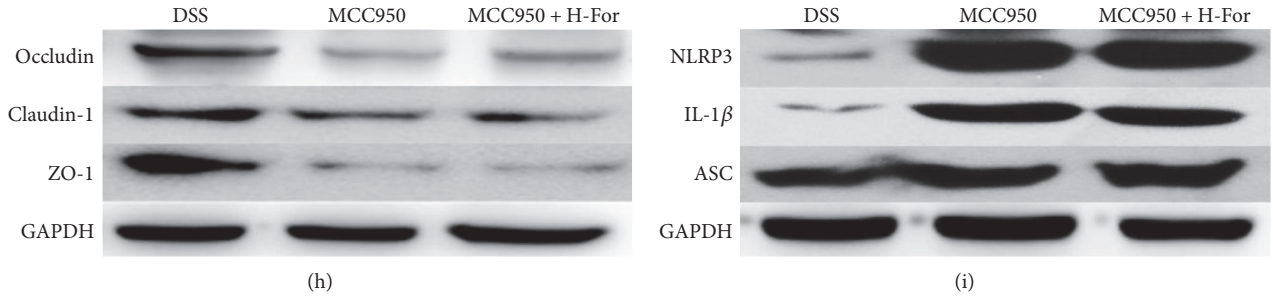

Figure 6: NLRP3 inhibitor MCC950 eliminated the protective effect of H-For on acute colitis in mice. (a) The experimental protocol with For and MCC950 in acute colitis model. (b) Body weights of mice and (c) disease activity index (DAI) during the disease process. (d) Morphological changes in the mice colons, (e) variations of colon length of mice, (f) representative HE staining, and (g) histological scores of colonic tissue. (h) Protein levels of claudin-1, occludin, and ZO-1 and (i) NLRP3, ASC, and IL-1β were analyzed by western blotting. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

[7] and ameliorate blood glucose levels in alloxan-induced hyperglycemia in mice [32]; Jin et al. revealed that formononetin (50 mg/kg or 100 mg/kg) dose-dependently mitigated the acetaminophen-induced hepatotoxicity in mice [33]. Therefore, we selected gradient doses of formononetin (25 mg/kg, 50 mg/kg, and 100 mg/kg) for animal experiments. Fortunately, the results showed that formononetin alleviated the inflammatory responses in mice colitis in a dose-dependent manner, which was confirmed by histopathological manifestation, the length of colon, and the clinical symptoms of mice. In addition, formononetin administration reduced the infiltration of neutrophils and macrophages into the colonic tissues. Collectively, these results suggested that formononetin could mitigate the inflammatory responses of mice colitis effectively.

Tight junction destruction plays a key role in the development and progression of IBD [25, 26]. In previous animal experiment [34], it has been found that the congenital epithelial cell tight junction protein knockout mice appeared with intestinal pathological changes which was similar with that of IBD after birth, implying that claudins were involved in the pathogenesis of IBD. Moreover, by adopting immunofluorescence and western blot, it could be observed that the expressions of claudin-1, occludin, and ZO-1 were downregulated in DSS-induced acute colitis model in rats [35, 36]. A large number of studies have shown that restoring and maintaining intestinal mucosal barrier function were beneficial to improve the defensive function of intestinal mucosa, promote disease remission, and reduce relapse times of IBD [37, 38]. Our result showed that the expressions of epithelial cell tight junction proteins claudin-1, occludin, and ZO-1 were reduced remarkably in DSS-induced colitis in vivo and in TNF-α-induced cell injury model in vitro, while after the administration of formononetin, we found that formononetin increased the expressions of claudin-1, occludin, and ZO-1 significantly, suggesting that formononetin could protect the colonic mucosal integrity and maintain the colonic epithelial barrier function of colitis.

NLRP3 presents as the most important member of pattern recognition receptor NLR family and recognizes the danger signals released by cellular pathogens or cells themselves [39], and it can combine with ASC to form a multimeric protein complex which is named NLRP3 inflammasome. Excessive activation of NLRP3 inflammasome has been proven to play a key role in a variety of inflammatory diseases, such as diabetes [40], Alzheimer's disease [41], and atherosclerosis [42]. Similarly, NLRP3 inflammasome also plays a critical role in the pathogenesis of IBD [17, 18]. Previous studies indicated that the expressions of NLRP3, ASC, and caspase-1 were remarkably elevated in colonic tissues of colitis both in patients and in animal models; meanwhile, the disease severities of colitis in NLRP3, ASC, and caspase-1 knockout mice tended to be much relieved in comparison with the wild-type mice, suggesting that NLRP3 inflammasome pathway exerted a great effect on the development and progression of colitis. More importantly, the inhibition of NLRP3 pathway could mitigate the disease severity of colitis [18]. In this study, formononetin showed great inhibitory effect on the expressions of NLRP3, ASC, and IL-1β and reduced the secretion of IL-1β significantly in colonic epithelial cells in vivo and vitro. Surprisingly, the NLRP3-specific inhibitor MCC950 could alleviate the DSS-induced acute colitis in mice; however, in the basis of administrating MCC950 to inhibit the activation of NLRP3, we failed to observe the protective effects of formononetin on acute colitis additionally. Based on the above results, our study for the first time demonstrated the inhibitory effect of NLRP3 inflammasome pathway by formononetin.

5. Conclusion

Through this study, we concluded that formononetin could protect colonic epithelial cells from injury to relieve the disease severity of colitis in mice via inhibition of NLRP3 inflammasome pathway. Formononetin may be a promising strategy for the clinical prevention and treatment of IBD in the future.

Conflicts of Interest

The authors declare that there are no competing interests.

Authors' Contributions

Dacheng Wu, Keyan Wu, and Qingtian Zhu contributed equally to this work.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (nos. 81471547 and 81671547) and the National Natural Science Foundation of Jiangsu Province (no. BK20161339).

Supplementary Materials

Figure S1: Chemical structure of formononetin. Figure S2: Cell viability was measured by MTS assay in different concentration of TNF-α (A) and formononetin (B) on HCT-166 cells. Data are shown as relative cell viability (mean % ± S.E. bar) as compared with that in control ($n = 5$). Figure S3: NLRP3 inhibitor MCC950 eliminated the protective effect of H-For on acute colitis in mice. Protein levels of (A) claudin-1, occludin, and ZO-1 and (B) NLRP3, ASC, and IL-1β were analyzed by western blotting. $^{*}p < 0.05$, $^{**}p < 0.01$. *(Supplementary Materials)*

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Hindawi
Mediators of Inflammation
Volume 2017, Article ID 3513651, 7 pages
https://doi.org/10.1155/2017/3513651

Research Article

Inhibiting Interleukin 17 Can Ameliorate the Demyelination Caused by *A. cantonensis* via iNOS Inhibition

Feng Ying,[1] Zheng Cunjing,[2] Feng Feng,[3] Wan Shuo,[4] Zeng Xin,[4] Xie Fukang,[2] and Wu Zhongdao[4]

[1]*Medical School of South China University of Technology, Guangdong 510006, China*
[2]*Histology and Embryology Department of Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou 510080, China*
[3]*The Department of Pharmacology and Toxicology, School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou 510080, China*
[4]*Parasitology Department of Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou 510080, China*

Correspondence should be addressed to Xie Fukang; frankxie2000@yahoo.com and Wu Zhongdao; wuzhd@mail.sysu.edu.cn

Received 3 August 2017; Revised 18 September 2017; Accepted 2 October 2017; Published 18 December 2017

Academic Editor: Helieh S. Oz

Angiostrongylus cantonensis (*A. cantonensis*) is an important food-borne parasitic disease. Previous study showed that *A. cantonensis* infection can cause demyelination in the central nerve system, but the mechanism of action has not been understood. To explore the mechanism and to look for effective therapeutic methods, interleukin 17A (IL-17A) and iNOS expressions were detected during *A. cantonensis* infection. In addition, IL-17A-neutralizing antibody was applied to treat *A. cantonensis*-infected mice. In our results, we found that IL-17A and iNOS RNA expressions increased gradually in the process of *A. cantonensis* infection. When infected mice were treated with IL-17A-neutralizing antibody, the pathologic changes of demyelination alleviated obviously, followed with the elevation of myelin basic protein (MBP) in the brain. In addition, the iNOS expression of the brain in infected animals also showed a decrease in astrocytes. Our study provided evidence that IL-17A may take part in the demyelination caused by *A. cantonensis* and inhibiting IL-17A expression can ameliorate the pathologic changes of demyelination. Moreover, the decreasing of iNOS expression may be the key reason for the effect of IL-17A inhibition on demyelination caused by *A. cantonensis*.

1. Introduction

Angiostrongylus cantonensis (*A. cantonensis*) is an important cause of food-borne diseases and eosinophilic encephalitis in humans [1]. *A. cantonensis* is a parasitic nematode from rats which invades the central nerve system (CNS) and causes eosinophilic encephalitis or meningoencephalitis [2]. During this process, neurons in CNS appear with obvious demyelination [3–5] (MBP is one component of myelin sheath) [6]. However, the reason for demyelination associated with *A. cantonensis* infection has not been fully known.

Cytokines of the interleukin 17 (IL-17) family are uniquely placed on the border between immune cells and tissue. As seen in psoriatic skin lesions or in joints of rheumatoid arthritis patients, high levels of IL-17 have been detected in CNS during inflammatory responses. Previous study showed that IL-17-induced Act1-mediated signaling cascades in CNS resident cells (astrocytes, oligodendrocytes, and neurons) might coordinately mediate CNS inflammation, demyelination, and neurodegeneration [7, 8]. But whether IL-17 is involved in the demyelination caused by *Angiostrongylus cantonensis* has never been studied.

Astrocytes probably represent the best-studied CNS resident cell type in the context of multiple sclerosis (MS) and EAE, which cause demyelination complications. Both human and mouse astrocytes (glial fibrillary acidic protein (GFAP) is the specific marker for astrocytes) express the IL-17RA, thereby allowing IL-17A ligation and consequently, the production of cytokines and chemokines, including IL-6, TNFα, and CCL2 [9–11]. Inducible nitric oxide (iNOS) is

involved in various physiological regulations and plays important roles in some CNS disease, such as brain ischemia, brain infections, and neurodegenerative diseases [12, 13].

In this study, we hypothesized that IL-17A expression is elevated during *A. cantonensis* infection and that anti-IL-17A antibody can ameliorate the demyelination in infected animals. In this study, we report that IL-17A expressions were detected during *A. cantonensis* infection. Moreover, IL-17A-neutralizing antibody protects against demyelination caused by *A. cantonensis* infection. Our results showed that IL-17A and iNOS RNA expressions increased gradually in the process of *A. cantonensis* infection, and IL-17A inhibition alleviated the demyelination caused by *A. cantonensis*. Furthermore, we also report that IL-17A inhibition may decrease the production of iNOS, which might be the key reason for the curative effect of IL-17A-neutralizing antibody on the demyelination caused by *A. cantonensis*. These findings explore a role of IL-17A on the demyelination caused by *A. cantonensis* and provide a new potential alternative therapy for this disease.

2. Materials and Methods

2.1. Infection of Mice with A. cantonensis Larvae and IL-17 Antibody Injection. Mice infected with *A. cantonensis* larvae BALB/c mice (20–40 g body weight) were purchased from the Animal Center Laboratory at Sun Yat-sen University (Guangzhou, China). The Institutional Animal Care and Use Committee approved all animal procedures. Larval collection: stage III larva (L3) of *A. cantonensis* were collected from giant African snails (*Achatina fulica*) via homogenization and digestion of minced snail tissue that was placed in a pepsin-HCl solution (pH 2.0, 500 IU pepsin/gram tissue) and incubated at 37°C for 2 h. L3 in the sediment were washed with phosphate-buffered saline (PBS) and counted under an anatomical microscope then given to experimental animals by gavage with 30 L3 per animal. The animals were divided into four groups: normal control group, normal control with IL-17 antibody, *A. cantonensis* infection group, and *A. cantonensis* infection group treated with IL-17 antibody. There are at least 5 mice in each group. The mice in the normal control treated with IL-17 antibody group and in the *A. cantonensis* infection treated with IL-17 antibody group were injected with IL-17-neutralizing antibody (per 0.05 mg/kg/day; eBioscience, USA) into the abdominal cavity at 0 d, 4 d, 8 d, 12 d, 16 d, and 20 d.

2.2. Transmission Electron Microscopy Observation. After anesthesia, the animals were euthanized by transcardial perfusion with 4% paraformaldehyde. Mice' optic nerves were crosscut into 15 μm sections at −20°C and mounted on glass slides. Optic nerves were quickly dissected and postfixed overnight in 2.5% glutaraldehyde. Next, optic nerve fragments were postfixed in a solution containing 1% osmium tetroxide (Sigma-Aldrich), then fragments were dehydrated in acetone series and embedded in SPIN-PON resin. Resin polymerization was performed at 60°C for three days. Semithin sections (0.5 μm thickness) were placed onto glass slides and stained with toluidine blue. Finally, demyelination detection was done by using a 300 kV transmission electronic microscope (FEI, USA).

2.3. Immunofluorescence. After fixing with 4% paraformaldehyde, brain sections were cut into 15 μm sections at −20°C and mounted on glass slides. Then, sections were blocked with 3% bovine serum albumin (BSA) at room temperature for 1 h before incubation with rabbit anti-iNOS (Abcam, UK) and anti-GFAP (Sigma-Aldrich, USA) monoclonal antibody in 1% BSA at 4°C overnight. Sections were washed three times in PBS, incubated with fluorescein isothiocyanate- (TRITC-) labeled and FITC-labeled (for others) secondary antibody(Abcam, Cambridge, UK), diluted 1 : 500 in 1% BSA at 37°C for 1 h, and washed again in PBS. Then, DAPI (1 : 1000 dilution, Beyotime Biotechnology) stained the nucleus for 5 min. Specimens stained without the primary antibody were used as negative controls. Then, the slides were observed under a confocal microscope.

2.4. RNA Isolation and Real-Time Quantitative PCR. Total RNA was extracted from the cerebrum with TRIzol reagent according to the manufacturer's instructions (Invitrogen). For cDNA synthesis, RNA was reverse transcribed with a PrimeScript RT reagent Kit (TaKaRa). The expression of the genes encoding IL-17A, and iNOS for mice by real-time PCR with SYBR Premix Ex Taq kit (TaKaRa). Relative quantification was applied to detect the mRNA expression of the above genes. The primer sequences as follows: 5′-TCATGTGGT GGTCCAGCTTTC-3′, 3′-CTCAGACTACCTCAACCGTT CC-5′ for IL-17A mice; 5′-CTGATGTTGCCATTGTTGG TG-3′, 3′-CTTTGACGCTCGGAACTGTAG-5′ for iNOS mice; and 5′-AAGAAGGTGGTGAAGCAGG-3′; 3′-GAAG GTGGAAGAGTGGGAGT-5′ for GAPDH mice as an internal reference. Amplification of cDNA was performed on an ABI Prism 7900 HT cycler (Applied Biosystems).

2.5. Western Blot Analysis. The brain tissue of mice in different groups were washed twice with cold PBS and lysed in extraction buffer (20 mM HEPES, pH 7.4, 2 mM EDTA, 50 mM 5-glycerophosphate, 1 mM dithiothreitol, 1 mM Na_3VO_4, 1% Triton X-100, and 10% glycerol) on ice. The lysates were centrifuged at 12,000 rpm for 15 min, and supernatants were collected. Protein (20–40 μg) was separated by SDS-PAGE and then transferred onto a nitrocellulose membrane (Pall Corporation, Ann Arbor, MI, USA). Transferred blots were incubated sequentially with a blocking agent (5% nonfat milk in TBS), and an anti-MBP antibody (1 : 125 dilution, Abcam, UK) and a HRP-conjugated secondary antibody (for 1 h at room temperature) were developed by the enhanced chemifluorescence detection kit on Hyperfilm (Fuji, Japan) according to the manufacturer's directions. The same blots were subsequently stripped and reblotted with internal referring antibodies β-actin and β-tubulin (Sigma-Aldrich, USA). Graphs of blots were obtained in the linear range of detection and were quantified for the level of specific induction by ImageJ System.

2.6. Statistical Analysis. One-way ANOVA was used to compare data of real-time PCR and graphs of blots in western

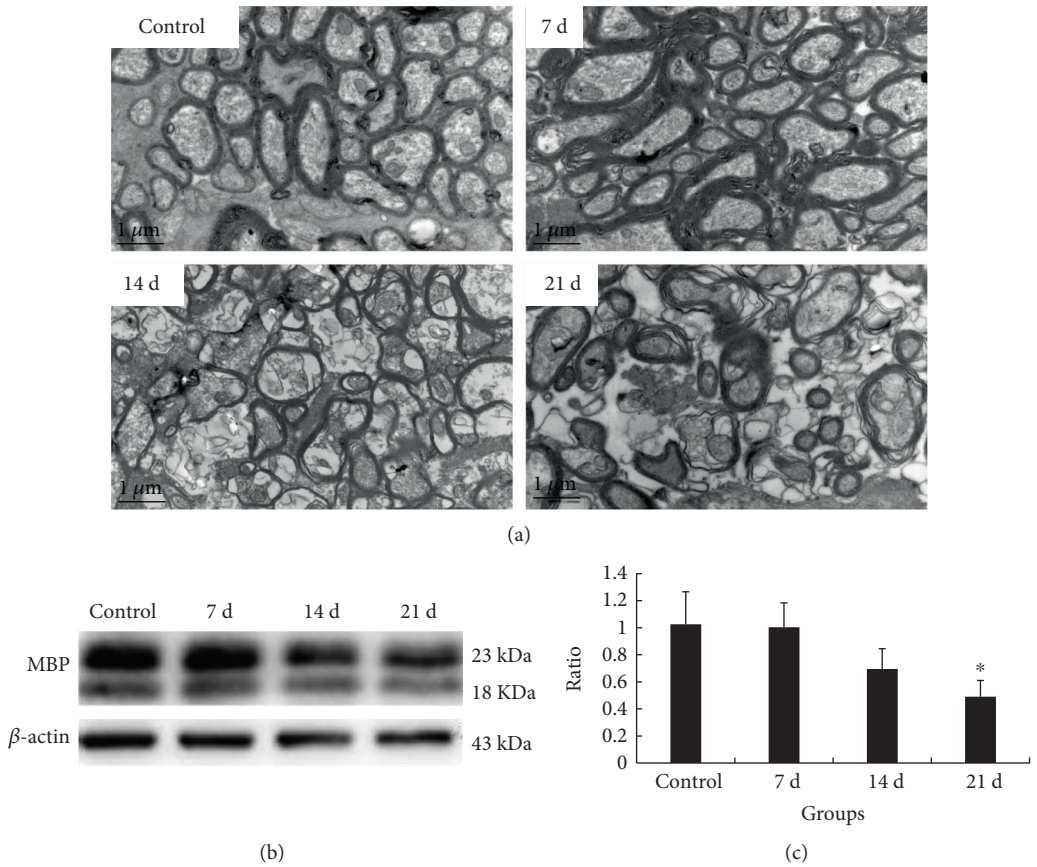

Figure 1: *A. cantonensis* infection caused demyelination in infected mice. (a) Transmission electronic image of the optic nerve at 0 d, 7 d, 14 d, and 21 d after *A. cantonensis* infection. Prominent demyelination (the black arrows point) can be observed in 14 d and 21 d. Scale bar = 1 μm. (b) The MBP protein expression of the brain via western blotting at 0, 7, 14, and 21 days after *A. cantonensis* infection. (c) The relative ration of MBP protein expression (β-actin was as the internal reference). These expressions decreased as time extended and had obvious difference with the control in 21 d of infection. Numerical results are presented as mean ± SEM. $n = 3$ animals per group, at least three fields were analyzed per section in at least two sections not next to each other per animal. $*$ represents statistically significant values when compared with the normal control ($P < 0.05$).

blotting among different groups. Statistics was performed using IBM SPSS statistics 19 (SPSS Inc., USA). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Demyelination Is of Serious Pathological Change in the Brain Tissue of Mice with A. cantonensis Infection. A. cantonensis invaded the central nerve system and caused demyelination. The resulting images of the transmission electronic microscope of the optic nerves showed that demyelination was obvious at 14 d and became serious on 21 d of infection of *A. cantonensis* (Figure 1(a)). Moreover, the MBP (myelin basic protein) expression also decreased gradually during *A. cantonensis* infection (Figures 1(b) and 1(c)). The above results proved that *A. cantonensis* can cause demyelination in the brain.

3.2. Increase of IL-17A and iNOS Is Significant in the Brain of the Infected Mice. We further explored whether the expression of IL-17A is altered in the process during *A. cantonensis* infection. We found that IL-17A RNA expression increased with the extension of the infection (Figure 2(a)). At the same time, the RNA expression of iNOS also increased gradually and peaked at 21 d of infection (Figure 2(b)). These findings suggest that IL-17A and iNOS may correlate with demyelination caused by *A. cantonensis*.

3.3. IL-17A Inhibition Can Lighten the Demyelination in the Brain of the Infected Mice. TEM and MBP protein expressions were applied to detect the alteration of demyelination. When *A. cantonensis* infected the mice for 21 d, TEM showed obvious demyelination. Moreover, MBP expression also decreased distinctly. After IL-17A antibody was injected in experimental mice, the results showed that IL-17A inhibition can restore the demyelination caused by *A. cantonensis* to normal levels (Figure 3(a)). In addition, levels of MBP and expression were elevated after IL-17A inhibition of *A. cantonensis* in infected mice but had no effect on normal mice (Figures 3(b) and 3(c)). These results displayed that IL-17A may be the key element for demyelination caused by *A. cantonensis*.

