D-limonene exhibits anti-inflammatory and antioxidant properties in an ulcerative colitis rat model via regulation of iNOS, COX-2, PGE2 and ERK signaling pathways

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Abstract. D-limonene has been demonstrated to have important immunomodulatory properties, including antitumor effects, and may alleviate asthma and allergies. In the present study, the anti-inflammatory effects of D-limonene were investigated in an ulcerative colitis (UC) rat model. Healthy male Sprague-Dawley rats were randomly divided into control, untreated UC, and treatment with 50 or 100 mg/kg D-limonene UC groups. In UC rats, disease activity and colonic mucosal damage were significantly reduced by the anti-inflammatory effects of D-limonene, via suppression of matrix metalloproteinase (MMP)-2 and -9 gene expression. In addition, treatment with D-limonene significantly increased antioxidant, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) protein expression levels in UC rats. A decrease in prostaglandin E2 (PGE2) production, transforming growth factor-β (TGF-β) gene expression and an increase phosphorylated-extracellular signal regulated kinase (ERK) 1/2 expression levels were observed in UC rats treated with D-limonene. In conclusion, D-limonene reduced MMP-2 and -9 mRNA expression levels via regulation of the iNOS, COX-2, PGE2, TGF-β and ERK1/2 signaling pathways in a UC rat model, indicating its potential antioxidant and anti-inflammatory properties.

Introduction

Ulcerative colitis (UC) is a non-specific type of inflammation with an unknown cause. Lesions exhibit continuous and diffuse distribution, primarily in the rectum and sigmoid colon of the large intestine (1). Typical clinical symptoms of UC include abdominal pain and diarrhea (2). As a systemic disease, it may lead to further manifestations in certain patients, including enteropathic arthritis, bowel disease, hepatobiliary diseases including primary sclerosing cholangitis, and eye and skin damage (3). UC has a worldwide incidence of 0.5-24.5 cases per 100,000. Notably, the incidence of UC is lowest in developing countries, and highest in North America and Western Europe (4). At present, the incidence of UC in Central Europe and Eastern Europe is increasing; however, it is steadily decreasing in Western Europe and Scandinavia (5). UC was first identified in 1859, yet its etiology remains unclear. A recent study on the underlying molecular mechanisms of the disease has furthered the understanding of the etiology of UC (6).

UC has numerous similarities with infectious enteritis and can cause microbial inflammation of the intestinal tract (7). However, no microorganism has yet been identified to be associated with UC. There may not be a single cause of the disease, as there is no evidence of infection in patients with UC. In countries with high incidences of UC the incidence of bowel infection is low and in developing countries with poor sanitation, the consumption of unprocessed food is a protective factor (8). The frequent use of antibiotics in childhood leads to an increased risk of UC, and antimicrobial agents are ineffective in the treatment of UC (9). Cultivation of feces from patients with UC has provided inconsistent results. Increasing evidence has indicated that there is an abnormal mucosal immune response between intestinal bacteria and the mucosal membrane in patients with UC (10). Molecular biology techniques have revealed that the adult intestinal space may accommodate >50 types of bacteria, that strains gradually increase in number along the small intestine and that Gram-negative bacteria predominate (11). There are up to ~10^{12} bacteria per cm in the large intestine. Currently, >50% of strains cannot be cultured by humans (12). D-limonene (Fig. 1) is a monoterpenoid, present in citrus and numerous other plants (13). It has been demonstrated that D-limonene may have broad anticancer properties. A previous study (14) revealed that D-limonene has significant inhibitory effects in...
animal models of breast, liver, lung, stomach and skin cancers, without clear adverse reactions. In addition, D-limonene may inhibit gastrointestinal reflux, promote healthy motility of the intestines, dissolve gallstones, relieve angina and prevent bacterial infection (15,16). The present study aimed to investigate the potential anti-inflammatory and antioxidant effects of D-limonene in a UC rat model, and the underlying mechanisms.