3.4. IL-17A Inhibition Can Cause the Downregulation of iNOS in Astrocytes in the Brain of the Infected Mice. From

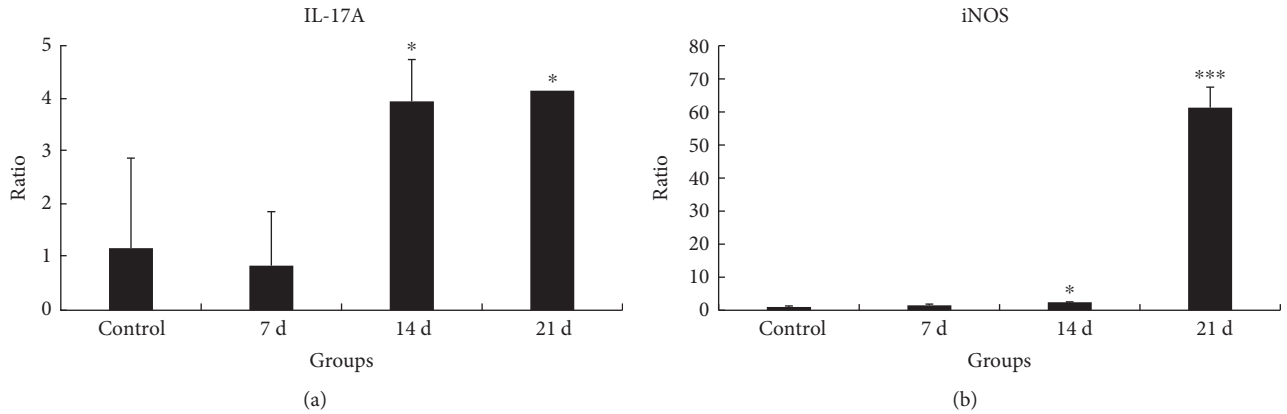

Figure 2: *A. cantonensis* induced the elevation of IL-17A and iNOS. The RNA expression of IL-17A and iNOS at 0 d, 7 d, 14 d, and 21 d after *A. cantonensis* infection (GAPDH was the internal reference for real-time PCR). (a) Expression of IL-17A increased gradually. $n = 3$ animals per group. (b) RNA expression of iNOS, which also elevated with infection time obviously. $*$ and $***$ represent statistically significant values when compared with normal control ($P < 0.05$ and $P < 0.001$, resp.).

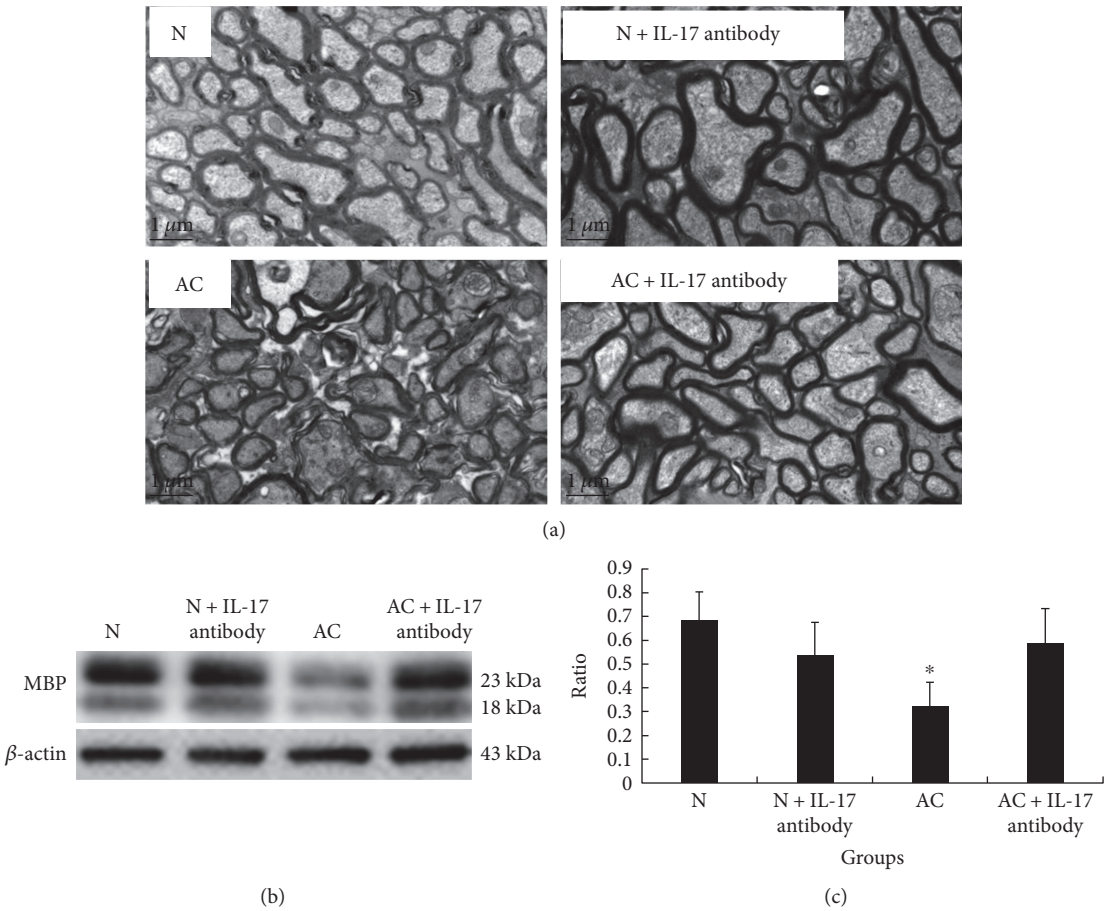

Figure 3: IL-17A inhibition can ameliorate the demyelination caused by *A. cantonensis*. (a) TEM picture of the optic nerve in the normal group, 21 d *A. cantonensis* infection group, normal mice with IL-17A antibody group, and 21 d *A. cantonensis* infection with IL-17A antibody group. IL-17A inhibition can restore the demyelination caused by *A. cantonensis* to the normal level. Scale bar = 1 μm. (b) The MBP protein expression of the brain via western blotting in the normal group, 21 d *A. cantonensis* infection group, normal mice with IL-17A antibody group, and 21 d *A. cantonensis* infection with IL-17A antibody group. (c) The semiquantification of MBP protein expression via western blotting in different groups (β-actin as the internal reference). IL-17A inhibition had no effect on MBP expression of the brain in the normal group but increased MBP expression in the 21 d *A. cantonensis* infection group. $n = 3$ animals per group, at least three fields were analyzed per section in at least two nonadjacent sections per animal. $*$ represents statistically significant values when compared with normal control ($P < 0.05$).

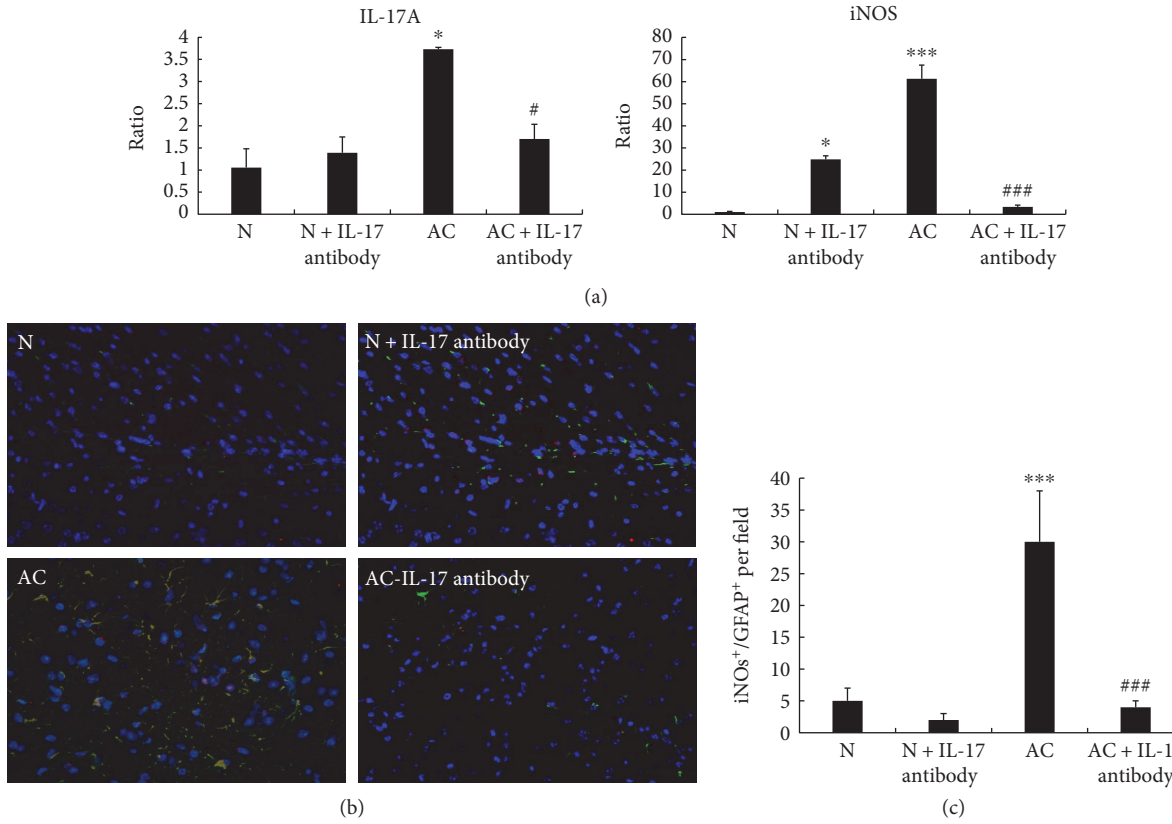

Figure 4: IL-17A inhibition causes the downregulation of iNOS in astrocytes after *A. cantonensis* infection. (a) The RNA expression of IL-17A and iNOS in the normal group, the 21 d *A. cantonensis* infection group, the normal mice with IL-17A antibody group, and the 21 d *A. cantonensis* infection with IL-17A antibody group. After IL-17A antibody was injected, the RNA expression of IL-17A was decreased in the *A. cantonensis*-infected group on day 21 but it had no effect on the normal group. IL-17A antibody increased the RNA expression of iNOS in the normal group, but levels were decreased in the 21 d *A. cantonensis* infection group. GAPDH was the internal reference for real-time PCR. (b) Brain sections stained with iNOS (red) and GFAP (green). Yellow color represented the double staining of iNOS and GFAP. (c) Percentage of $iNOS^+/GFAP^+$ cells per field of the brain section. $^*P < 0.05$, $^{***}P < 0.001$, compared with the normal group; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$, compared with the 21 d *A. cantonensis* infection group. $n = 3$ mice.

above results, we found that IL-17A inhibition can alleviate demyelination caused by *A. cantonensis*, but the mechanism was still unknown. To resolve this question, real-time PCR and immunofluorescence were applied to examine the expression of IL-17A and iNOS. Our results showed when *A. cantonensis* infected the mice for 21 d, IL-17A and iNOS elevated obviously. IL-17A-neutralizing antibody can decrease the expression of IL-17A, which proved that the inhibition of IL-17A in *A. cantonensis*-infected mice was effective, but it cannot influence the IL-17A expression of normal animals. More importantly, iNOS expression was also decreased obviously during IL-17A inhibition. Moreover, we found that when IL-17A-neutralizing antibody was applied, iNOS expression in normal mice was elevated (Figure 4(a)). The results of immunofluorescence also proved that when *A. cantonensis* infected the mice for 21 d, the double labeling of iNOS and GFAP (astrocytes marker) both increased, whereas IL-17A inhibition can decrease the protein expression of iNOS in astrocytes (Figures 4(b) and 4(c)). The above results showed that IL-17A inhibition may alleviate demyelination caused by *A. cantonensis* through decreasing the iNOS expression in astrocytes.

4. Discussion

A. cantonensis is an important cause of food-borne diseases in humans. *A. cantonensis* infection is endemic in the Pacific Islands, Southeast Asia, China, and Hawaii. Sporadic infections are reported in Southern United States and Florida [14]. Outbreaks of eosinophilic meningitis have been reported due to the consumption of raw snails harboring L3 or associated with the consumption of contaminated raw vegetable juice in Taiwan [15, 16]. Rat is a known definitive host which can tolerate the worms. In large numbers (240), *A. cantonensis* cause significant cardiovascular and neurological impairment in rats [17].

In this study, we detected IL-17A expression in the brain tissue of the infected mice and applied IL-17A-neutralizing antibody to treat the infected mice to investigate the process of brain demyelination. Our findings demonstrated that *A. cantonensis* infection promotes the IL-17A expression in the brain as well as IL-17A inhibition to alleviate the demyelination caused by *A. cantonensis* in mice. Moreover, IL-17A inhibition may decrease the production of iNOS, which might be the reason for the curative effect for

IL-17A-neutralizing antibody on demyelination caused by *A. cantonensis*. The evidence was as follows.

Firstly, TEM detection of the optic nerve and western blotting of MBP proved that *A. cantonensis* infection actually caused demyelination. Moreover, the RNA expression of IL-17A and iNOS increased gradually following with the prolonged *A. cantonensis* infection. IL-17 is a mediator of communication between immune cells and tissues. IL-17 is the founding member of a family of 6 cytokines, IL-17A–F, and IL-17A is the most investigated one. IL-17A has recently emerged as an attractive target, especially for the treatment of T cell-mediated autoimmune diseases [18, 19]. Previous study showed that IL-17A expression alone was able to activate glial cells and enhance neuroinflammatory responses, thus showing that CNS cells express a functional IL-17RA/C receptor complex [6]. In our study, IL-17A expression elevated with demyelination during *A. cantonensis* infection. Similarly, we observed elevation of iNOS associated with demyelination in experimental animals. These findings indicate that IL-17A along with iNOS may take part in the demyelination caused by *A. cantonensis*.

Next, the IL-17A-neutralizing antibody was administered in the infected mice to explore the function of IL-17A on demyelination caused by *A. cantonensis*. Our results proved that IL-17A inhibition can alleviate demyelination obviously observed via TEM of the optic nerve and western blotting of MBP after *A. cantonensis* infection. In the previous study, there were strong indications pointing to a role for IL-17 in the pathogenesis of MS and EAE, major causes of demyelination diseases [20, 21]. As a result, we applied IL-17A-neutralizing antibody to treat the demyelination caused by *A. cantonensis* and the effect proved our presumption.

Finally, we explored the mechanism by which IL-17A-neutralizing antibody alleviated demyelination caused by *A. cantonensis*. Our results showed that IL-17A-neutralizing antibody inhibited the RNA expression of IL-17A and also decreased the production of iNOS induced by *A. cantonensis* infection. Moreover, IL-17A-neutralizing antibody also inhibited the protein expression of iNOS in astrocytes. Astrocytes probably represent the best-studied CNS resident cell type in the context of MS and EAE. Both human and mouse astrocytes express IL-17RA, thereby allowing for IL-17A ligation and consequently, the production of cytokines and chemokines, including IL-6, TNFα, and CCL2 [6, 7]. iNOS is involved in various physiological regulations and plays important roles in some CNS disease, such as brain ischemia, brain infections, and neurodegenerative diseases [22, 23]. As a result, we speculated that IL-17A-neutralizing antibody alleviated demyelination via inhibiting iNOS production in astrocytes. Previous study showed that exogenous IL-17A significantly induced iNOS expression and hence cardiomyocyte apoptosis [24]. Our study was partly in accordance with these findings. To the best of our knowledge, there has been no previous report that IL-17A inhibition would decrease the production of iNOS in demyelination caused by *A. cantonensis*. In this line, we will further investigate iNOS using knockout mice in future studies.

In conclusion, IL-17A may play important roles in demyelination caused by *A. cantonensis* and the present study suggested that IL-17A-neutralizing antibody may be an effective method to treat the demyelination caused by *A. cantonensis*. Furthermore, iNOS inhibition is the possible mechanism for the therapeutic effect. As a result, our study provides a new potential alternative therapy for demyelination caused by *A. cantonensis*.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Feng Ying, Zheng Cunjing, Feng Feng, and Wan Shuo carried out the experiments and performed the statistical analyses. Feng Ying drafted the manuscript. Wu Zhongdao and Xie Fukang conceived the study and coordinated the project. All authors read and approved the final manuscript. Feng Ying and Zheng Cunjing contributed equally to this work.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (81401688, 81271855, and 81261160324), the National Research and Development Plan of China (no. 2016YFC1200500), the Central College basic research operating funding of the South China University of Technology (2017MS090), the South China University of Technology scientific research funding (D6172910), and the South China University of Technology School of Medicine scientific research project funding (yxy2016007).

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Hindawi
Mediators of Inflammation
Volume 2017, Article ID 5801768, 11 pages
https://doi.org/10.1155/2017/5801768

Research Article

Different Dietary Proportions of Fish Oil Regulate Inflammatory Factors but Do Not Change Intestinal Tight Junction ZO-1 Expression in Ethanol-Fed Rats

Yi-Wen Chien,[1,2] Hsiang-Chi Peng,[1,2] Ya-Ling Chen,[3] Man-Hui Pai,[4] Hsiao-Yun Wang,[1] Hsiao-Li Chuang,[5] and Suh-Ching Yang[1,2]

[1]*School of Nutrition and Health Sciences, Taipei Medical University, Taipei 110, Taiwan*
[2]*Research Center of Geriatric Nutrition, College of Nutrition, Taipei Medical University, Taipei 110, Taiwan*
[3]*Department of Nutrition and Health Sciences, Chang Gung University of Science and Technology, Taoyuan 333, Taiwan*
[4]*Department of Anatomy, Taipei Medical University, Taipei 110, Taiwan*
[5]*National Applied Research Laboratories, National Laboratory Animal Center, Taipei 115, Taiwan*

Correspondence should be addressed to Suh-Ching Yang; sokei@tmu.edu.tw

Received 17 July 2017; Revised 30 September 2017; Accepted 15 October 2017; Published 13 December 2017

Academic Editor: Helieh S. Oz

Sixty male Wistar rats were fed a control or an ethanol-containing diet in groups C or E. The fat compositions were adjusted with 25% or 57% fish oil substituted for olive oil in groups CF25, CF57, EF25, and EF57. Hepatic thiobarbituric acid-reactive substance (TBARS) levels, cytochrome P450 2E1 protein expression, and tumor necrosis factor- (TNF-) α, interleukin- (IL-) 1β, IL-6, and IL-10 levels, as well as intracellular adhesion molecule (ICAM)-1 levels were significantly elevated, whereas plasma adiponectin level was significantly reduced in group E ($p < 0.05$). Hepatic histopathological scores of fatty change and inflammation, in group E were significantly higher than those of group C ($p < 0.05$). Hepatic TBARS, plasma ICAM-1, and hepatic TNF-α, IL-1β, and IL-10 levels were significantly lower, and plasma adiponectin levels were significantly higher in groups EF25 and EF57 than those in group E ($p < 0.05$). The immunoreactive area of the intestinal tight junction protein, ZO-1, showed no change between groups C and E. Only group CF57 displayed a significantly higher ZO-1 immunoreactive area compared to group C ($p = 0.0415$). 25% or 57% fish oil substituted for dietary olive oil could prevent ethanol-induced liver damage in rats, but the mechanism might not be related to intestinal tight junction ZO-1 expression.

1. Introduction

Excessive or chronic alcohol consumption can lead to liver damage through various pathogenic mechanisms. Three primary types of alcohol-induced liver damage include fatty liver, hepatitis, and cirrhosis [1]. Alcohol-induced liver damage is related to an increased NADH/NAD^+ ratio which promotes fatty acid synthesis and lipid accumulation in liver cells, oxidative stress caused by increased CYP2E1 activity, and an increased endotoxin level which triggers Kupffer's cell activation and inflammatory processes [2–4]. However, the pathogenic mechanisms are complicated and remain obscure.

There is an emerging theory that chronic ethanol abuse dislocates the tight junction (TJ) structure of the intestinal epithelium, which allows bacterial translocation from the intestines into the in vivo circulation thereby inducing hepatic inflammation [5]. It was indicated that higher endotoxin levels were observed in alcoholic liver disease (ALD) patients, and gut leakage seemed to be the main cause [6, 7]. Endotoxins, also known as lipopolysaccharides (LPSs), are derived from the cell walls of gram-negative bacteria. Animal studies also showed that ALD could be prevented when the intestinal microflora was removed by antibiotics [8–10]. Our previous studies also indicated that epidermal

growth factor or synbiotics exhibited hepatoprotective effects through ameliorating the intestinal permeability and microbiota in rats under chronic ethanol feeding [11, 12]. Those previous findings powerfully indicated that intestinal barrier disturbances caused by ethanol abuse are the principal pathway of endotoxemia in ALD.

The consumption level and type of dietary fat can influence the progression of liver injury in ALD. It was indicated that diets rich in saturated fatty acids (SFAs) or medium-chain triglycerides (MCTs) protect against liver injury in rats and mice under chronic ethanol feeding, but diets containing polyunsaturated fatty acids (PUFAs) aggravate liver damage induced by ethanol intake [13–15]. However, there were some limitations of those previous studies. First, only one type of fat was used in each experimental diet. Second, the effects on other organs or tissues were not detected.

Fish oil contains abundant levels of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are known as n-3 PUFAs. Based on a majority of studies, fish oil (or n-3 PUFAs) is considered to have beneficial effects, including immune regulation, vascular protection, and lipid metabolism modulation [16–18]. However, few studies have discussed the relationship between fish oil and ALD, particularly those focused on intestinal integrity. According to our earlier study, substituting fish oil for olive oil under ethanol exposure improved the fecal microbiota composition; however, effects on intestinal pathological changes in ethanol-fed rats are still unclear. Thus, we hypothesized that fish oil may have a hepatoprotective effect in ethanol-fed rats by means of maintaining the epithelial barrier function in the intestines and further inhibiting the appearance of endotoxin in the circulation. This animal study was performed to investigate the proposed hypothesis.

2. Materials and Methods

2.1. Animals. Sixty male Wistar rats (8-weeks old, 160~180 g) provided by BioLASCO Taiwan (Ilan, Taiwan) were acclimatized in individual cages at 22 ± 2°C with 50%~70% humidity and a 12 h light/dark cycle for 1 week with a standard rodent diet (LabDiet 5001 Rodent Diet; PMI Nutrition International, St. Louis, MO, USA). The Institutional Animal Care and Use Committee of Taipei Medical University approved all procedures in this study.

2.2. Study Protocol. Rats were divided into groups according to their plasma aspartate transaminase (AST) and alanine transaminase (ALT) activities after 1 week of acclimation in order to ensure there was no significant difference among groups in plasma AST and ALT activities at the beginning of the study. Rats were fed with either a control diet or ethanol diet, in which the fat composition of both diets was adjusted with 25% (7.1 g fish oil/kg diet, 6% of total calories) or 57% (16.2 g fish oil/kg diet, 15% of total calories) fish oil substituted for olive oil. Thus, there were six groups in this study: C (control), CF25 (control with 25% fish oil), CF57 (control with 57% fish oil), E (ethanol), EF25 (ethanol with 25% fish oil), and EF57 (ethanol with 57% fish oil). Rats in groups E, EF25, and EF57 were fed an ethanol-containing liquid diet (35% of calories from ethanol) which was modified from Lieber-DeCarli formula [19], while rats in groups C, CF25, and CF57 were pair-fed with an isoenergetic diet without ethanol by substituting ethanol-derived calories with maltodextrin [16]. One gram of fish oil (VIVA Omega-3™) which was provided by Viva Life Science (Costa Mesa, CA, USA) contains 350 mg EPA and 250 mg DHA. Monounsaturated fatty acid (MUFA)/PUFA ratios of the diets without fish oil and with 25% and 57% fish oil substitutions were 0.4, 0.7, and 1.5, respectively [16].

Rats were anesthetized and sacrificed after 8 weeks. Blood samples were collected via the ventral aorta into heparin-containing tubes and centrifuged at 1200 ×g for 15 min (at 4°C); then plasma was collected and stored at −80°C until analysis. Liver tissues were rapidly excised, and a small portion of the liver specimen was cut and fixed in a 10% formaldehyde solution. The remaining liver tissues were stored at −80°C for further analysis. Moreover, jejunum tissue (2 cm of the middle section) of the small intestine was excised and fixed in a 10% formaldehyde solution.

2.3. Measurements and Analytical Procedures

2.3.1. Liver Function Indicators. The most commonly used indicators of liver damage are plasma AST and ALT activities which were measured with the ADVIA® 1800 Chemistry System (Siemens Healthcare Diagnostics, Eschborn, Germany) in this study.

2.3.2. Hepatic Histopathological Examination. Liver tissues were fixed in a 10% formaldehyde solution and embedded in paraffin. Paraffin sections were cut and stained with hematoxylin and eosin (H&E) and trichrome stains. Experienced pathologists blinded to the experimental data carried out the semiquantitative histological evaluation of liver specimens according to the degree of tissue damage, which was scored on a scale of 0 = absent, 1 = trace, 2 = mild, 3 = moderate, and 4 = severe.

2.3.3. Hepatic Antioxidative Status

(1) Plasma and Hepatic Lipid Peroxidation. One gram of liver tissue was added to 4 mL of buffer containing 0.25 mM phenylmethylsulfonyl fluoride, 0.25 mM sucrose, and 10 mM Tris-HCl (pH 7.4) and then homogenized and centrifuged at 10^4 ×g for 15 min at 4°C. Supernatants of the liver homogenate and plasma sample were analyzed for lipid peroxidation by measuring the concentration of thiobarbituric acid-reactive substances (TBARSs) as described previously [20].

(2) CYP2E1 Protein Expression. The method of microsome preparation from liver tissues was described previously [19]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 10%) was used to separate microsomal proteins (30 μg). Proteins were electroblotted onto polyvinylidene difluoride transfer membranes, and the membranes were separately incubated with mouse monoclonal anti-rat CYP2E1 (Oxford Biomedical Research, Oxford, MI, USA) or mouse anti-actin monoclonal antibodies (Chemicon

International, Temecula, CA, USA), then samples were treated with goat anti-mouse immunoglobulin G (IgG)-horseradish peroxidase (HRP) (Chemicon International) and detected with a Western Lightning kit (PerkinElmer Lifesciences, Boston, MA, USA). An Image-Pro Plus 4.5 software analysis was used to quantify the bands.

2.3.4. Inflammatory Response

(1) Cytokine Measurements. Ice-cold buffer (1.5 mL) containing 50 mM Tris (pH 7.2), 150 mM NaCl, 1% Triton-X, and 0.1% protease inhibitor was added to the liver tissue (0.5 g) and then homogenized and shaken on ice for 90 min. The homogenized solution was centrifuged at 3000 ×g and 4°C for 15 min. A DuoSet® rat TNF-α kit, a rat IL-1β/IL-1F2 kit, a rat IL-6 kit, and a rat IL-10 kit (R&D Systems, Minneapolis, MN, USA) were used to analyze the supernatant according to assay kit instructions. A microplate reader (Molecular Devices, Sunnyvale, CA, USA) was used to read the optical density (OD) at 450 nm for all cytokines.

(2) Plasma Adiponectin Concentration. An enzyme-linked immunosorbent assay (ELISA) kit (AssayMax Rat Adiponectin ELISA kit Assaypro, St. Charles, MO, USA) was used to measure the plasma adiponectin concentration. The OD was the same as for the cytokine measurements.

(3) Cell Adhesion Molecule Measurement. Plasma VCAM-1 and ICAM-1 levels were, respectively, determined with a rat ICAM-1/CD54 Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA) and Cell Adhesion Molecule 1 Assay Kit (USCN Life Science, Wuhan, China). Procedures followed the manufacturer's instructions. The OD was the same as for the cytokine measurements.

2.3.5. Small-Intestinal Histopathological Examination

(1) H&E Dye Staining. Jejunum tissue (2 cm of the middle section) was fixed in 10% formaldehyde and embedded in paraffin. Paraffin sections were cut and stained with H&E dye. A semiquantitative histological evaluation was carried out by a trained pathologist who was blinded to the treatment groups and visually evaluated the degree of tissue injury, according to Chiu's Score Classification of Small-Intestinal Injury [21]. The grading ranges 0~5, the same as described by Yuan et al. [22].

(2) TJ Protein ZO-1 Immunohistochemical Staining. The method of ZO-1 immunohistochemical (IHC) staining was described previously [23]. Tissue sections were deparaffinized and incubated with a primary antibody against ZO-1 (1 : 300, Abcam, Cambridge, UK) overnight at 4°C, followed by incubation with a biotinylated secondary antibody (1 : 300, Nippon Chemi-Con, Tokyo, Japan) for 1 h at room temperature. After carrying out the reaction with the peroxidase-linked avidin-biotin complex (Vector) for 1 h at room temperature, a diaminobenzidine solution kit (Vector) was used to detect ZO-1 immunoreactivity. The "count/size" and "area" commands were used to determine the intensity of ZO-1 immunoreactivity.

(3) Plasma Endotoxin Levels. Plasma endotoxin levels were measured using a Limulus Amebocyte Lysate Kit (Associates of Cape Cod, East Falmouth, MA, USA). A microplate reader (Molecular Devices) was used to read the OD at 405 nm.

2.4. Statistical Analysis. Data are presented as the mean ± standard error of the mean (SEM). SAS software vers. 9.4 (SAS Institute, Cary, NC, USA) and Student's *t*-tests were used to determine statistical differences between groups C and E. A one-way analysis of variance (ANOVA) followed by Duncan's new multiple range test was used to determine statistical differences among groups C, CF25, and CF57 and groups E, EF25, and EF57. A two-way ANOVA was used to confirm the interaction between ethanol and fish oil. p values of <0.05 were regarded as statistically significant.

3. Results

3.1. Food Intake and Ethanol Consumption. No difference was found in food intake among the six groups (group C: 74.8 ± 3.9 kcal/day; group CF25: 74.1 ± 3.9 kcal/day; group CF57: 74.3 ± 4.1 kcal/day; group E: 76.4 ± 3.2 kcal/day; group EF25: 72.0 ± 3.2 kcal/day; and group EF57: 70.1 ± 3.0 kcal/day). The average ethanol consumption in groups E, EF25, and EF57 was 11.4 ± 0.2, 11.3 ± 0.2, and 11.1 ± 0.2 g/kg BW/day, respectively. There was no difference among these ethanol-intake groups.

3.2. Body Weight and Relative Liver Weight. Final body weights are shown in Table 1. There was no difference in final body weights between groups C and E. However, final body weights in groups EF25 and EF57 were significantly lower than that of group E ($p < 0.05$). The relative liver weight in group E was significantly higher compared to that of group C ($p < 0.05$). However, the relative liver weights exhibited no differences among groups E, EF25, and EF57.

3.3. Hepatic Histopathological Examination. After 8 weeks of feeding, plasma AST and ALT activities of group E were significantly higher than those of group C ($p < 0.05$, Table 2). However, plasma AST activities in groups EF25 and EF57 were significantly lower compared to those of group E ($p < 0.05$).

Histopathological scores of the livers are presented in Table 3. Fatty changes (including macrovesicular and microvesicular), inflammatory cell infiltration, and cell degeneration and necrosis were observed in group E; however, fatty changes, inflammation, and cell degeneration and necrosis were significantly lower in groups FE25 and FE57 than those in group E ($p < 0.05$). According to Figure 1, H&E staining showed hepatocyte degeneration and necrosis accompanied by fat accumulation and inflammatory cell infiltration.

3.4. Oxidative Stress. TBARS concentrations and CYP2E1 expressions are considered indicators for evaluating the hepatic antioxidative status. Results of plasma and hepatic TBARS concentrations are given in Table 4. Plasma and hepatic TBARS concentrations were significantly higher in group E ($p < 0.05$); however, both plasma and hepatic TBARS concentrations were significantly lower in groups

Table 1: Final body weights and relative liver weights in each group[1,2,3].

		—	F25	F57	Ethanol* and fish oil
Final weight (g)	C	409.5 ± 5.4	410.5 ± 3.6	413.6 ± 6.3	0.0338
	E	397.1 ± 4.4^{e}	374.5 ± 5.5^{f}	368.0 ± 10.4^{f}	
Liver weight (g)	C	10.1 ± 0.2^{b}	10.8 ± 0.4^{ab}	11.7 ± 0.3^{a}	0.3006
	E	$12.1 \pm 0.3^{*}$	12.3 ± 0.8	12.3 ± 0.5	
Relative liver weight (%)	C	2.5 ± 0.0^{c}	2.6 ± 0.1^{b}	2.8 ± 0.0^{a}	0.8601
	E	$3.0 \pm 0.1^{*}$	3.3 ± 0.2	3.3 ± 0.1	

[1]Values are expressed as the mean ± SEM. Means between groups C and E with * significantly differ ($p < 0.05$). Means among groups C, CF25, and CF57 with different superscript letters (a, b, c) significantly differ ($p < 0.05$). Means among groups E, EF25, and EF57 with different superscript letters (e, f) significantly differ ($p < 0.05$). [2]Relative liver weight: (liver weight/body weight) × 100%. [3]C: control group; CF25: control diet with fish oil substituted for 25% of olive oil; CF57: control diet with fish oil substituted for 57% of olive oil; E: ethanol group; EF25: alcohol-containing diet with fish oil substituted for 25% of olive oil; EF57: alcohol-containing diet with fish oil substituted for 57% of olive oil.

Table 2: Final plasma aspartate transaminase (AST) and alanine transaminase (ALT) activities in each group[1,2].

(U/L)		—	F25	F57	Ethanol* and fish oil
ALT	C	48.4 ± 4.4	45.5 ± 2.4	49.1 ± 2.3	0.5782
	E	$87.9 \pm 12.5^{*}$	75.9 ± 5.7	73.6 ± 4.8	
AST	C	83.4 ± 2.5	84.9 ± 3.0	91.5 ± 2.5	0.3965
	E	$185.3 \pm 18.6^{*e}$	131.0 ± 13.5^{f}	156.0 ± 17.4^{f}	

[1]Values are expressed as the mean ± SEM. Means between groups C and E with * significantly differ ($p < 0.05$). Means among groups E, EF25, and EF57 with different superscript letters (e, f) significantly differ ($p < 0.05$). [2]Details are the same as those described in the footnotes of Table 1.

Table 3: Hepatic histopathology scores in each group[1,2].

		—	F25	F57	Ethanol* and fish oil
Fatty change (macrovesicular)	C	1.6 ± 0.2	1.2 ± 0.2	1.2 ± 0.2	0.1779
	E	$2.8 \pm 0.2^{*e}$	1.8 ± 0.2^{f}	1.6 ± 0.2^{f}	
Fatty change (microvesicular)	C	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	<0.0001
	E	$1.8 \pm 0.2^{*e}$	0.0 ± 0.0^{f}	0.0 ± 0.0^{f}	
Inflammatory cell infiltration	C	1.6 ± 0.2^{a}	1.8 ± 0.2^{a}	0.6 ± 0.2^{b}	0.0635
	E	$2.8 \pm 0.2^{*e}$	1.8 ± 0.2^{f}	1.6 ± 0.4^{f}	
Cell degeneration and necrosis	C	1.4 ± 0.2^{a}	1.0 ± 0.0^{ab}	0.8 ± 0.2^{b}	0.4831
	E	$3.0 \pm 0.0^{*e}$	2.4 ± 0.2^{f}	2.0 ± 0.0^{f}	
Bile duct hyperplasia	C	1.4 ± 0.2	1.4 ± 0.2	1.0 ± 0.3	0.1288
	E	1.0 ± 0.0	1.4 ± 0.2	1.6 ± 0.2	
Fibrosis	C	0.8 ± 0.4	1.4 ± 0.2	0.8 ± 0.2	0.7725
	E	1.0 ± 0.3	1.4 ± 0.2	0.6 ± 0.2	

[1]Values are expressed as the mean ± SEM. Means between groups C and E with * significantly differ ($p < 0.05$). Means among groups C, CF25, and CF57 with different superscript letters (a, b) significantly differ ($p < 0.05$). Means among groups E, EF25, and EF57 with different superscript letters (e, f) significantly differ ($p < 0.05$). [2]Details are the same as those described in the footnotes of Table 1.

EF25 and EF57 than those in group E ($p < 0.05$). As shown in Figure 2, CYP2E1 expression in group E was significantly higher than that in group C ($p < 0.05$); however, there were no differences among groups E, EF25, and EF57.

3.5. Inflammatory Responses. Rats in group E showed significantly elevated TNF-α, IL-1β, IL-6, and IL-10 concentrations compared to rats in group C ($p < 0.05$, Table 5). Hepatic TNF-α, IL-1β, IL-6, and IL-10 levels were significantly lower in groups EF25 and EF57 than those in group E ($p < 0.05$).

In addition, group E showed the significantly lowest plasma adiponectin concentration among all groups ($p < 0.05$, Table 6). Further, plasma adiponectin levels were significantly higher in groups EF25 and EF57 than that in group E ($p < 0.05$).

Plasma VCAM-1 and ICAM-1 levels in each group are shown in Table 7. Plasma VCAM-1 and ICAM-1 levels of group E were significantly higher than those of group C ($p < 0.05$). However, plasma VCAM-1 concentrations were significantly lower in groups EF25 and EF57

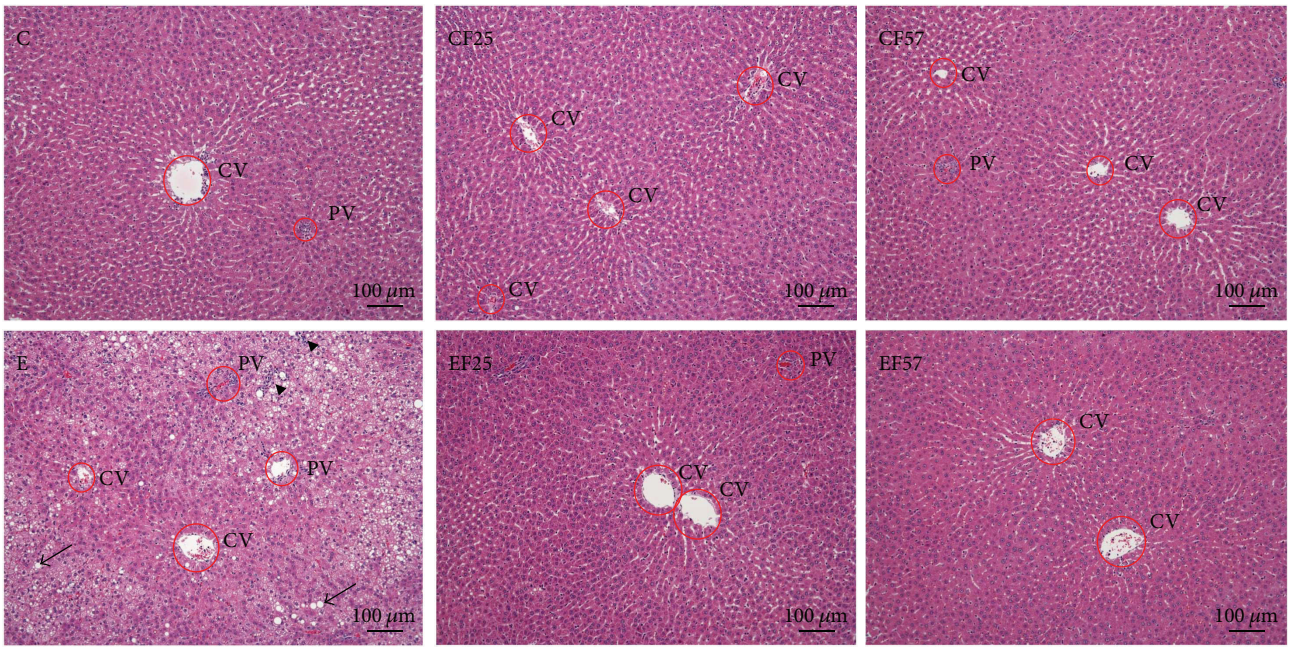

Figure 1: Effects of fish oil on H&E staining of liver tissue sections in rats with chronic ethanol feeding. CV: central vein; PV: portal vein; C: control group; CF25: control diet with fish oil substituted for 25% of olive oil; CF57: control diet with fish oil substituted for 57% of olive oil; E: ethanol group; EF25: alcohol-containing diet with fish oil substituted for 25% of olive oil; EF57: alcohol-containing diet with fish oil substituted for 57% of olive oil. H&E staining showed hepatocyte degeneration and necrosis accompanied by inflammatory cell infiltration (triangle) in group E. Moreover, fatty changes (arrow) were also found in group E.

Table 4: Thiobarbituric acid-reactive substance (TBARS) concentrations in each group[1,2].

		—	F25	F57	Ethanol* and fish oil
Plasma TBARS	C	15.4 ± 0.6	16.5 ± 0.8	15.1 ± 0.5	0.0009
(μM)	E	$20.4 \pm 0.5^{*e}$	16.3 ± 0.7^{f}	16.0 ± 0.8^{f}	
Hepatic TBARS	C	615.4 ± 17.5^{a}	532.3 ± 17.8^{b}	463.0 ± 25.0^{c}	0.328
(nmol/g liver)	E	$804.3 \pm 29.1^{*e}$	637.7 ± 26.2^{f}	594.1 ± 7.4^{f}	

[1]Values are expressed as the mean ± SEM. Means between groups C and E with * significantly differ ($p < 0.05$). Means among groups C, CF25, and CF57 with different superscript letters (a, b, c) significantly differ ($p < 0.05$). Means among groups E, EF25, and EF57 with different superscript letters (e, f) significantly differ ($p < 0.05$). [2]Details are the same as those described in the footnotes of Table 1.

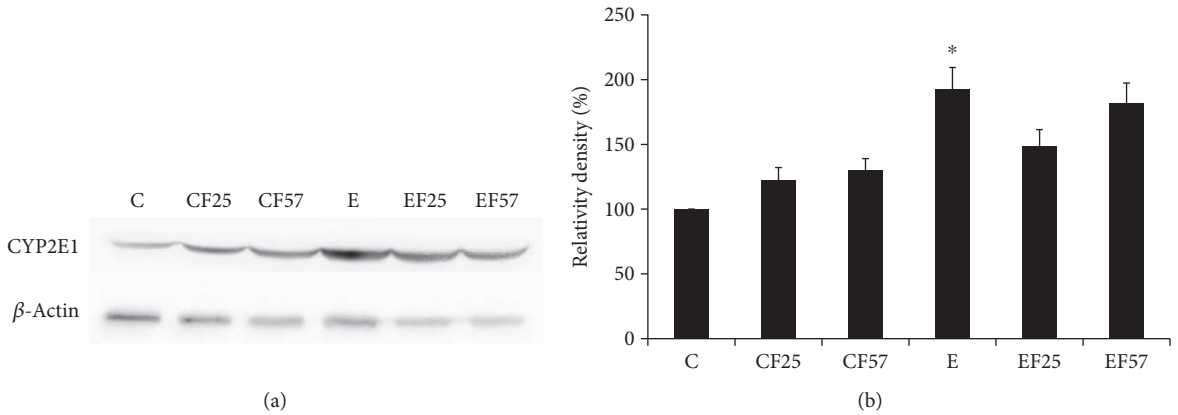

Figure 2: Hepatic CYP2E1 protein expressions in each group. Values are expressed as the mean ± SEM. C: control group; CF25: control diet with fish oil substituted for 25% of olive oil; CF57: control diet with fish oil substituted for 57% of olive oil; E: ethanol group; EF25: alcohol-containing diet with fish oil substituted for 25% of olive oil; EF57: alcohol-containing diet with fish oil substituted for 57% of olive oil. Bars with * significantly differ between groups C and E at the $p < 0.05$ level according to Student's t-tests.

Table 5: Hepatic tumor necrosis factor- (TNF-) α, interleukin- (IL-) 1β, IL-6, and IL-10 levels in each group[1,2].

(pg/mg liver)		—	F25	F57	Ethanol* and fish oil
TNF-α	C	62.2 ± 5.5	69.7 ± 3.6	62.7 ± 3.8	0.0118
	E	$86.1 \pm 4.5^{*e}$	65.3 ± 5.4^{f}	67.8 ± 4.3^{f}	
IL-1β	C	60.4 ± 3.0^{ab}	66.9 ± 2.2^{a}	58.6 ± 1.3^{b}	0.0012
	E	$76.5 \pm 1.9^{*e}$	61.3 ± 2.4^{f}	64.6 ± 4.5^{f}	
IL-6	C	95.0 ± 4.5	94.1 ± 3.2	87.5 ± 4.9	0.0974
	E	$120.6 \pm 6.2^{*e}$	97.2 ± 5.8^{f}	100.0 ± 5.3^{f}	
IL-10	C	88.2 ± 3.2	89.5 ± 4.9	75.9 ± 5.6	0.0263
	E	$115.8 \pm 4.4^{*e}$	92.4 ± 4.4^{f}	92.8 ± 3.3^{f}	

[1]Values are expressed as the mean ± SEM. Means between groups C and E with $*$ significantly differ ($p < 0.05$). Means among groups C, CF25, and CF57 with different superscript letters (a, b) significantly differ ($p < 0.05$). Means among groups E, EF25, and EF57 with different superscript letters (e, f) significantly differ ($p < 0.05$). [2]Details are the same as those described in the footnotes of Table 1.

Table 6: Plasma adiponectin levels in each group[1,2].

		—	F25	F57	Ethanol* and fish oil
Adiponectin	C	15.0 ± 0.5	16.0 ± 0.5	16.5 ± 0.8	0.1805
	E	$8.2 \pm 0.7^{*f}$	12.5 ± 1.2^{e}	11.2 ± 1.1^{ef}	

[1]Values are expressed as the mean ± SEM. Means between groups C and E with $*$ significantly differ ($p < 0.05$). Means among groups C, CF25, and CF57 with different superscript letters significantly differ ($p < 0.05$). Means among groups E, EF25, and EF57 with different superscript letters (e, f) significantly differ ($p < 0.05$). [2]Details are the same as those described in the footnotes of Table 1.

compared to group E ($p < 0.05$). The plasma ICAM-1 concentration in group EF25 was significantly lower than that in group E ($p < 0.05$).

3.6. Small-Intestinal Histopathological Examination and the TJ Protein ZO-1 Distribution. According to the Chiu's Score Classification of Small-Intestinal Injury, scores of small-intestinal injury are shown in Figure 3(b). There were no differences among all groups, but groups E, EF25, and EF57 showed a higher trend compared to group C. Scores ranged 2~4, which means the presence of cellular lysis, increased spacing among villosities, structural destruction of the villosities, and so forth (Figure 3(a)). ZO-1 expression in the small-intestinal mucosa was examined by IHC, which revealed that the epithelial structure differed among groups (Figure 4). In group C, the epithelium of the small-intestinal mucosa was intact. Compared to groups C and CF25, group CF57 displayed a significantly larger ZO-1 immunoreactive area ($p < 0.05$). However, there was no change between groups C and E and even among the ethanol-intake groups.

3.7. Plasma Endotoxin Level. As shown in Table 8, the plasma endotoxin level was significantly higher in group E compared to that of group C ($p < 0.05$). However, groups EF25 and EF57 presented significantly lower plasma endotoxin concentrations compared to group E ($p < 0.05$).

4. Discussion

Similar to our previous studies, the average ethanol intake was 11.1~11.4 g/kg BW/day in the ethanol-intake groups, which would be comparable to heavy drinkers in humans (more than 50~60 g/day of absolute alcohol) after conversion of animal doses to a human equivalent based on body surface areas [16, 17].

Rats fed with the ethanol-containing liquid diet (group E) for 8 weeks showed a slight loss of body weight. However, when rats simultaneously consumed ethanol and fish oil (groups EF25 and EF57), the final body weights significantly decreased (Table 1). Fish oil is associated with a body weight-loss effect in high-fat diet-induced obese animal studies [24, 25]. The potential antibody fat mechanisms of fish oil were suggested to include increased plasma adiponectin levels [25], increased adipocyte apoptosis [26], and altered fat oxidation [27]. Therefore, effects of ethanol and fish oil on adipose tissues should be checked in future studies.

Higher AST and ALT activities, hepatic lipid accumulation, and inflammatory cell infiltration were observed in group E rats (Tables 2 and 3). Ethanol abuse induces hepatic fatty liver and inflammation as proven by hundreds of studies [28] and also by our previous studies [11, 12, 20]. Ethanol-induced pathological alterations in the liver are caused by abnormal lipid metabolism, an imbalance between pro- and anti-inflammatory cytokines, and an elevated plasma endotoxin level [20]. In the present study, fish oil displayed hepatoprotective effects in rats fed with ethanol-containing liquid diets based on the lower ALT activity and hepatic histopathological scores (Tables 2 and 3). We speculated that the protective mechanisms of fish oil in rats with ethanol-induced liver injuries might be associated with antilipid accumulation, antioxidative stress (Table 4), and immunoregulatory effects (Table 5). The antioxidative potential of fish oil is controversial. Ramaiyan et al. suggested that fish oil which was added to the AIN-70 diet (50 g/kg diet, 2.5 g/kg body weight) decreased hepatic TBARS contents in rats [29]. On the contrary, Tsuduki et al. indicated that the consumption of a fish oil diet (fish oil: safflower oil ratio of 50 : 50 g/kg of diet, 5.53 g/kg body weight) for 28 weeks significantly increased plasma and hepatic TBARS contents in male SAMP8 mice [30]. In the present study, fish oil intake levels were 1.07 and 2.43 g/kg body weight in rats fed with fish oil, which were similar to levels in Kikugawa et al.'s study [27]. Therefore, appropriate proportions of SFAs, MUFAs, and PUFAs are very

Table 7: Plasma vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecular (ICAM)-1 levels of rats in each group[1,2].

(ng/mL)		—	F25	F57	Ethanol* and fish oil
VCAM-1	C	124.21 ± 12.16	147.56 ± 16.23	132.45 ± 16.57	0.0085
	E	187.81 ± 33.07^{e}	86.13 ± 9.35^{f}	83.95 ± 5.29^{f}	
ICAM-1	C	28.64 ± 1.24^{a}	23.86 ± 1.23^{b}	26.02 ± 0.72^{ab}	0.1612
	E	36.58 ± 1.27^{*e}	26.56 ± 0.79^{f}	32.9 ± 1.79^{e}	

[1]Values are expressed as the mean ± SEM. Means between groups C and E with * significantly differ ($p < 0.05$). Means among groups C, CF25, and CF57 with different superscript letters (a, b) significantly differ ($p < 0.05$). Means among groups E, EF25, and EF57 with different superscript letters (e, f) significantly differ ($p < 0.05$). [2]Details are the same as those described in the footnotes of Table 1.

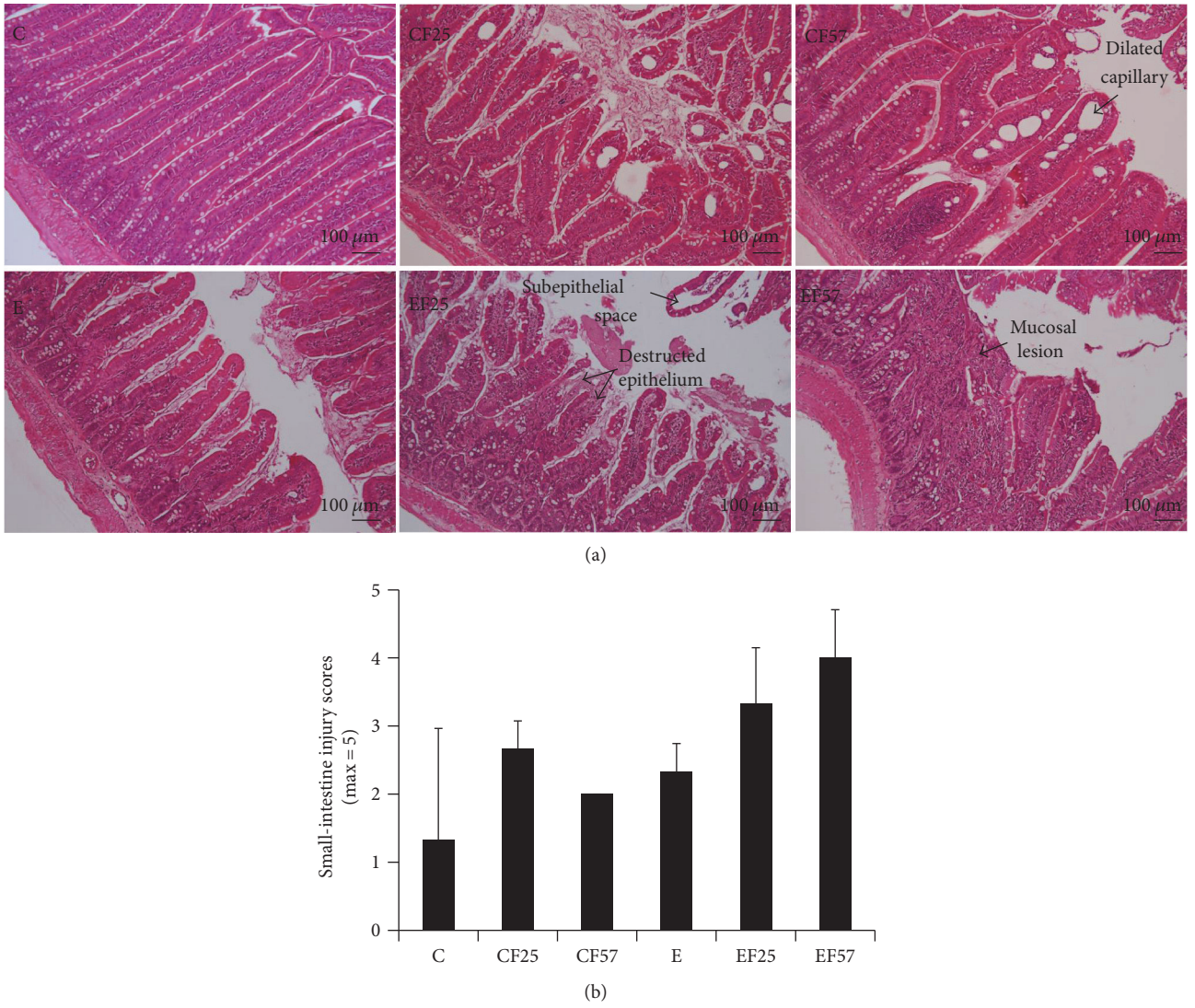

Figure 3: Score of small-intestinal injury in each group. C: control group; CF25: control diet with fish oil substituted for 25% of olive oil; CF57: control diet with fish oil substituted for 57% of olive oil; E: ethanol group; EF25: alcohol-containing diet with fish oil substituted for 25% of olive oil; EF57: alcohol-containing diet with fish oil substituted for 57% of olive oil. (a) Representative histological images of rats in all groups at 100x magnification. (b) Quantification of the small-intestinal injury score among groups.

important for preventing diseases induced by oxidative stress [31]. On the other hand, several studies substantiated that the anti-inflammatory effects of fish oil were related to the production of E-series resolvins (from EPA) and D-series resolvins (from DHA) through the cyclooxygenase (COX)-2 pathway [32]. In our previous study, we also found that fish oil normalized hepatic pro- and anti-inflammatory cytokine secretions in rats under chronic ethanol abuse [20].

Adiponectin inhibits expressions of ICAM-1 and VCAM-1 through inhibiting nuclear factor (NF)-κB activation and has several antiatherogenic and anti-inflammatory properties [33]. Moreover, several animal models indicated

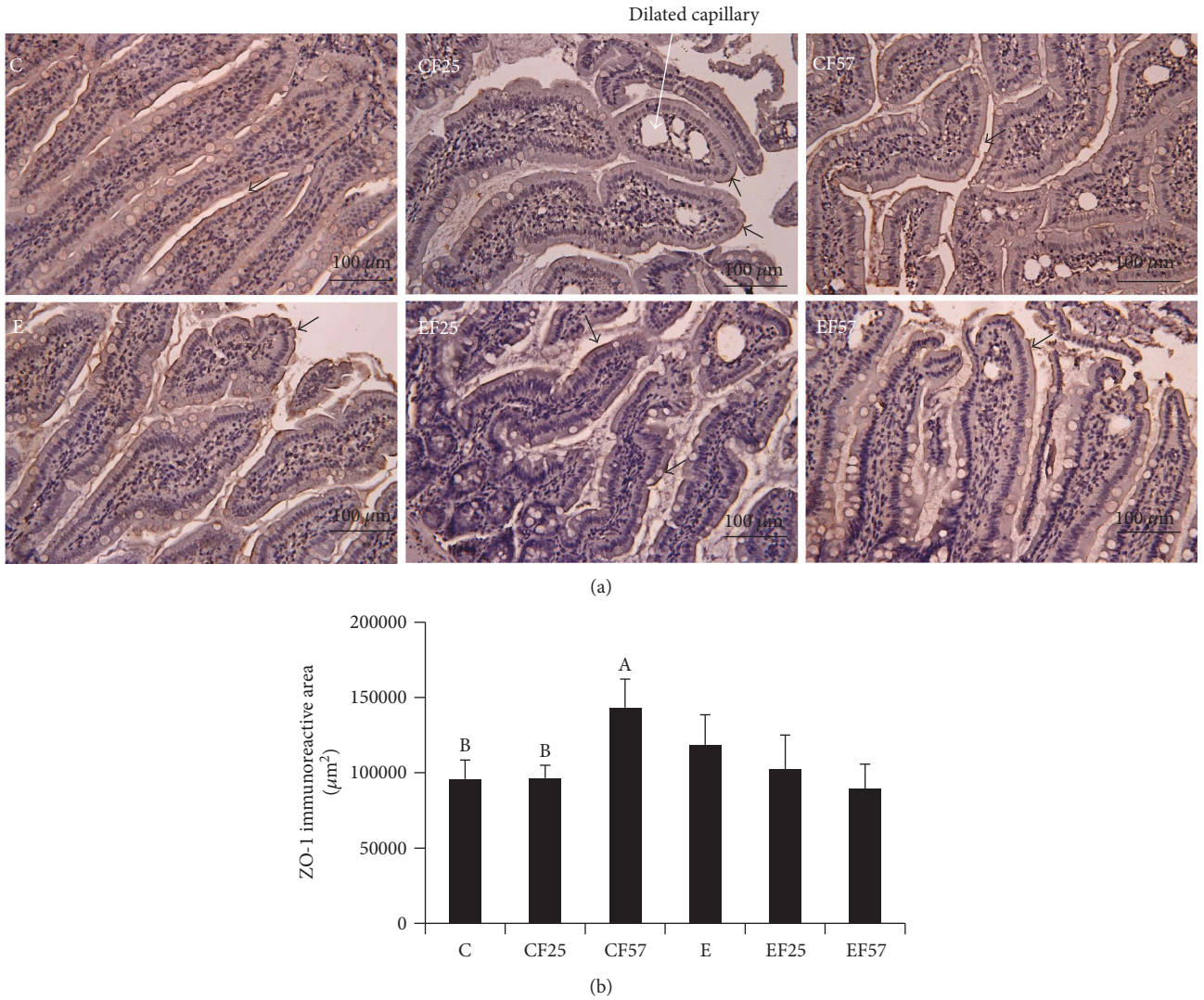

Figure 4: IHC staining of the tight junction protein, ZO-1, in the small-intestinal mucosa in each group. C: control group; CF25: control diet with fish oil substituted for 25% of olive oil; CF57: control diet with fish oil substituted for 57% of olive oil; E: ethanol group; EF25: alcohol-containing diet with fish oil substituted for 25% of olive oil; EF57: alcohol-containing diet with fish oil substituted for 57% of olive oil. (a) Representative histological images of rats in all groups at 200x magnification. Arrows indicate ZO-1-positive areas. The normal small intestine exhibited intact epithelium with marked dark-brown ZO-1 expression. (b) Quantification of ZO-1-immunoreactive areas among groups. Bars with different letters (A, B) significantly differ among groups C, CF25, and CF57 at the $p < 0.05$ level according to a one-way analysis of variance (ANOVA) followed by Duncan's new multiple range test.

Table 8: Plasma endotoxin levels in each group[1,2].

(EU/ml)		—	F25	F57	Ethanol* and fish oil
Endotoxin	C	20.71 ± 0.27	19.36 ± 0.82	18.95 ± 0.84	0.0064
	E	$24.67 \pm 1.22^{*e}$	17.8 ± 1.87^{f}	16.18 ± 1.12^{f}	

[1]Values are expressed as the mean ± SEM. Means between groups C and E with * significantly differ ($p < 0.05$). Means among groups E, EF25, and EF57 with different superscript letters (e, f) significantly differ ($p < 0.05$). [2]Details are the same as those described in the footnotes of Table 1.

that hypoadiponectinemia and altered hepatic adiponectin signaling induced by chronic ethanol intake are associated with steatosis and inflammation [34]. We also found that the plasma adiponectin level significantly decreased; in contrast, plasma ICAM and VCAM levels increased in rats fed with ethanol only (group E in Tables 6 and 7). However, when ethanol-fed rats ingested fish oil, lower plasma adiponectin levels were ameliorated; in addition, plasma ICAM and VCAM levels were reduced (groups EF25 and EF57 in Tables 6 and 7). Dietary intake of omega-3 (n-3) PUFAs has emerged as an important way to modify cardiovascular risk by regulating the endothelial expression of adhesion

molecules and adipokines, such as ICAM, VCAM, and adiponectin in cardiovascular diseases and diabetes [35, 36]. To our best knowledge, this is the first study to find that fish oil substitution in the diet can increase plasma adiponectin levels and decrease plasma adhesion molecules in rats under chronic ethanol feeding. Further studies are necessary to clarify the relationship between fish oil and lipid metabolism-related molecular factors of the adiponectin regulatory pathway.

A previous study indicated that ethanol and its metabolites (such as acetaldehyde) destroy intestinal epithelial TJ proteins, including ZO-1 and occludin, and thus cause poor integrity of the gut barrier in a chronic ethanol-intake animal model [37]. In this study, no differences in small-intestinal injury or the ZO-1 immunoreactive area were found in rats fed with ethanol (group E in Figures 3 and 4); nevertheless, rats that were fed with ethanol chronically for 8 weeks (group E) showed significantly higher plasma endotoxin levels (Table 8). Thus, data on intestinal histopathology in this study were insufficient to explain the hyperendotoxinemia in rats exposed to chronic ethanol intake. The other TJ protein, occludin, or the intestinal permeability regulator, zonulin, should be measured in future studies [38]. Interestingly, when fish oil was substituted for olive oil in the nonethanol-containing diet (group CF57), a significantly larger ZO-1 immunoreactive area was detected (Figure 4). In contrast, no obvious change in the ZO-1 immunoreactive area was observed in rats fed with fish oil and an ethanol-containing diet (group EF57, Figure 4). The feeding pattern which mixed fish oil into the ethanol-containing liquid diet might be a possible reason for the weakened protective effect on the intestinal epithelium by fish oil supplements. However, we still found that fish oil ameliorated high plasma endotoxin levels in rats under chronic ethanol-intake (groups EF25 and EF57, Table 8). Mani et al. indicated that postprandial serum endotoxin concentrations increased after a meal rich in SFAs and decreased with higher n-3 PUFA intake in a pig model [39]. A previous study also demonstrated that the signaling and transport processes for endotoxin are initiated in specialized membrane microdomains called lipid rafts, and oil rich in n-3 PUFAs may unsettle lipid rafts that inhibit greater endotoxin transport [39, 40]. Thus, we propose that the mechanism of n-3 PUFA-enriched fish oil inhibiting endotoxin transport across the intestinal epithelium may be associated with fatty acid regulation of intestinal membrane lipid rafts rather than the structural integrity.

In this study, no dose-response effect of fish oil substitution levels on alcohol-induced liver damage was observed in the hepatic histopathological score or inflammatory factors, including cytokines, adhesion molecules, and adipokines. Therefore, based on our data, taking more fish oil supplements cannot provide greater protective effects against alcoholic liver injuries in rats.

5. Conclusions

In conclusion, chronic ethanol feeding elevated the plasma endotoxin level that may trigger inflammatory responses and consequently contribute to liver injury. Moreover, fish oil substituted for olive oil under ethanol exposure inhibited the appearance of endotoxin in the circulation, thus decreasing inflammatory responses which exert a hepatoprotective potential in rats under chronic ethanol feeding. However, the mechanism of decreased plasma endotoxin levels by fish oil supplementation might not be related to improved intestinal structural integrity.

Conflicts of Interest

The authors declare that there are no conflicts of interests regarding the publication of this paper.

Authors' Contributions

Dr. Yi-Wen Chien is the first author. Yi-Wen Chien and Suh-Ching Yang designed the study. Hsiang-Chi Peng and Ya-Ling Chen carried out the experiments. Man-Hui Pai conducted the small-intestinal histopathological examination. Hsiao-Li Chuang measured plasma endotoxin levels. Suh-Ching Yang, Hsiang-Chi Peng, and Hsiao-Yun Wang wrote the original manuscript.

Acknowledgments

This study was funded by the Ministry of Science and Technology, Taiwan (NSC100-2320-B-038-023-MY3; MOST104-2320-B-038-041-MY3). The authors thank VIVA Life Science, Taiwan branch, for providing the fish oil.

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Hindawi
Mediators of Inflammation
Volume 2017, Article ID 4265898, 8 pages
https://doi.org/10.1155/2017/4265898

Research Article

Reclamation of Herb Residues Using Probiotics and Their Therapeutic Effect on Diarrhea

Fanjing Meng,[1] Tingtao Chen,[1,2] Dongwen Ma,[1] Xin Wang,[2,3] Xiaoxiao Zhao,[1] Puyuan Tian,[1,2] Huan Wang,[1,3] Zhiwen Hai,[1] Liang Shen,[4] Xianyao Tang,[1] Xiaolei Wang,[1] and Hongbo Xin[1]

[1]*Institute of Translational Medicine, Nanchang University, Nanchang, Jiangxi 330031, China*
[2]*State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang, Jiangxi 330047, China*
[3]*School of Life Sciences, Nanchang University, Nanchang, Jiangxi 330047, China*
[4]*Department of Obstetrics and Gynaecology, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, Shandong 250012, China*

Correspondence should be addressed to Tingtao Chen; chentingtao1984@163.com and Hongbo Xin; hongboxin@yahoo.com

Received 18 March 2017; Revised 3 August 2017; Accepted 11 October 2017; Published 29 November 2017

Academic Editor: Helieh S. Oz

Residues from herbal medicine processing in pharmaceutical plants create a large amount of waste (herb residues), which consists mainly of environmental pollution and medicinal waste. In order to resolve this problem, probiotics of *Bacillus (B.) subtilis*, *Aspergillus (A.) oryzae*, and *Lactobacillus (L.) plantarum* M3 are selected to reuse herb residue of Jianweixiaoshi tablets (JT), and an antibiotic-associated diarrhea (AAD) mouse model was established to evaluate the therapeutic effects of the herb residue fermentation supernatant. Our results indicated that the fermentation supernatant had scavenged 77.8% of 2,2-diphenyl-1-picrylhydrazyl (DPPH), 78% of $O_2^{\bullet-}$, 36.7% of $^{\bullet}OH$, 39% of Fe^{2+} chelation, and 716 mg/L reducing power. The inhibition zones for *Salmonella (S.) typhimurium*, *S. enteritidis*, *Shigella (Sh.) flexneri*, *Escherichia (E.) coli*, *Listeria (L.) monocytogenes*, *Sh. dysenteriae* 301, and *Staphylococcus (S.) aureus* were 17, 14, 19, 18, 20, 19, and 20 mm, respectively. The in vivo results indicated that the fermentation supernatant resulted in a high diarrhea inhibition rate (56%, $p < 0.05$), greatly enhanced the disruption of bacterial diversity caused by antibiotics, and restored the dominant position of *L. johnsonii* in the treatment and recovery stages. Therefore, the combination of the herb residue and probiotics suggests a potential to explore conversion of these materials for the possible development of therapies for AAD.

1. Introduction

Traditional Chinese herbal medicine (TCHM) is an essential part of the healthcare system in China, Hong Kong, and several other Asian countries, whereas it is considered as a complementary or alternative medical system in most Western countries [1]. At present, approximately 12,000,000 tons of herb residues are generated annually by 1500 Chinese medicine enterprises in China [2].

The active ingredients of TCHM are the secondary metabolites of plants, and the low decoction efficiency leaves approximately 30%–50% of the medicinally active substances in their herb residues [1]. In addition, herb residues are mostly disposed of through stacking in the open, sanitary burial, or burning, causing serious environmental pollution, especially affecting water quality in China [3]. Therefore, the huge amounts of herb residues produced by the continuous development of the Chinese herbal medicine industry have become a serious problem for large pharmaceutical companies.

The microorganism fermentation theory suggests that the digestive enzymes (e.g., cellulase, protease, pectinase and lignin enzymes, and lipase) produced by microorganisms could effectively degrade plant cell walls, expand the intercellular region, and improve the extraction yield of active ingredients [4, 5]. Moreover, probiotics (microorganisms) are now

accepted as useful in the prevention and/or treatment of certain pathological conditions, especially diarrhea, when administered in adequate amounts [6–9]. *Bacillus (B.) subtilis* is one of the bacterial champions in secreted enzyme production as an immunostimulatory agent to aid treatment of gastrointestinal tract diseases [10]; *Aspergillus (A.) oryzae* has been widely used in various traditional fermented foods and endow them a great taste and aroma [11]; *Lactobacillus (L.) plantarum* is commonly found in many fermented food products, and it can help suppress the growth of gas producing bacterium in the intestines and may have benefit in some patients who suffer from intestinal tract diseases [12]. Therefore, the combination of probiotics of *B. subtilis*, *A. oryzae*, and *L. plantarum* M3 not only participate the digestion, absorption, and metabolism of protein, carbohydrate, and fat via synthesizing the nutrients of vitamins and folic acids but also endow their probiotic characteristics into the fermentation [13].

Antibiotic-associated diarrhea (AAD) is clearly one of the most common side effects encountered with antimicrobial treatment, which is caused by the intestinal microbiota changes and overgrowth of potentially pathogenic organisms [14]. Jianweixiaoshi is a TCHM constituted of *Pseudostellaria heterophylla* root tuber (Tai Zi Shen), *Dioscorea opposita* rhizome (Shan Yao), *Hordeum vulgare* fruit (Mai Ya), *Crataegus pinnatifida* fruit (Shan Zha), *Citrus reticulata* pericarp (Chen Pi), and Jianweixiaoshi tablets (JT) (a trademark ® Z20013220) approved by the Ministry of Public Health as treatment for intestinal diseases. In the present study, probiotics were used to ferment the herbal residues in JT as therapeutic potential against AAD in an in vivo model.

2. Materials and Methods

2.1. Antioxidative and Antibacterial Activity of the Fermentation Supernatant. Herb residue of JT was obtained from River Pharmaceutical Co. Ltd. and mashed using a pulper within 2 h. The bacteria *B. subtilis*, *A. oryzae*, and *L. plantarum* M3 (10^8 cfu/mL) were used as an inoculum for preparing the herb residue fermentation supernatant. In short, *B. subtilis* and *A. oryzae* were added to the fermentation substrate for 24 h, and then *L. plantarum* M3 was added for another 24 h. Then, the clearance of 2,2-diphenyl-1-picrylhydrazyl (DPPH), $O_2^{\bullet-}$, and·OH; Fe^{2+} chelation; and the redox activity of the fermentation supernatant were measured exactly as described in reference [15].

For antimicrobial activity, overnight (12 h) cultures of pathogenic microorganisms including *Salmonella (S.) typhimurium* ATCC 13311, *S. enteritidis* ATCC13076, *Shigella (Sh.) flexneri* ATCC 12022, *Escherichia (E.) coli* 44102, *Listeria (L.) monocytogenes* ATCC 19111, *Sh. dysenteriae* 301, and *Staphylococcus (S.) aureus* Cowan 1 were spread on the surface of LB agar plates, and the culture supernatant (200 μL) was loaded into an Oxford cup (outer diameter 7.8 ± 0.1 mm, inner diameter 6.0 ± 0.1 mm, and height 10.0 ± 0.1 mm), which was placed on the surface of the agar. The size of the inhibition zone was measured until the formation of a clear zone around the Oxford cup. The experiment was carried out in duplicate [16, 17].

2.2. Diarrhea Model and Treatment. The study was approved by the Ethical Committee of the Second Affiliated Hospital of Nanchang University, and all methods were conducted in accordance with the approved guidelines.

Specific pathogen-free 6- to 8-week-old male C57BL/6 mice were housed and fed a commercial diet, with water ad libitum. To establish the diarrhea model, 0.15 mL/day lincomycin hydrochloride (40 mg/mL) were administered to mice via orogastric inoculation for 5 days. All noninfected control animals were inoculated with the same volume of phosphate buffered saline (PBS). Then, mice were divided into three groups as follows: modeling group ($n = 10$), modeling mice only given PBS; probiotics + drug residues group ($n = 10$), modeling mice given herb residue fermentation supernatant; and JT group ($n = 10$), modeling mice given JT.

The feces of mice were collected in the control stage (day 0, with no treatment), modeling stage (day 5, with the inoculation of lincomycin hydrochloride), treatment stage (day 10, with the drug treatment), and recovery stage (day 17, with no management). Then, the feces of three mice in the modeling group, probiotics + drug residues group, and JT group were randomly chosen for analysis by denaturing gradient gel electrophoresis (DGGE).

2.3. Determination of the Diarrhea Indexes. On the second day of treatment (day 7), mice were placed in cages and the cage bottoms lined with filter paper to observe the occurrence of diarrhea. Mouse feces were divided into five types: 1, normal feces; 2, normal shape with wateriness; 3, soft feces with normal shape; 4, watery stool; and 5, mucous stool. The normal feces and normal-shaped feces with wateriness were deemed normal feces, and the normal-shaped soft feces, watery stool, and mucous stool were regarded as diarrhea. Filter papers were changed once the diarrhea occurred, and the loose stool rate and diarrhea inhibition rate were counted within 6 h. The loose stool rate (%) = (number of loose stools for each mouse/total feces number of each mouse) × 100; diarrhea inhibit rate (%) = ((number in control group with diarrhea − number in treatment group with diarrhea)/number in control group with diarrhea)H × 100.

2.4. DGGE Analysis. DNA was isolated by a bead-beating method, and the bacterial and *Lactobacillus* primers were used for DGGE analysis [18, 19]. The bands of interest in DGGE gels were excised using a sterile blade and incubated overnight at 4°C in TE buffer (pH 8.0) to allow DNA diffusion for further amplifications. PCR products for sequencing were purified using the QIAquick PCR purification kit and subcloned using the pMD18-T vector system I (Takara) according to the manufacturer's instructions, and the transformants were randomly picked and sequenced by Invitrogen (Shanghai, China) [20, 21].

2.5. Data Analysis. Data are reported as means ± SD, and results were analyzed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA) by means of an independent one-way ANOVA test at each sampling point. The differences between the three groups were assessed by means of the least significant difference (LSD) multiple comparison test ($P < 0.05$).

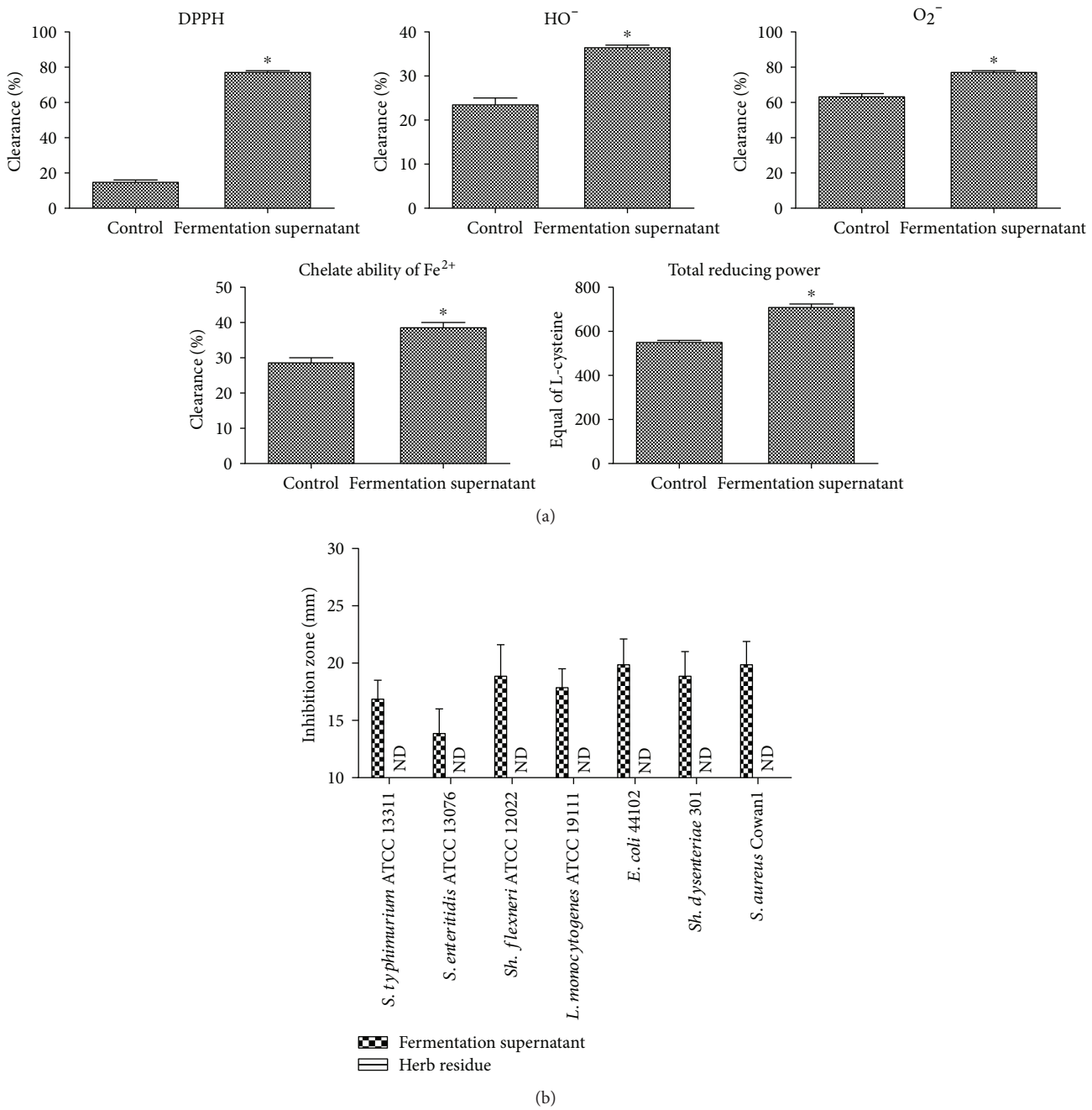

Figure 1: Antioxidative (a) and antibacterial activity (b) of fermentation supernatant against selected foodborne pathogens compared with the control group (the herb residues). Data are shown as the mean ± SD. $^{*}p < 0.05$.

3. Results

3.1. Antioxidative and Antibacterial Activity of Herb Residue Fermentation Supernatant. Compared with the herb residues (control group), the fermentation supernatant (probiotics + drug residues group) had significantly enhanced DPPH clearance, OH clearance, $O_2^{\bullet-}$ clearance, and Fe^{2+} chelation and reduction activity (Figure 1, $p < 0.05$). Interestingly, no antimicrobial effect was observed using herb residues, while the addition of probiotics conferred 100% inhibitory activity against all pathogens tested on the fermentation supernatant, for example, *S. typhimurium* ATCC 13311 (inhibition zone diameter: 17 mm), *S. enteritidis* ATCC13076 (IZD: 14 mm), *Sh. flexneri* ATCC 12022 (IZD: 19 mm), *E. coli* 44102 (IZD: 18 mm), *L. monocytogenes* ATCC 19111 (IZD: 20 mm), *Sh. dysenteriae* 301 (IZD: 19 mm), and *S. aureus* Cowan 1 (IZD: 20 mm) (Figure 1).

3.2. Diarrhea Model and Treatment. Compared with the modeling group, both the fermentation supernatant group and JT group showed significant inhibition of the average diarrhea frequency and ratio of diarrhea ($p < 0.05$), of which the fermentation supernatant possessed the highest diarrhea inhibition rate (56%) (Table 1).

Table 1: The effects of fermentation supernatant on the diarrhea of mice.

Groups	Total diarrhea frequency	Average diarrhea frequency	Ratio of diarrhea (%)	Inhibition ratio of diarrhea (%)
Modeling group	72	7.2 ± 0.31	78 ± 2.67	/
Probiotics + drug residues group	32	$3.2 \pm 0.23^*$	$39 \pm 1.24^*$	56
JT group	58	$5.8 \pm 0.24^*$	$61 \pm 2.31^*$	19

Note: data are shown as the mean ± SD. $^*p < 0.05$ (compared with the modeling group).

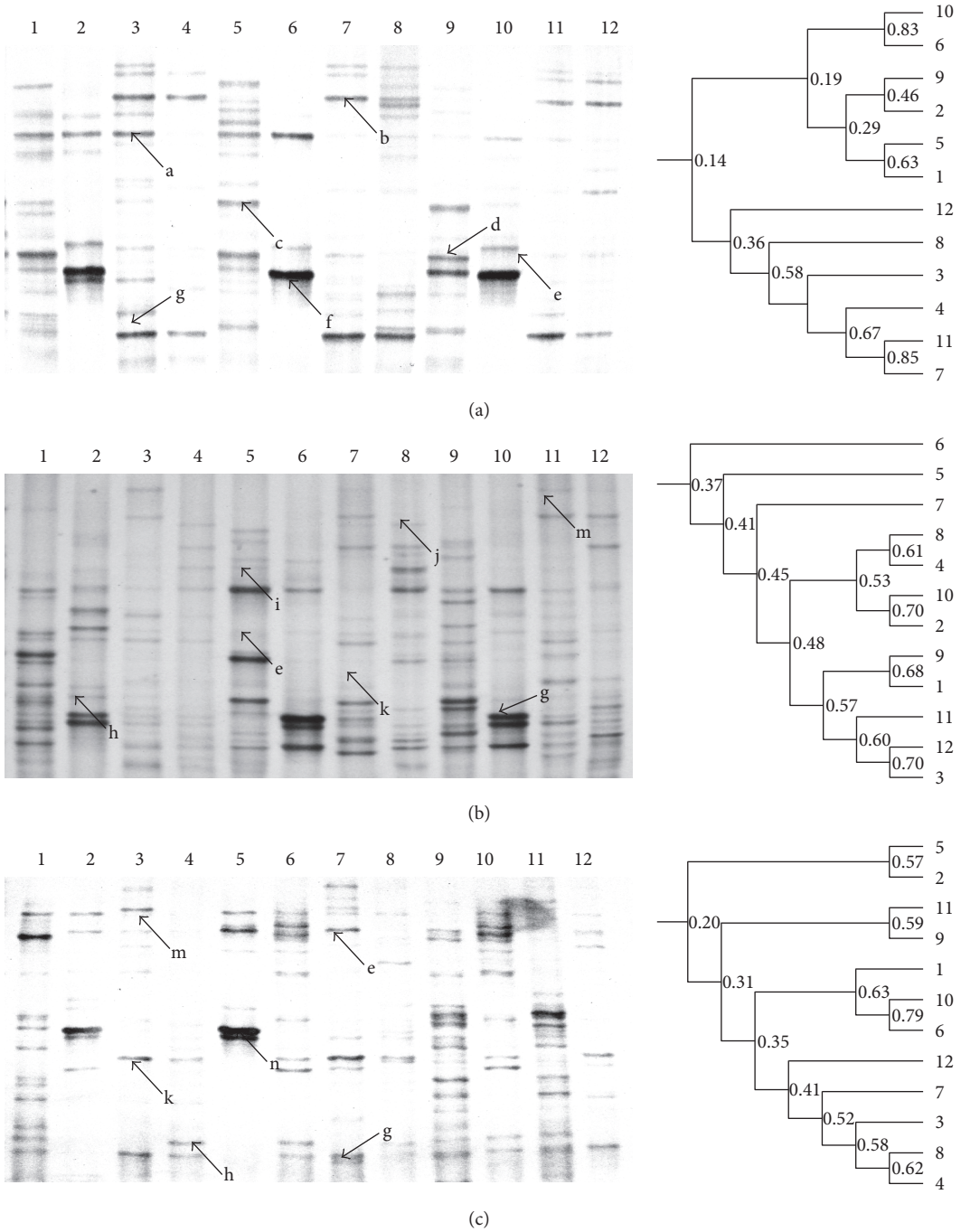

Figure 2: DGGE profile and UPGMA analysis of the fecal microbiota using bacterial primers. (a, b, c) refer to the modeling group, probiotics + drug residues group, and JT group; L1–L3 from the control stage, L4–L6 from the modeling stage, L7–L9 from the treatment stage, and L4–L6 from recovery stage. The corresponding strains were seen in Table 2.

3.3. Effects of Herb Residue Fermentation Supernatant on Bacterial Diversity in the Intestine. The DGGE results indicated that bands b (uncultured bacterium) and g (uncultured *Bacteroidetes* bacterium) occupied the dominant positions in the modeling group and appeared in all stages. Band a (*Enterococcus* sp.), the dominant bacterium in the control stage, disappeared or weakened after antibiotic treatment (Figure 2(a)). For the fermentation supernatant and JT groups, bands m (uncultured bacterium) and g (uncultured *Bacteroidetes*) were the dominant bacteria and existed in all stages, and the administration of fermentation supernatant selectively enhanced bands e (*L. johnsonii*), j (uncultured bacterium), k (*Enterococcus* sp.), and m (uncultured bacterium), which became the dominant bacteria in the treatment and recovery stages (Figure 1(b) and Table 2).

Moreover, the DGGE profile indicated that antibiotic administration severely reduced bacterial diversity (band numbers), while the administration of fermentation supernatant and JT prevented the decreasing trends and enhanced bacterial richness in mouse intestines (Figure 2). The unweighted pair-group method with arithmetic means (UPGMA) results showed that orally administered antibiotics had seriously changed the bacterial composition, reduced bacterial diversity, and could not restore bacterial diversity to its original level, even after the recovery stage. For the probiotics + drug residues and JT groups, both the fermentation supernatant and JT greatly enhanced the reduced bacterial diversity caused by antibiotics, and the greater similarity of lanes 2 and 10 (70%), lanes 1 and 9 (68%), and lanes 3 and 12 (70%) indicated that the combination of probiotics and herb residues were the most effective at restoring the destroyed intestinal bacteria to the original levels (Figure 2).

3.4. Effects of Herb Residue Fermentation Supernatant on Bacillus Diversity in the Intestine. For bacillus DGGE profiles, the antibiotics eliminated band b (*L. johnsonii*) in the treatment and recovery stages in the modeling group, and the same strain regained its position as the dominant bacterium in both the fermentation supernatant and JT groups (Figure 3). Moreover, the addition of fermentation supernatant made band e (*Clostridium* sp.) the dominant bacterium in the treatment and recovery stages (Figure 3(b)).

Table 2: Strains identified from mouse intestine by denaturing gradient gel electrophoresis using bacterial primers and bacillus primers.

Strain number	Closest relatives	Similarity (%)	GeneBank number
Bacterial primers			
a	*Enterococcus* sp.	100	AB602933.1
b	Uncultured bacterium	100	HQ321987.1
c	Uncultured bacterium	100	GQ001435.1
d	Uncultured *Bacilli*	100	EF698450.1
e	*Lactobacillus johnsonii*	100	CP002464.1
f	*Helicobacter pullorum*	100	GU902714.1
g	Uncultured *Bacteroidetes*	100	HM442510.1
h	*Clostridium paraputrificum*	100	AB627080.1
i	Uncultured bacterium	100	EU505174.1
j	Uncultured bacterium	100	EU656086.1
k	*Enterococcus* sp.	100	JF910016.1
m	Uncultured bacterium	100	GU606372.1
n	Uncultured bacterium	100	JF837882.1
Bacillus primers			
a	Uncultured bacterium	100	HM363549.1
b	*Lactobacillus johnsonii*	100	CP002464.1
c	Uncultured bacterium	99	HM363550.1
d	Uncultured bacterium	100	FJ881122.1
e	*Clostridium* sp.	99	Y10584.1
f	Uncultured bacterium	100	EU006396.1
g	Uncultured bacterium	100	EU475615.1
h	*Enterococcus faecium*	100	HQ384298.1
i	Uncultured bacterium	100	EU491355.1
j	Uncultured bacterium	100	EU006313.1

4. Discussion

AAD is a form of diarrhea that occurs during or shortly after administration of an antibiotic, with an occurrence rate in the range of 1%–44% depending on the population and type of antibiotic [22, 23]. Overgrowth of potentially pathogenic organisms, and the changes in carbohydrate metabolism with decreased short-chain fatty acid absorption result in diarrhea [14], which can be treated with traditional Chinese medicine and probiotics [24, 25].

In China, JT generate more than 1.2 billion RMB of income for businesses each year, but they also produce approximately 100,000 tons of herb residue. *Pseudostellaria heterophylla* root tuber (Tai Zi Shen), *Dioscorea opposita* rhizome (Shan Yao), *Hordeum vulgare* fruit (Mai Ya), *Crataegus pinnatifida* fruit (Shan Zha), and *Citrus reticulata* pericarp (Chen Pi) contained in JT are useful for digestion, anorexia, abdominal distension, invigorating the stomach, and restoring tone to the spleen. It is claimed that JT promote gastrointestinal peristalsis and gastric secretion of digestive juices and enhance pepsin activity, physique, and immune function, and no side effect of diarrhea is reported. Moreover, probiotics are now accepted as useful in the prevention and/or treatment of certain pathological conditions [17]. At present, the most studied probiotics are lactic acid-producing bacteria, particularly *Lactobacillus* species [26], which are proven to be useful in the treatment of several gastrointestinal diseases, such as acute infectious diarrhea or pouchitis, and a metastudy suggested that probiotics might be beneficial for AAD prevention [25]. Therefore, a combination of the spleen-stomach strengthening effect (herb residues) and the diarrhea prevention effect (probiotics) might be a perfect choice for diarrhea treatment. In our previous study, we found that the herb residues fermented by *L. plantarum* (HM218749)

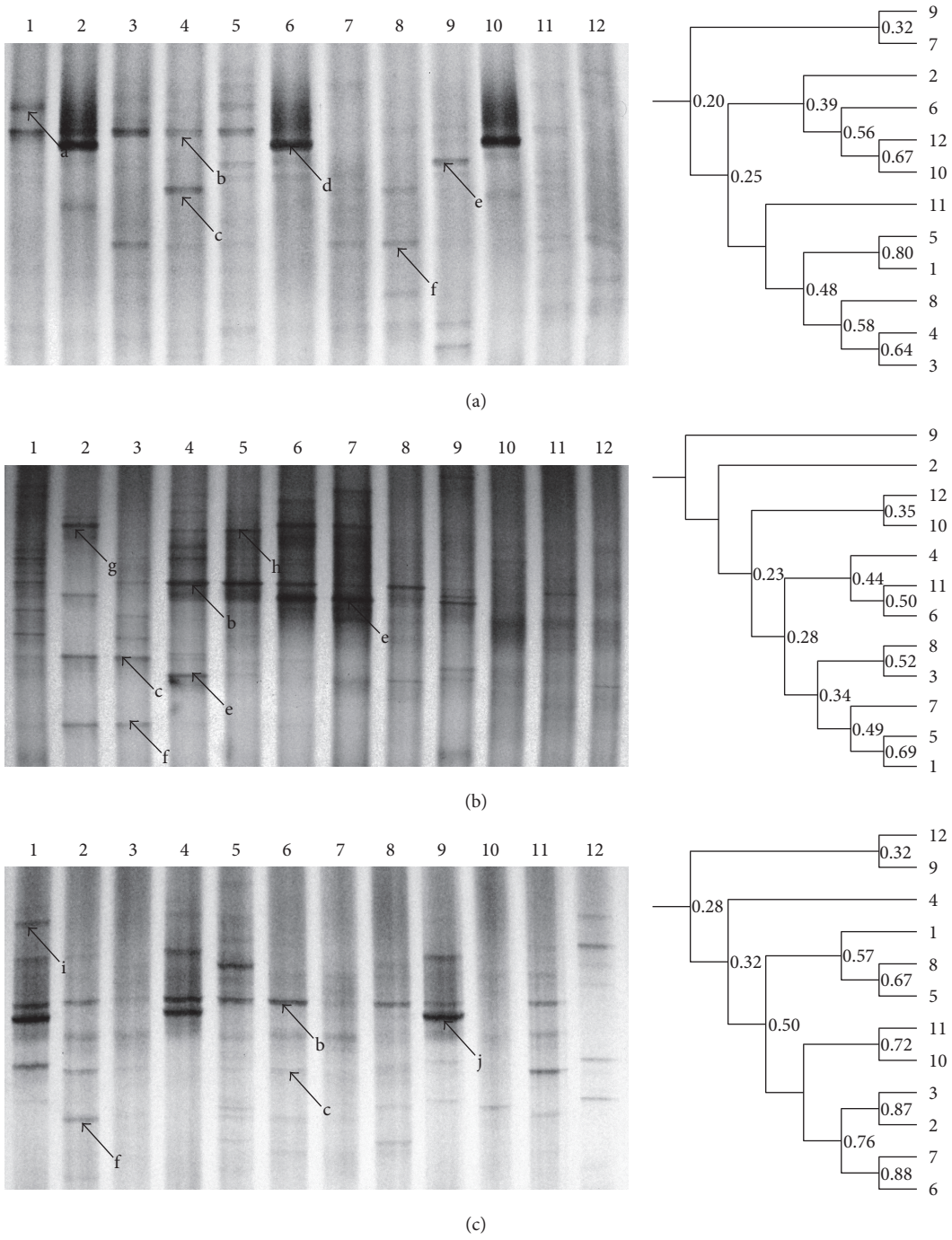

Figure 3: DGGE profile and UPGMA analysis of the fecal microbiota using bacillus primers. (a, b, c) refer to the modeling group, probiotics + drug residues group, and JT group; L1–L3 from the control stage, L4–L6 from the modeling stage, L7–L9 from the treatment stage, and L4–L6 from the recovery stage. The corresponding strains were seen in Table 2.

had significantly inhibited urease activity and slowed cell infiltration and the inflammatory factors in blood of the mouse model of *Helicobacter pylori* infection [17], and we further discussed the antidiarrhea effect of the herb residue fermentation supernatant in this study.

As we know, diarrhea is characterized by an overgrowth of opportunistic pathogens and a drastic reduction of probiotics (e.g., *Lactobacilli*, *Bacteroides*, and *Bifidobacteria*), and the microbial imbalance will conversely lower nutrient absorption and immune capability and decrease resistance to colonization by pathogens, which further aggravates the illness [13]. Therefore, the sound clearance of DPPH (77.8%), OH (36.7%), and $O_2^{\bullet-}$ (78%) and Fe^{2+} chelation (39%) and reduction activity (716 mg/L), together with the 100% inhibition of all tested pathogens exhibited by the fermentation supernatant, indicated a promising antidiarrheal

effect. Moreover, antibiotics seriously lowered the mice's spirits and significantly increased the total frequency of diarrhea (72), average diarrhea frequency (7.2), and diarrhea ratio (78), even 2 days after the modeling, while the fermentation supernatant significantly inhibited the diarrhea rate (56%, $p < 0.05$) (Table 1).

As the gut microbiome plays a major role in the production of vitamins, enzymes, and other compounds that digest and metabolize food and regulate the host immune system, it can be considered as an extra organ with remarkable dynamics and a major impact on host physiology [27], and the ratio of probiotics to pathogens has been regarded as one of the important standards to evaluate human health in Chinese hospitals. Therefore, DGGE was used to monitor microbial diversity in vivo. As shown in Figure 3 and Table 2, bacterial DGGE profiles indicated that the use of antibiotics severely decreased microbial diversity, and the reduction of bands in the modeling group indicated fewer choices for the host to defend itself against external invasion. Moreover, the enhanced diversity in the fermentation supernatant and JT groups indicated their strong recovery ability to guard host intestinal health. Moreover, the high similarity of the UPGMA index between the control and recovery stage in the probiotics + drug residues group indicated that the fermentation supernatant possessed a powerful capability to restore intestinal balance to its formal levels (Figure 2).

Moreover, the bacillus DGGE profiles also confirmed that antibiotics eliminated band b (*L. johnsonii*), while treatment with fermentation supernatant and JT restored the dominance of this bacterium in the treatment and recovery stages. *L. johnsonii* belongs to the class of lactic acid bacteria (LAB), which is evidenced by their generally recognized as safe (GRAS) status, due to their ubiquitous appearance in food and their contribution to the healthy microflora of human mucosal surfaces. Therefore, the recovery of the dominant *L. johnsonii* indicated good health status in mouse intestines.

In the present study, we report the conversion of herb residues of JT by probiotics to an antidiarrheal fermentation supernatant. This ingredient was shown to be effective against diarrhea and to maintain intestinal health in mice. Therefore, the combination of herb residues and probiotics may provide a novel method to resolve the environmental pollution problem and reuse the waste ingredients from herbal medicine.

Conflicts of Interest

The authors declare no competing financial interests.

Authors' Contributions

Fanjing Meng and Tingtao Chen contributed equally to this study.

Acknowledgments

The present study was supported by grants from the National Natural Science Foundation of China (Grant nos. 81503364, 91639106, 81270202, and 91339113), the National Basic Research Program of China (Grant no. 2013CB531103), and grants from Jiangxi Province (Grant nos. 20171BCB23028 and 20175526).

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Hindawi
Mediators of Inflammation
Volume 2017, Article ID 4856095, 12 pages
https://doi.org/10.1155/2017/4856095

Research Article

Antilipotoxicity Activity of *Osmanthus fragrans* and *Chrysanthemum morifolium* Flower Extracts in Hepatocytes and Renal Glomerular Mesangial Cells

Po-Jung Tsai,[1] Mei-Ling Chang,[2] Ching-Mei Hsin,[1] Chung-Chieh Chuang,[1] Lu-Te Chuang,[3] and Wen-Huey Wu[1]

[1]*Department of Human Development and Family Studies, National Taiwan Normal University, Taipei, Taiwan*
[2]*Department of Food Science, Nutrition and Nutraceutical Biotechnology, Shih Chien University, Taipei, Taiwan*
[3]*Department of Biotechnology and Pharmaceutical Technology, Yuanpei University of Medical Technology, Hsinchu, Taiwan*

Correspondence should be addressed to Wen-Huey Wu; t10005@ntnu.edu.tw

Received 27 July 2017; Accepted 10 October 2017; Published 20 November 2017

Academic Editor: Sung-Ling Yeh

The excess influx of free fatty acids (FFAs) into nonadipose tissues, such as those of liver and kidney, induces lipotoxicity leading to hepatic steatosis and renal dysfunction. The aim of this study was to investigate the protective effects of methanolic flower extracts of *Osmanthus fragrans* (OF) and *Chrysanthemum morifolium* (CM) against FFA-induced lipotoxicity in hepatocytes (human HepG2 cells) and renal glomerular mesangial cells (mouse SV40-Mes13 cells). The results showed that OF and CM significantly suppressed FFA-induced intracellular triacylglycerol accumulation via partially inhibiting the gene expression of sterol regulatory element-binding protein-1c (SREBP-1c) and glycerol-3-phosphate acyltransferase (GPAT) in HepG2 cells. Both extracts inhibited reactive oxygen species (ROS) generation by FFA-stimulated HepG2 cells. OF and CM also suppressed the mRNA expression of interleukin- (IL-) 1β, IL-6, IL-8, tumor necrosis factor- (TNF-) α, and transforming growth factor- (TGF-) β by HepG2 cells treated with conditioned medium derived from lipopolysaccharide-treated THP-1 monocytes. Furthermore, OF and CM effectively inhibited oleate-induced cellular lipid accumulation, TGF-β secretion, and overexpression of fibronectin in mesangial cells. In conclusion, OF and CM possess hepatoprotective activity by inhibiting hepatic fat load and inflammation and renal protection by preventing FFA-induced mesangial extracellular matrix formation.

1. Introduction

Lipotoxicity is generally defined as an increased concentration of harmful lipids, leading to cellular dysfunction and disruption of tissue function. The different classes of free fatty acids are known to trigger toxic effects and inflammation in numerous cell types [1]. Lipotoxicity may occur in several target organs via direct effects of triggering inflammation pathways and through indirect effects of alterations in the gut microbiota associated with endotoxemia [2]. Lipotoxicity plays a critical role in the pathogenesis of nonalcoholic fatty liver disease (NAFLD) and renal diseases [3, 4]. Nonalcoholic steatohepatitis (NASH), an advanced form of NAFLD that may progress to cirrhosis, is caused by lipid-mediated toxicity and inflammatory responses [3]. Intracellular lipid accumulation in NAFLD results from increased fatty acid uptake, increased de novo lipogenesis, and decreased fatty acid oxidation followed by esterification for the triacylglycerol (TG) synthesis. Lipogenesis is controlled primarily at the transcriptional level. Sterol regulatory element-binding protein 1c (SREBP-1c) and carbohydrate response element-binding protein (ChREBP) have been described as major transcription factors for increased de novo lipogenesis in NAFLD [5]. Glycerol-3-phosphate acyltransferase (GPAT) which catalyzes the esterification of glycerol-3-phosphate with fatty acid to generate lysophosphatidic acids is the rate-limiting enzymes of TG synthesis, and its gene expression is activated by SREBP-1c [5].

High level of plasma lipids may contribute to renal lipid accumulation, generation of reactive oxygen species (ROS),

mesangial expansion, and development of glomerulosclerosis [4]. Many studies indicate that transforming growth factor β (TGF-β) plays a central role in the pathogenesis of renal fibrosis and is an important marker of early renal fibrosis. TGF-β exerts profibrotic activity through stimulation of fibroblast proliferation and epithelial-mesenchymal transition (EMT). The increased TGF-β stimulates glomerular ECM accumulation by stimulating mesangial cells to produce type I, III, and IV collagen; laminin; and fibronectin and by blocking matrix degradation [6].

Flowers of *Osmanthus fragrans* and *Chrysanthemum morifolium* are commonly used as folk medicine and additives for teas, beverages, and foods in Taiwan. *O. fragrans* flowers (also known as Kwai-fah in Chinese) have been used to relieve pain, coughing, stomachache, diarrhea, and hepatitis in traditional Chinese medicine (TCM). Various compounds have been isolated from *O. fragrans* flowers, including flavonoids, phenolic acids, tyrosyl acetate, phillygenin, ligustroside, and verbascoside [7–9]. *O. fragrans* flower extract and its bioactive components have shown anti-inflammatory, antioxidant, and neuroprotection activity, alleviating diabetic pathological conditions and attenuating acetaminophen-induced hepatotoxicity [9–11]. *Chrysanthemum morifolium* flowers (also known as Ju-hua in Chinese) have been used in TCM as a medication for common cold, dim eyesight, dizziness, and skin itch. This flower is also widely used as a food supplement, or herbal tea, and is considered a healthy food by many consumers [12]. There are several cultivars of *C. morifolium* flowers available in herb markets in Taiwan; "Taiwan Hang Ju" is often used as herbal tea or beverage. *C. morifolium* flowers contain many phenolic compounds such as flavonoids, caffeic acid derivatives, hydroxycinnamoylquinic acids, and triterpenoid compounds [12]. Our previous study showed that selective phenolics, including chlorogenic acid, quercetin, myricetin, and caffeic acid, were identified in the methanol extracts of *C. morifolium* and *O. fragrans* flowers [7]. *C. morifolium* flower extract and its components also possess a variety of biological characteristics such as antioxidant, anti-inflammatory, antivirus, anti-HIV, antimutagenic, anticarcinogenic, and antiaging activities [12, 13]. In addition, polyphenol-rich *C. morifolium* extract ameliorated high-fat/drug-induced fatty liver in mice by orally feeding emulsion containing 10% cholesterol, 20% lard, and 0.2% propylthiouracil [14]. Propylthiouracil is known to cause liver injury and acute liver failure [15].

Despite numerous known biological functions of flower extracts of *O. fragrans* and *C. morifolium*, limited information is available on their effects on FFA-induced hepatic and renal lipotoxicity. In this study, we examined the effect of extracts of *O. fragrans* and *C. morifolium* flowers on lipotoxicity in FFA-overloaded hepatocytes (human HepG2 cells) and renal glomerular mesangial cells (mouse SV40-Mes13 cells).

2. Materials and Methods

2.1. Materials. HepG2 cells (BCRC RM60025; human hepatoblastoma cell line), THP-1 cells (BCRC 60430; human monocytic cell line), and mesangial SV40-Mes13 cells (BCRC 60366; mouse glomerular mesangial cell line) were obtained from the Bioresource Collection and Research Center, Hsinchu, Taiwan. HepG2 cells were cultured in DMEM/high glucose (Gibco, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco), 1% nonessential amino acid (Gibco), 1% L-glutamin (Gibco), and 1% penicillin/streptomycin (Gibco). Monocytic THP-1 cells were maintained in RPMI 1640 (Gibco) supplemented with 10% heat-inactivated FBS (Gibco) and 1% penicillin/streptomycin (Gibco). Mesangial cells were cultured in DMEM/low glucose/F12 medium (Gibco) supplemented with 5% FBS (Gibco) and 1% penicillin/streptomycin (Gibco). These cell lines were at 37°C in a humidified atmosphere with 5% CO_2.

BSA (Sigma-Aldrich, St. Louis, MO, USA) solutions (10%) were prepared using phosphate-buffered saline (PBS, pH 7.2). All free fatty acids (FFAs) were purchased from Sigma-Aldrich. FFA-bovine serum albumin (FFA/BSA) complex solution was prepared as reported previously [16]. The FFA/BSA or oleic acid (OA)/BSA complex solution was sterile-filtered through 0.22 μm sterile filters (Millipore S.A.S., Molsheim, France) and then stored at −20°C until use.

2.2. Preparation of Flower Extracts. Flowers of *Osmanthus fragrans* and *Chrysanthemum morifolium* (Taiwan Hang Ju) were, respectively, collected from Shiding, New Taipei, and Tongluo, Miaoli, Taiwan. The voucher specimens were deposited in the Department of Human Development and Family Studies, National Taiwan Normal University. The voucher specimen of the plant was authenticated by Dr. Po-Jung Tsai. The air-dried flowers were milled into powder and extracted twice with ten volumes of methanol. The filtration was performed in an evaporated and concentrated manner under vacuum at 45°C to obtain the methanolic flower extract of *O. fragrans* (OF) and the methanolic flower extract of *C. morifolium* (CM). The yields of OF and CM were, respectively, 25 and 24% (based on the weight of dried and ground plant materials). OF and CM then were redissolved in dimethyl sulfoxide (DMSO; RDH Chemical Co., Spring Valley, CA, USA) to 200 mg/mL of stock solution for the sequential experiments.

2.3. Effects of OF and CM on Lipid Deposition and Inflammatory Responses in HepG2 Cells

2.3.1. FFA-Treated HepG2 Cells and Determination of Cellular Viability. To mimic lipid exposures during in vivo high-fat-diet condition, FFA mixtures were used to induce lipotoxicity in HepG2 cells as previously reported by Lin et al. [17]. Stock solutions of 50 mM FFA mixtures consisting of palmitic acid, oleic acid, linolic acid, linoleic acid, and arachidonic acid (in proportions of 40 : 25 : 15 : 15 : 5) were prepared in culture medium containing 1% BSA [16, 17]. HepG2 cells (5×10^4 cells/well) were cultured in 96-well culture plates and 24 h later treated with various concentrations of OF or CM for the indicated times. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. MTT solution (Sigma-Aldrich; 100 μL, 0.5 mg/mL) was added to each well and incubated at 37°C for 3 h. The reaction was terminated by

Table 1: List of primer pairs used for quantitative PCR.

Primer	Sequence (5′ to 3′)	Product length (bp)	Binding site
SREBP-1 forward	CGG AGA AGC TGC CTA TCA AC	379	Exon 5
SREBP-1 reverse	GGT CAG TGT GTC CTC CAC CT		Exon 7
GPAT forward	AGT GAG GAA TGG GGT GAG TG	300	Exons 3 and 4
GPAT reverse	CAG TCA CAT TGG TGG CAA AC		Exon 6
IL-1β forward	CAC ATG GGA TAA CGA GGC TT	147	Exon 5
IL-1β reverse	TTG TTG CTC CAT ATC CTG TCC		Exon 5
IL-6 forward	CTC AGC CCT GAG AAA GGA GA	310	Exons 2 and 3
IL-6 reverse	CAG GGG TGG TTA TTG CAT TCT		Exon 5
IL-8 forward	GTG CAG TTT TGC CAA GGA GT	195	Exon 2
IL-8 reverse	CTC TGC ACC CAG TTT TCC TT		Exon 3
TNF-α forward	CAC TAA GAA TTC AAA CTG GGG C	165	Exon 4
TNF-α reverse	GAG GAA GGC CTA AGG TCC AC		Exon 4
TGF-β forward	GGG ACT ATC CAC CTG CAA GA	420	Exon 1
TGF-β reverse	CAC GTG CTG CTC CAC TTT TA		Exon 2
GAPDH forward	AAA GGA TCC ACT GGC GTC TTC ACC ACC	206	Exon 5
GAPDH reverse	GAA TTC GTC ATG GAT GAC CTT GGC CAG		Exon 7

replacing the MTT-containing medium with 500 μL of DMSO, and the formazan salts were dissolved by gentle shaking for approximately 5 min at room temperature. The optical density (OD) of each well was measured at 550 nm using a microplate reader (Tecan, Männedorf, Switzerland). Each assay was completed in triplicate wells, and each experiment was repeated three times.

2.3.2. Determination of Intracellular Lipid Content in HepG2 Cells. HepG2 cells (5×10^4 cells/well) were cultured in 96-well culture plates for 24 h. Cells were then incubated for another 24 h with the indicated concentrations of OF and CM or 0.1% DMSO (as vehicle cells) in the presence of 1 mM FFAs/BSA. Control cells were incubated with 1% BSA alone. The total intracellular lipid content was evaluated by Oil Red O staining. Briefly, the cells were fixed in 4% paraformaldehyde in PBS for 1 h, stained with Oil Red O (Sigma-Aldrich) for 1 h at room temperature, and then rinsed with ddH_2O few times to remove the excess stain. After washing and drying completely, 100 μL of isopropanol was added to each well and the mixtures were incubated for 10 min, followed by gentle vibration to release Oil Red O. The extraction solution was then transferred to another 96-well plate for the measurement of OD at 510 nm by a microplate reader (BioTek, Nevada, USA).

2.3.3. Determination of Cellular Cholesterol and Triacylglycerol Contents. HepG2 cells (1×10^6 cells/well) were cultured in 6-well culture plates and 24 h later treated with 1 mM FFAs/BSA alone (as vehicle cells) or in combination with various concentrations of OF or CM for 24 h. Cells from each well were harvested by addition of lysis buffer, and cell proteins were assessed by the Lowry protein assay (Bio-Rad, Hercules, CA, USA). The total cholesterol (TC) and total triacylglycerol (TG) contents of whole cell lysates were measured with the colorimetric assay kit (Randox, Crumlin, Antrim, UK). Their masses in HepG2 cells were calculated and normalized to total cellular protein content.

2.3.4. Effect of OF and CM on mRNA Levels of SREBP-1c and GPAT in FFA/BSA-Treated HepG2 Cells. HepG2 cells (1×10^6 cells/well) were cultured in 6-well culture plates and 24 h later treated with FFAs/BSA alone (as vehicle cells) or in combination with various concentrations of OF and CM. After 24 h incubation, HepG2 cells were collected and total RNA was extracted from cells using the TRizol reagent (Invitrogen, Carlsbad, CA), and complementary DNA (cDNA) was generated from 2 μg of total RNA, with the oligo (dT) primer and 1 μL of reverse transcriptase (Promega, Madison, WI, USA). Real-time PCRs were conducted in an iCycler iQ Real-Time detection system (Bio-Rad, Hercules, CA, USA) using iQ™ SYBR Green Supermix (Bio-Rad). The relative amounts of the PCR products were analyzed by iQ5 optical system software, vers. 2.1. As shown in Table 1, primer sequences for SREBP-1c and GPAT were used in this study. The messenger RNA (mRNA) level of each sample for each gene was normalized to that of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Fold expression was defined as the fold increase relative to control cells.

2.3.5. Measurement of ROS Production. The probe 2,7-dichlorofluorescein diacetate (H_2DCF-DA; Sigma-Aldrich) was used to monitor the intracellular ROS generation. HepG2 cells (5×10^4 cells/well) were seeded in a 96-well plate in DMEM medium for 24 h. Cells were then incubated for another 24 h with the indicated concentrations of OF or CM or 0.1% DMSO (as vehicle cells) in the presence of 1 mM FFAs/BSA. Control cells were incubated with 1% BSA in the absence of 1 mM FFAs/BSA. After incubation, cells were washed with PBS and incubated with 10 μM H_2DCF-DA at 37°C for 2 h. The formation of the oxidized fluorescent derivative dichlorofluorescein (DCF) was monitored at 475 nm

excitation and 525 nm emission using a Synergy™ HT Multi-Mode Microplate Reader (BioTek, Nevada, USA). All procedures were performed in the dark.

2.3.6. Determination of Proinflammatory Cytokine mRNA Levels in Conditioned Medium-Treated HepG2 Cells. For the preparation of conditioned medium, THP-1 cells were treated with 0.5 μg/mL lipopolysaccharide (LPS) (Sigma-Aldrich) for 24 h; the medium was collected and centrifuged to remove cell debris. Culture supernatants derived from THP-1 monocytes were referred to as THP-1/LPS/conditioned media (TLPS/CM) and stored at −20°C until use. HepG2 cells were initially maintained in DMEM with 10% FBS. The RPMI 1640 medium was then gradually increased to replace DMEM, and the cells were routinely passaged when confluence was achieved. Afterwards, HepG2 cells were grown in RPMI 1640 medium supplemented with 10% FBS for the subsequent experiments. HepG2 cells (1×10^6 cells/well) were seeded in a 6 cm dish with serum-free RPMI 1640 medium. HepG2 cells were cultured in RPMI 1640 medium (as control cells) and 50% TLPS/CM alone (as vehicle cells) and coincubated with 400 μg/mL of OF or CM in the presence of 50% TLPS/CM. After 48 h incubation, HepG2 cells were collected and total RNA was extracted from cells using the TRizol reagent (Invitrogen, Carlsbad, CA), and complementary DNA (cDNA) was generated from 2 μg of total RNA, with the oligo (dT) primer and 1 μL of reverse transcriptase (Promega, Madison, WI, USA). Primer sequences were used in this study (Table 1). Real-time PCR analyses were conducted as described above.

2.4. Effects of OF and CM on Oleic Acid-Induced Lipotoxicity in Renal Glomerular Mesangial Cells

2.4.1. OA-Treated Mesangial SV40-Mes13 Cells and Determination of Cellular Viability. Mishra and Simonson [18] found that treatment of 200 μM oleate/BSA induced a myofibroblast-like phenotype in mesangial cells, which is an implication for renal fibrosis. So, we used the same methodology to examine the possible nephroprotective properties of OF and CM. Stock solutions of 50 mM OA/BSA were also prepared as previously described [16].

Mesangial SV40-Mes13 cells (1×10^4 cells/well) were seeded in a 96-well plate for 24 hours and then growth-arrested for another 24 h in FBS-free medium. Mesangial cells were then incubated in DMEM/F12 medium containing 200 μM OA/BSA and coincubated with various concentrations of OF or CM (50, 100, and 200 μg/mL) for another 48 h. Vehicle cells were incubated with 0.1% DMSO in the presence of OA/BSA. Control cells were incubated with 1% BSA alone. Cell viability was determined by the Alamar blue assay (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol.

2.4.2. Determination of Intracellular Lipid Content in Mesangial Cells. Mesangial SV40-Mes13 cells (3×10^5 cells/well) were seeded in 6-well plates for 24 h and then growth-arrested for another 24 h in FBS-free medium. Cells were treated with OA/BSA (200 μM) alone or in combination with different concentrations of OF or CM for 12 h. The intracellular lipid content of mesangial cells was also evaluated by Oil Red O staining as described above.

2.4.3. Determination of Cellular Cholesterol and Triacylglycerol Contents in Mesangial Cells. Mesangial SV40-Mes13 cells (3×10^5 cells/well) were seeded in 6-well plates for 24 hours and then growth-arrested for another 24 h in FBS-free medium. Cells were treated with OA/BSA (200 μM) alone or in combination with different concentrations of OF and CM for 12 h. Whole cell lysates were collected. The contents of TC and TG in mesangial cells were also measured as described above.

2.4.4. Measurement of Protein Levels of TGF-β and Fibronectin. Mesangial cells were seeded at 3×10^4 cells/well in 24-well plates with DMEM/F12 medium with 5% FBS. Subconfluent mesangial cells were made quiescent by serum deprivation for 24 h before treatment. Cells were then treated with OA/BSA (200 μM) alone or in combination with different concentrations of OF or CM for 6 h incubation (for the determination of TGF-β) and for 24 h incubation (for the determination of fibronectin). Cell-free supernatants were collected, and the concentrations of TGF-β and fibronectin in supernatants were quantified using the commercial TGF-β ELISA kit (Bender MedSystems GmbH, Vienna, Austria) and the fibronectin ELISA kit (Assaypro, Winfield, MO, USA), respectively, following the protocols from the manufacturers.

2.4.5. Immunofluorescence Staining. Mesangial SV40-Mes13 cells (2×10^4 cells/well) were seeded onto 8-well Lab-Tek II chamber slides (NUNC, Rochester, NY). After serum starvation, cells were treated with OA/BSA (200 μM) alone or in combination with 200 μg/mL of OF or CM. After 24 h incubation, mesangial cells were washed with cold PBS, fixed with acetone/methanol (1/1; *v*/*v*), and then stained. After 1 h blocking with 3% BSA in PBS with 0.1% Triton X-100 (PBST), the cells were incubated with a primary antibody (rabbit anti-mouse fibronectin antibody, Epitomics, CA, Burlingame, USA) overnight, washed with PBS, and incubated with the DyLight 488-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) for 2 h. The slides were mounted with 87% glycerol and imaged using a DeltaVision® Core live-cell microscope (Applied Precision Inc., WA, USA).

2.4.6. Quantitative RT-PCR Analysis of Fibronectin mRNA Level. Mesangial SV40-Mes13 cells (1×10^6 cells/well) were seeded in a 6 cm dish for 24 h incubation. After serum starvation, cells were treated with OA/BSA (200 μM) alone or in combination with various concentrations of OF or CM. After 24 h incubation, cells were lysed in the TRIzol reagent (Invitrogen) and total RNA was isolated. Then, RNA was analyzed by RT-PCR as previously described. The upstream and downstream PCR primers for fibronectin were designed as 5′-GCT TCA TGC CGC TAG ATG T-3′ and 5′-GTG TGG ATT GAC CTT GGT AGA G-3′, respectively. In this experiment, the gene of β-actin was selected as a reference. The sequences of the upstream and downstream PCR primers for β-actin were 5′-GGA CTC CTA TGT GGG TGA CG-3′

and 5′-CTT CTC CAT GTC GTC CCA GT-3′, respectively. These primer pairs amplified a 99 bp fragment of the fibronectin cDNA and a 102 bp fragment of the β-actin cDNA, respectively. The fibronectin mRNA level was normalized to that of β-actin mRNA. Fold expression was defined as the fold increase relative to control cells.

2.5. Statistical Analysis. All data are presented as the mean ± standard deviation (SD). Statistical analyses were performed using the SPSS 23.0 statistical package (Chicago, IL, USA). Student's *t*-test or one-way ANOVA and Duncan's multiple comparison test were used to compare between-group differences. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Inhibitory Effect of OF and CM on Lipid Accumulation in FFA/BSA-Treated HepG2 Cells. A cellular hepatic model to study hepatic steatosis *in vitro* has been established by treating human HepG2 cells with FFAs. Herein, we used a mixture of different proportions of saturated and unsaturated FFAs as described by Lin et al. [17] to examine the antilipotoxic effect of OF and CM. To determine whether the treatment of OF or CM on HepG2 cells has an apparent toxic effect, the cell viability of HepG2 cells treated with various concentrations of OF or CM in the presence of FFAs/BSA (1 mM) was determined. The results showed that after 24 h, treatment with OF or CM at concentrations up to 200 μg/mL did not significantly cause cell death with respect to control cells (Figure 1(a)). Hence, the concentrations of 25 and 100 μg/mL for OF and CM were used in subsequent experiments.

As shown in Figure 1(b), OF and CM attenuated FFA/BSA-induced intracellular lipid deposition in HepG2 cells. Next, we investigated whether both extracts inhibited intracellular accumulation of TG and cholesterol. OF and CM treatment significantly reduced FFA/BSA-induced cellular triacylglycerol accumulation (Figure 1(c)). OF treatment (100 μg/mL) significantly reduced cholesterol content, while the reductions by CM treatment did not reach statistical significance (Figure 1(d)).

Since both extracts possessed a significantly inhibitory effect of lipid accumulation, we tried to find out the molecular target(s) of OF and CM in the lipogenesis-related genes. As shown in Figure 2, treatment of OF or CM significantly inhibited the mRNA expression of SREBP-1c and GPAT as compared to the vehicle-alone treatment.

3.2. Inhibitory Effect of OF and CM on FFA/BSA-Induced ROS Production in HepG2 Cells. Excess ROS accumulation plays a causative role in a variety of lipotoxic disorders. After 24 h of treatment, vehicle cells exhibited a significant increase in ROS accumulation as measured by DCF fluorescence, while coincubation of HepG2 cells with OF or CM (25 and 100 μg/mL) resulted in a significant decrease in comparison with vehicle cells, suggesting that the antilipotoxicity effect of both extracts was also related to their antioxidant properties (Figure 3).

3.3. Inhibitory Effect of OF and CM on Conditioned Medium-Induced Proinflammatory Cytokine Expression in HepG2 Cells. Besides accumulation of triglycerides in hepatocytes, chronic hepatic inflammation is also closely associated with the pathogenesis of NAFLD. Since activated monocytes/macrophages release a wide range of proinflammatory mediators leading to induction of inflammation. In this study, the conditioned medium derived from LPS-stimulated THP-1 monocytes (TLPS/CM) was used to induce inflammatory responses in HepG2 cells. Treatment with TLPS/CM and cotreatment of TLPS/CM with OF or CM (200 and 400 μg/mL) did not significantly affect cell viability with respect to control cells (Figure 4(a)). So, the concentration of 400 μg/mL was used for the subsequent experiments to examine the anti-inflammatory activity of both extracts. To determine the effect of OF and CM on the mRNA expression of proinflammatory cytokines, HepG2 cells were treated with both extracts in the presence of TLPS/CM. As shown in Figures 4(b), 4(c), 4(d), 4(e), and 4(f), the mRNA expression of genes encoding proinflammatory cytokines TNF-α, IL-6, IL-8, IL-1β, and TGF-β was significantly increased after TLPS/CM treatment. OF or CM treatment decreased the mRNA expression of these inflammatory cytokines.

3.4. OF and CM Reduced Cholesterol and Triacylglycerol Contents in OA/BSA-Treated Mesangial Cells. The cytotoxicity of OF and CM on mesangial cells was assessed, indicating that both extracts at the concentrations of 50, 100, or 200 μg/mL did not affect cell viability (Figure 5(a)). Therefore, these concentrations of both extracts were used for further studies. As shown in Figure 5(b), OF and CM at the concentrations of 100 and 200 μg/mL significantly attenuated OA/BSA-induced intracellular lipid deposition in mesangial cells. In addition, OF (50, 100, and 200 μg/mL) markedly reduced TG and cholesterol contents, while CM also showed a significantly suppressive effect on TG and cholesterol accumulation but only at 200 μg/mL and to a less extent (Figures 5(c) and 5(d)).

3.5. Effect of OF and CM on TGF-β and Fibronectin Levels in OA/BSA-Treated Mesangial Cells. Given the known importance of TGF-β in the induction of matrix production by mesangial cells in glomerular sclerosis, we examined the inhibitory effect of OF and CM on OA/BSA-induced TGF-β secretion. As shown in Figure 6(a), both extracts markedly decreased TGF-β levels. Immunofluorescence staining showed increased presence of fibronectin in OA/BSA-treated mesangial cells. Treatments of mesangial cells with OF or CM (200 μg/mL) inhibited fibronectin levels as compared to the vehicle treatment (Figure 6(b)). Hence, the effect of both extracts on fibronectin protein level was evaluated. As shown in Figure 6(c), when mesangial cells were treated with OA/BSA, fibronectin protein level was markedly elevated in the supernatant. Consistent with the immunofluorescence staining results, both extracts also reduced the secretion of fibronectin induced by OA/BSA (Figure 6(c)). Next, we observed that cotreatment with OF or CM resulted in a significant reduction in fibronectin mRNA level in comparison to the treatment with OA alone (Figure 6(d)).

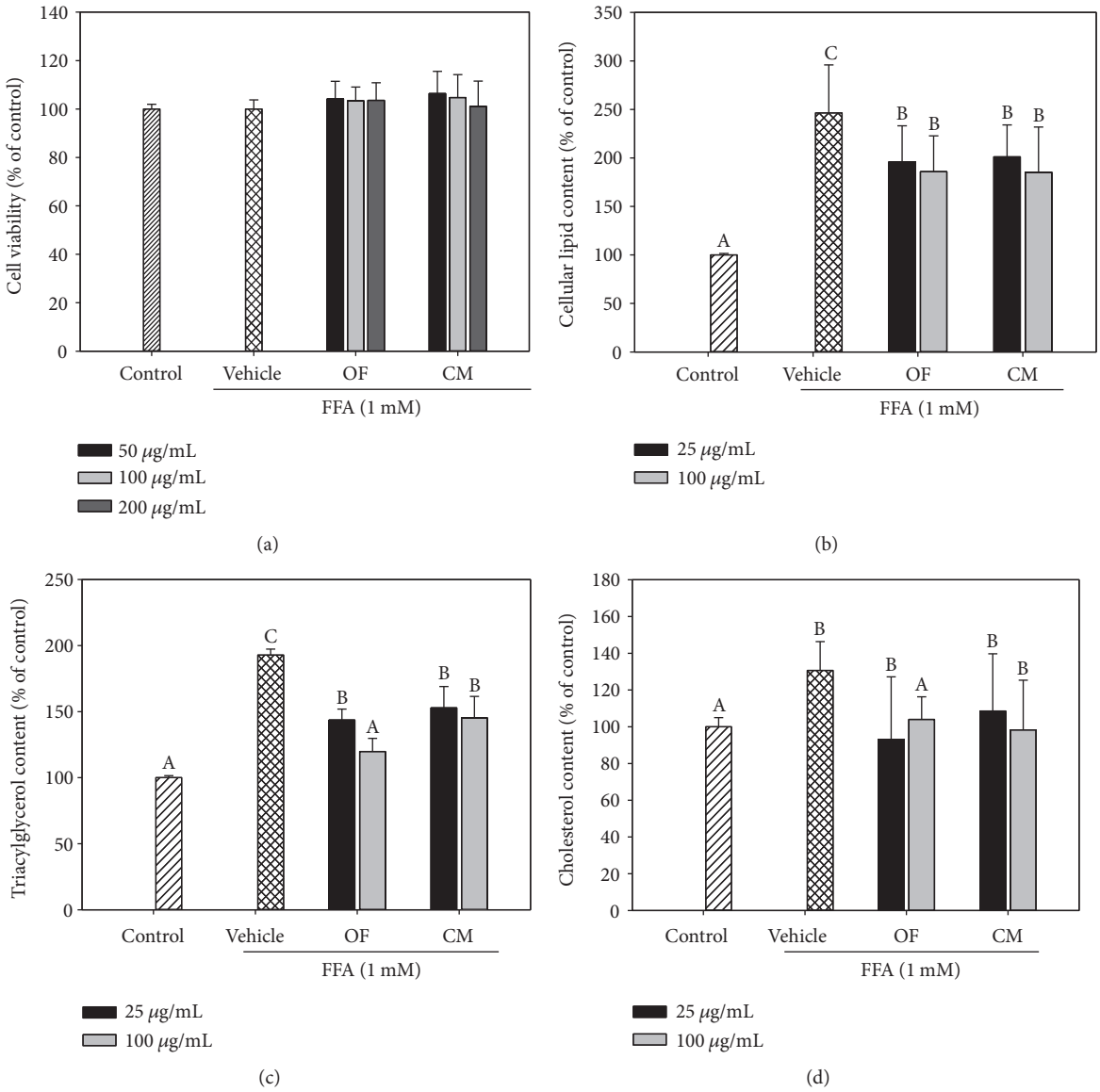

Figure 1: Effects of OF and CM on lipid accumulation in free fatty acid-overloaded HepG2 cells. HepG2 cells were incubated with 1 mM FFAs/BSA and cotreated with various concentrations of OF and CM for 24 h. Vehicle cells were incubated with 0.1% DMSO in the presence of FFAs/BSA. Control cells were incubated with 1% BSA. Cell viability was measured by the MTT assay (a). Quantitative analysis of lipid deposition in cells by the $OD_{500\ nm}$ values using Oil Red O staining (b). Intracellular triglyceride (c) and cholesterol (d) contents were determined in cell lysates by an enzymatic colorimetric method using a commercially available kit. Total cholesterol and TG levels of the control cells were 25.3 ± 7.1 and 28.7 ± 2.7 μg/mg of cellular protein, respectively. Data were presented as mean ± SD of three independent experiments. Values not sharing common superscripts are significantly different ($p < 0.05$).

These data suggested that both extracts inhibited oleic acid-induced mesangial cell activation.

4. Discussion

Consistent with previous studies [17], treatment of 1 mM FFAs caused lipid accumulation but not cytotoxicity toward HepG2 cells (Figure 1). Lee et al. [19] reported that the methanol extract of *C. morifolium* flowers significantly inhibited lipid accumulation in 3T3-L1 adipocyte cells during differentiation. In the present study, we observed that OF and CM had an inhibitory effect on lipid accumulation in FFA-treated HepG2 cells (Figures 1(b), 1(c), and 1(d)). We next examined whether OF and CM could influence lipid metabolism through the transcriptional regulation of SREBP-1c and GPAT. SREBP-1c is a lipogenic transcription factor which upregulates acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), which catalyze de novo fatty acid synthesis contributing to hepatic steatosis [20]. GPAT catalyzes the first and rate-limiting step in glycerolipid synthesis. It contributes to TG biosynthesis and lipid droplet formation [21]. Both OF and CM downregulated SREBP-1c and GPAT gene expression in FFA-overloaded HepG2 cells (Figure 2). Cui et al. [14] reported that polyphenol-rich *C. morifolium*

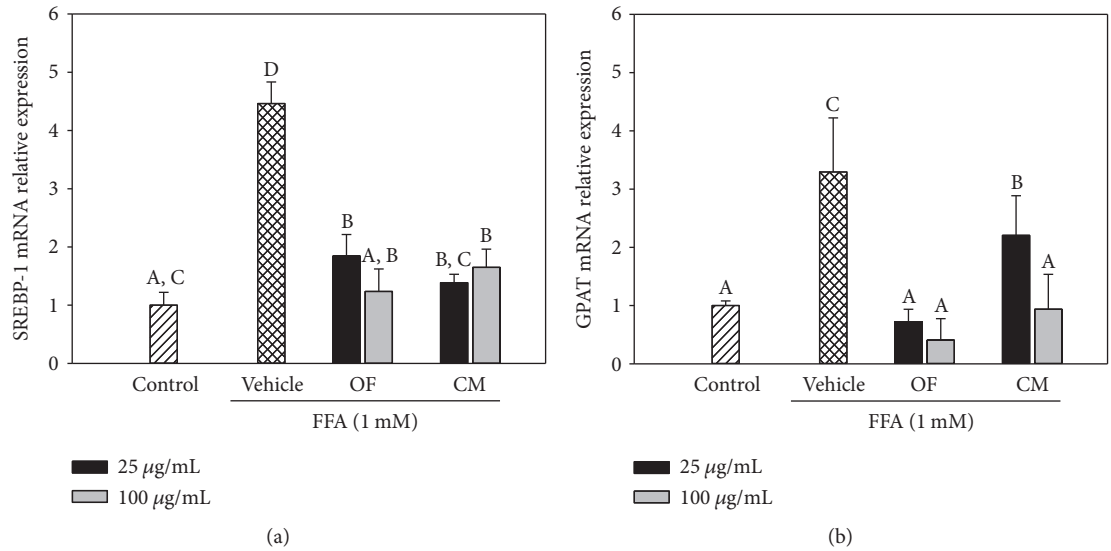

Figure 2: Effects of OF and CM on the mRNA expressions of lipogenesis-related genes. Real-time RT-PCR analysis of sterol regulatory element-binding protein-1 (*SREBP-1c*) (a) and glycerol-3-phosphate acyltransferase (*GPAT*) (b) mRNA levels in 1 mM FFA/BSA-treated HepG2 cells. All data were normalized to *GAPDH* mRNA, and the fold changes in expression were calculated relative to control cells (treated with 1% BSA). Each experiment was independently performed three times. Data were presented as the mean ± SD. Values not sharing common superscripts are significantly different ($p < 0.05$).

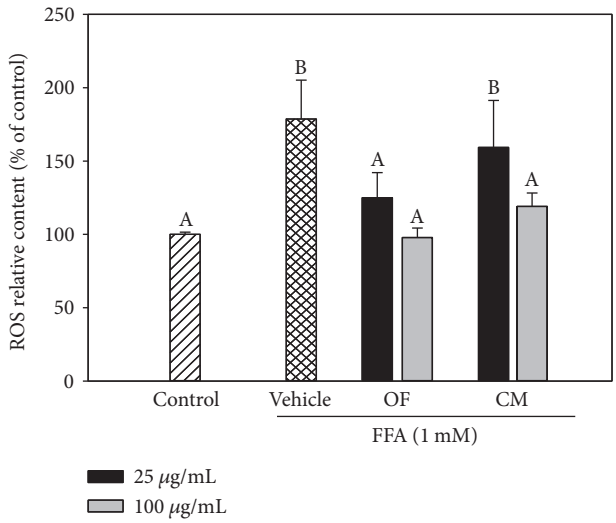

Figure 3: Effect of OF and CM on FFA-induced ROS production. HepG2 cells were incubated with 1 mM FFAs/BSA for 24 h in the presence of OF or CM. Vehicle cells were incubated with 0.1% DMSO in the presence of FFAs/BSA. Control cells were incubated with 1% BSA. Intracellular ROS production was quantified using the fluorescent probe DCFDA. Data were presented as mean ± SD of three independent experiments. Values not sharing common superscripts are significantly different ($p < 0.05$).

extract prevented fatty liver by decreasing the expression of SREBP-1c and its target gene FAS in high-fat/propylthiouracil-fed mice. Thus, the results suggested that the lipid-lowering effect of OF and CM might be partially mediated by the downregulation of SREBP-1c and GPAT gene expression. Notably, although OF or CM treatment resulted in a significant downregulation of SREBP-1c at the transcriptional level (Figure 2(a)), further study will be still required to investigate whether both extracts regulate proteolytic processing of the inactive endoplasmic reticulum membrane-bound SREBP-1c precursor to yield its transcriptionally active N-terminal form.

Crude methanol or ethanol extract of *O. fragrans* flowers exerted antioxidant activity *in vitro* [7, 22]. The 75% ethanolic extract of *O. fragrans* flowers showed antioxidant activity by the reduction of hepatic lipid peroxidation in acetaminophen-fed mice [11]. The total flavonoids of *C. morifolium* reversed lipid peroxidation and protected the liver and kidney against lead-induced oxidative damage in mice [23]. Consistent with these previous studies, OF and CM possessed antioxidant capacity to reduce FFA-induced ROS production (Figure 3).

The intestinal microorganisms play a critical role in normal gut function and health maintenance, and the dietary composition can affect the nature of microbial colonization [2]. Evidences indicate that a high-fat diet affects gut microbiota composition accompanied with elevating plasma endotoxin levels. These alterations have been associated with hepatic steatosis and obesity [24–26]. LPS in the circulation (endotoxemia) can activate NF-κB and then trigger proinflammatory signaling pathways. THP-1 cells respond with a similar transcriptional pattern as peripheral blood mononuclear cell- (PBMC-) derived macrophages after stimulation with LPS [27]. After LPS stimulation, THP-1 cells secrete increased amounts of TNF-α, IL-1β, IL-6, IL-8, and IL-10 [28, 29]. Therefore, we used a TLPS/CM-treated HepG2 cell model to mimic endotoxemia-mediated inflammation in this study. Our results showed that OF or CM treatment effectively inhibited TLPS/CM-induced mRNA expressions of proinflammatory cytokines, such as IL-1β, IL-6, IL-8, TNF-α, and TGF-β in HepG2 cells (Figure 4). We

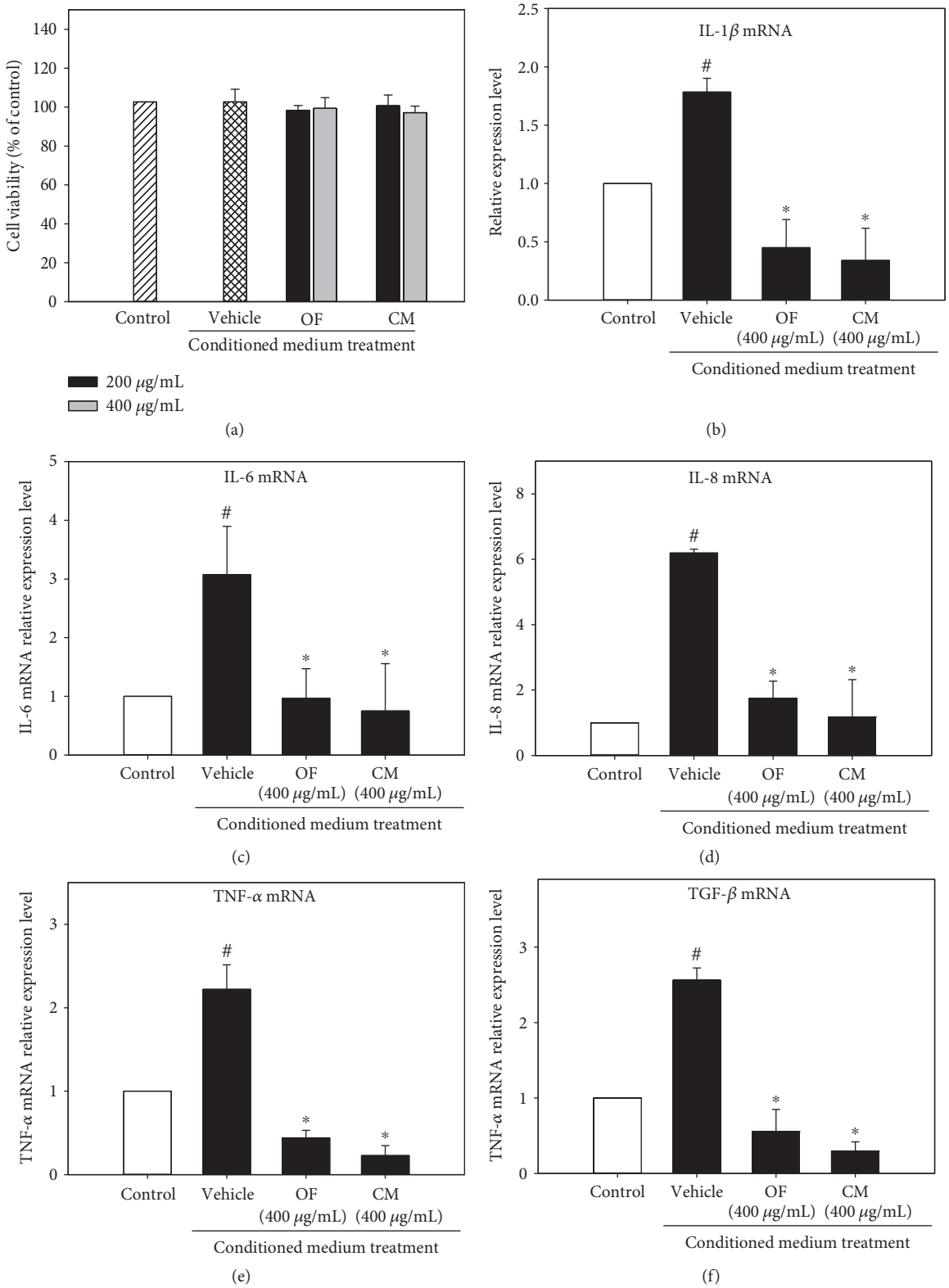

Figure 4: OF and CM suppressed proinflammatory cytokine mRNA expression induced by conditioned medium derived from LPS-stimulated THP-1 cells (TLPS/CM). Cell viability was measured by the MTT assay (a). Real-time RT-PCR analysis of mRNA levels of IL-1β (b), IL-6 (c), IL-8 (d), TNF-α (e), and TGF-β (f) in HepG2 cells cultured with RPMI medium (control cells) and 50% TLPS/CM (vehicle cells) or coincubated with 400 μg/mL of OF or CM in 50% TLPS/CM. All data were normalized to GAPDH mRNA, and the fold changes in expression were calculated relative to control cells. Each experiment was independently performed three times. Data were presented as the mean ± SD. $^{\#}p < 0.05$ versus control cells; $^{*}p < 0.05$ versus vehicle cells.

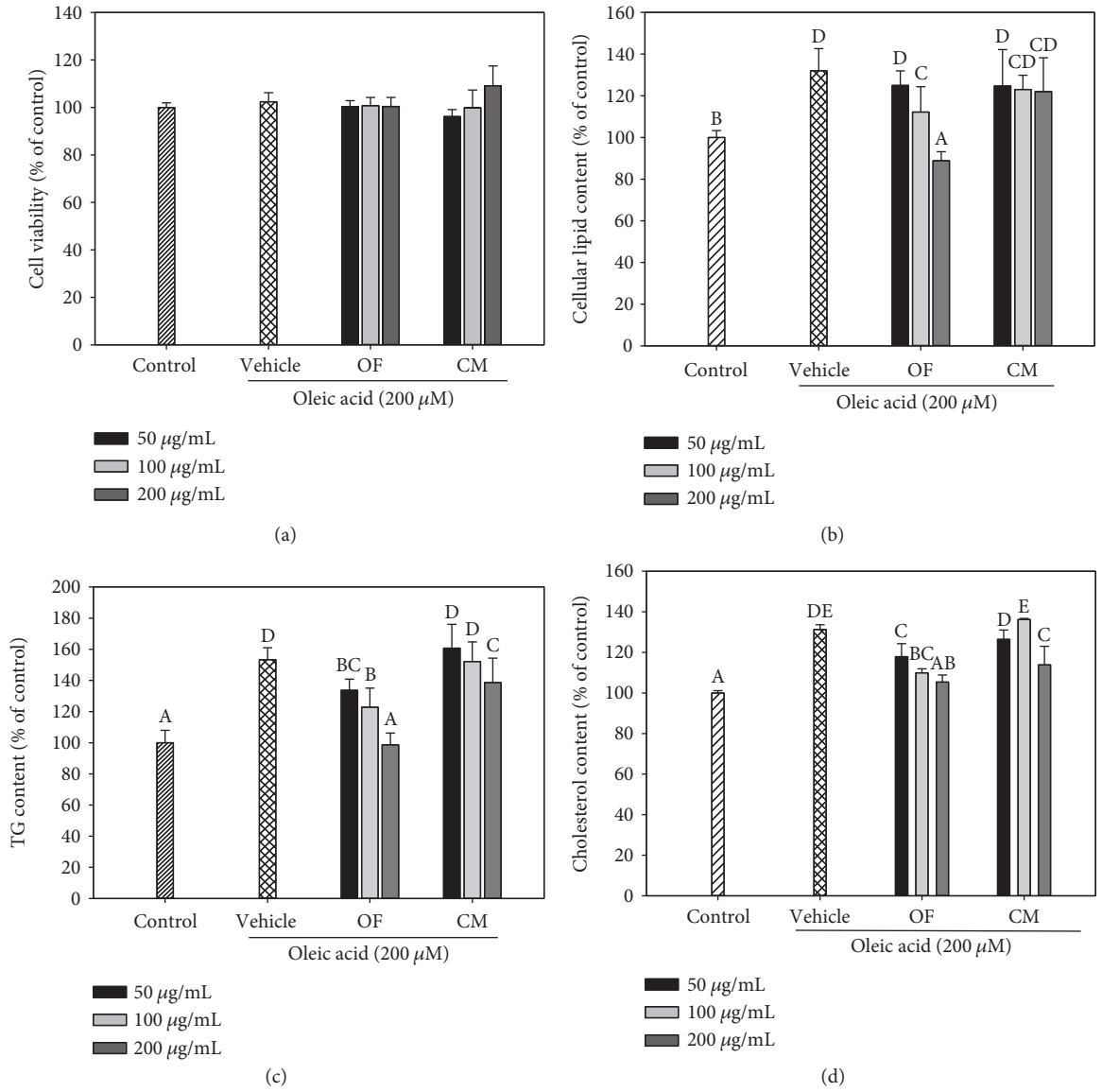

Figure 5: Effects of OF and CM on lipid deposit in oleic acid- (OA-) stimulated mesangial SV40-Mes13 cells. Mesangial cells were incubated with 200 μM OA/BSA and cotreated with various concentrations of OF and CM for 12 h. Vehicle cells were incubated with 0.1% DMSO in the presence of OA/BSA. Control cells were incubated with 1% BSA. Cell viability was measured by the Alamar blue assay (a). Quantitative analysis of lipid deposition in cells by the $OD_{500\ nm}$ values using Oil Red O staining (b). Intracellular triglyceride (c) and cholesterol (d) contents were determined in cell lysates by an enzymatic colorimetric method using a commercially available kit. Total cholesterol and TG contents of the control cells were 9.8 ± 0.1 and 7.5 ± 0.2 μg/mg cellular protein, respectively. Data were presented as mean $\pm$ SD of three independent experiments. Values not sharing common superscripts are significantly different ($p < 0.05$).

previously reported that water extracts of *O. fragrans* and *C. morifolium* also possessed anti-inflammatory capacity to attenuate LPS-induced nitric oxide (NO) production by RAW 264.7 macrophages [30]. Ethanol extract of *O. fragrans* flowers has been shown to obviously reduce the expression levels of the proinflammatory mediators IL-6, IL-8, and NO in LPS-stimulated human periodontal ligament cells [31]. The *n*-hexane soluble form and the nonsaponifiable lipid fractions of *C. morifolium* flower extract and its components showed marked anti-inflammatory activity against 12-O-tetradecanoylphorbol-13-acetate- (TPA-) induced ear edema in mice [32]. Taken together, OF and CM could have hepatoprotective activity by modulating fat deposition in hepatocytes and regulating the inflammatory responses to decrease the progression of steatohepatitis.

Notably, OF and CM treatments significantly suppressed TGF-β level induction by TLPS/CM. Since TGF-β inhibitors may be nephroprotective [33], we next examined the possible nephroprotective properties of OF and CM in oleate-treated mesangial cells. The prevalence of obesity-related glomerulopathy is increasing, that includes increases in mesangial matrix, thickening of the glomerular basement membrane, and glomerulosclerosis. The changes may be independent on high blood pressure and glucose, or may precede the

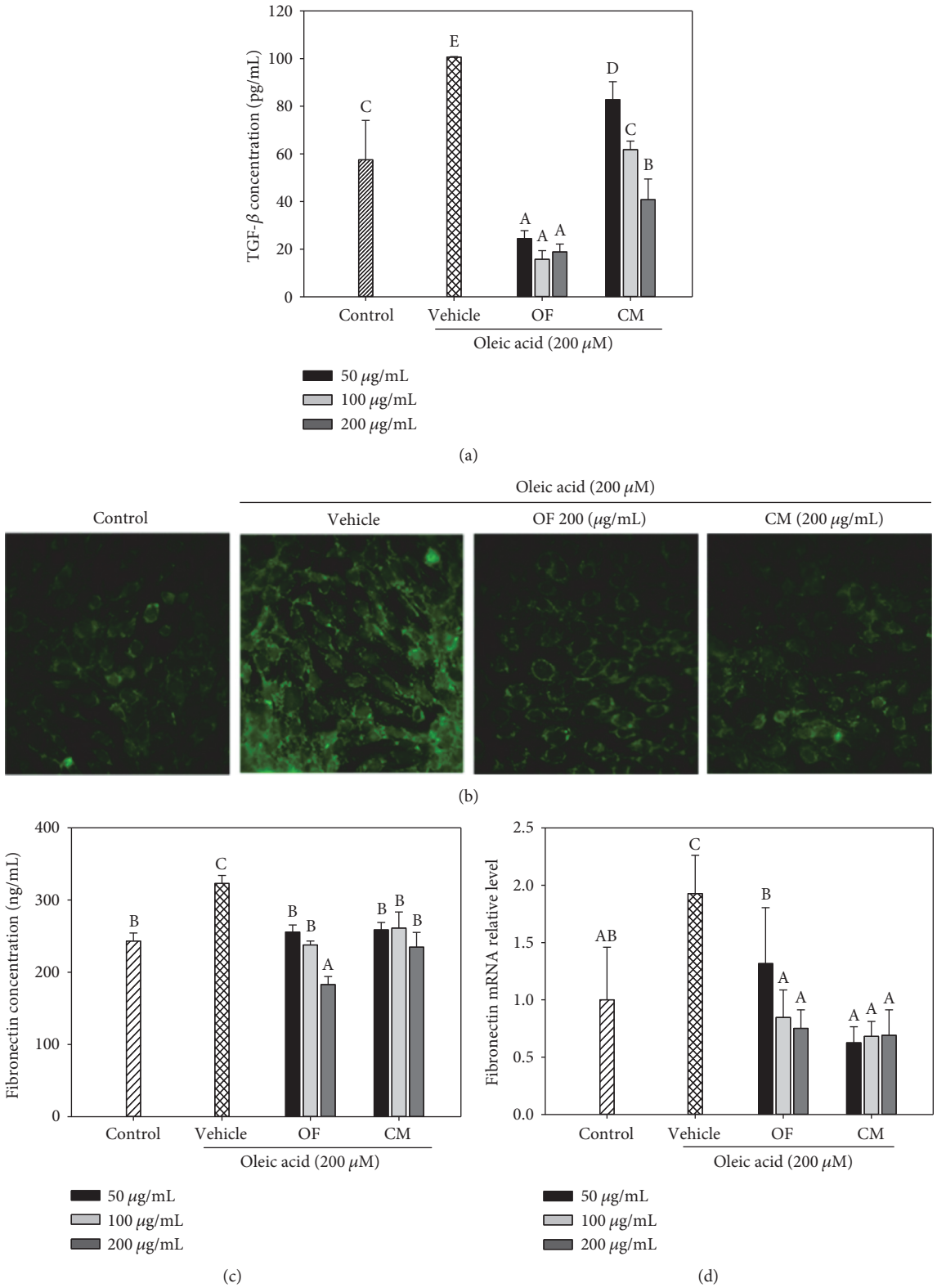

Figure 6: Effects of OF and CM on TGF-β and fibronectin expression in oleic acid- (OA-) stimulated mesangial SV40-Mes13 cells. TGF-β protein level in cell supernatant was measured by the ELISA method (a). Fibronectin deposition was observed by immunofluorescence staining (b). OF and CM suppressed OA-induced fibronectin protein level (c) and mRNA expression (d). Data were presented as mean ± SD of three independent experiments. Values not sharing common superscripts are significantly different ($p < 0.05$).

emergence of them, and may be attributed to lipid accumulation in mesangial cells [34]. OF and CM also showed a lipid-lowering effect on OA/BSA-treated renal glomerular mesangial cells (Figure 5). Thus, the TG-lowering effect of OF and CM might contribute to the nephroprotective potential.

Mishra and Simonson [18] demonstrated that oleate raises secretions of TGF-β, collagen I, and fibronectin and can induce a myofibroblast phenotype in mesangial cells. Consistent with their findings, 200 μM OA/BSA treatment significantly elevated TGF-β and fibronectin protein levels (Figure 6). Hung et al. [35] reported that the water extract of *O. fragrans* attenuated TGF-β1-induced intercellular/extracellular original fibronectin in human lung fibroblast cells and exerted antifibrotic activity against lung fibrosis. The hot-water extract of *C. morifolium* flowers was considered to be beneficial for type 2 diabetes [36]. In this study, we demonstrated that OF and CM can reverse the increased expression of TGF-β and the increased deposition and secretion of fibronectin in high-OA-treated mesangial cells (Figure 6). Taken together, OF and CM may prove beneficial in the development of natural agents for the prevention or treatment for TGF-β-mediated fibrosis disorders. Besides TGF-β, monocyte chemoattractant protein-1 (MCP-1) also contributes to ECM accumulation in the pathogenesis of diabetic nephropathy (DN) [37]. Our preliminary data showed that CM treatment (100, 200, and 400 μg/mL) significantly abrogated high-glucose-induced MCP-1 protein level in mesangial cells (data not shown). Thus, further investigation of the potential protective role of OF and CM in DN will be studied in the future.

In conclusion, both flower extracts of *O. fragrans* and *C. morifolium* ameliorated FFA-induced lipid deposit and ROS and possessed anti-inflammatory activity in HepG2 cells. In addition, both extracts inhibited lipid accumulation, induction of TGF-β, and extracellular matrix accumulation in OA-overloaded mouse mesangial cells. These results demonstrate that both flower extracts of *O. fragrans* and *C. morifolium* may have a protective effect on nonalcoholic steatohepatitis and renal fibrosis.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Acknowledgments

This work was supported by the Ministry of Science and Technology, Taiwan under Grant no. MOST 97-2321-B-003-001 (to Wen-Huey Wu) and Grant no. MOST 105-2320-B-003-002 (to Po-Jung Tsai).

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