Materials and methods

Materials. 2,4,6-trinitrobenzenesulfonic acid solution and D-limonene were obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Tumor necrosis factor-α (TNF-α; R019), interleukin (IL)-1β (H002), IL-6, nuclear factor-kB (NF-κB; H202), superoxide dismutase (SOD; A001-3), glutathione (GSH; A006-2) and prostaglandin (PG) E2ELISA kits were obtained from the Nanjing Jiangcheng Bioengineering Institute (Jiangsu, China). A bicinchoninic acid (BCA) assay kit was obtained from Fermentas; Thermo Fisher Scientific, Inc. (Waltham, MA, USA).

Animal treatment and grouping. Healthy male Sprague-Dawley rats (weight, 220‑300 g; age, 8-10 weeks; n=32) were purchased from Changzhou Cavens Laboratory Animal Co., Ltd. (Changzhou, China), housed at 23-24°C, 50-60% humidity, light/dark cycle (7:00-19:00) with free access to food and water, and randomly divided into control, UC model, and treatment groups, UC model rats were administered with 50 or 100 mg/kg D-limonene groups (n=8/group). The control group rats were subjected to enema and oral gavage with normal saline. The UC model was established by administration of 2% DSS for 7 days. For the D-limonene-treated groups, UC model rats were administered with 50 or 100 mg/kg D-limonene by gastric lavage for 7 days (17). After treatment with D-limonene, rats were sacrificed using decapitation under anesthesia (2% pentobarbital sodium; Sigma-Aldrich; Merck KGaA).

Disease Activity Index (DAI) and Colonic Mucosa Damage Index (CMDI) scoring. Body weight, stool consistency, behavior and fecal blood in the stools of the rats were recorded daily. The scores were assigned as follows: Body weight reduction (0, no alteration; 1, 0.1-15%; 2, 15-20%; 3, 20-25%; 4, >25%); stool consistency (0, typical; 1, loose; 2, diarrhea); and the presence of fecal blood (0, typical; 2, positive occult blood test; 4, visible bleeding). The DAI was calculated as the sum of these scores. The entire colon was excised from the cecum of rats, and macroscopic damage was evaluated using the CMDI scoring system (18), with slight modifications: 0, no inflammation; 1, local hyperemia without ulcers, and/or stool consistency; 2, ulceration without hyperemia; 3, ulceration and adhesions at one site; 4, two or more sites of inflammation and ulceration extending >1 cm; 5, ulceration >2 cm.

Inflammatory cytokine, antioxidant and PGE2 production. Serum was obtained from a peripheral vessel and centrifuged at 1,200 x g for 10 min at room temperature. Serum protein expression levels of TNF-α, IL-1β, IL-6, NF-κB, SOD, GSH and PGE2 were measured using ELISA kits, and the absorbance was measured at a wavelength of 450 nm using an ELISA reader.

Matrix metalloproteinase (MMP)-2, -9 and transforming growth factor-β (TGF-β) gene expression. Total RNA was extracted from colonic mucosa tissue samples using TRizol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Equal quantities of total RNA were used to synthesize cDNA using a RNA Polymerase Chain Reaction (PCR) kit (Avian Myeloblastosis Virus 3.0; Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer's protocol. Following this, quantitative PCR (qPCR) was performed using a SYBR®-Green JumpStart™ Taq ReadyMix™ (Sigma-Aldrich; Merck KGaA), SYBR®-Green PCR Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) and iCycler IQ™ Real-Time PCR Detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The sequences for gene-specific primers are presented in Table I. The thermocycling conditions for MMP-2 were as follows: Predenaturation at 95°C for 10 min, followed by 35 cycles of denaturation for 30 sec at 94°C, annealing at 90°C for 30 sec and extension at 72°C for 90 sec. The thermocycling conditions for MMP-9 were as follows: Predenaturation at 94°C for 10 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 62°C for 30 sec and extension at 72°C for 90 sec. The thermocycling conditions for MMP-2, MMP-9 and TGF-β were as follows: Predenaturation at 94°C for 10 min, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 58°C for 30 sec and extension at 72°C for 90 sec. Relative quantitation values were calculated using the 2-ΔΔCq method (19).

Western blot analysis of inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2 and extracellular signal-regulated kinase (ERK) 1/2. For western blot analysis, colonic mucosa tissue samples were obtained and homogenized with radioimmunoprecipitation assay buffer (EMD Millipore, Billerica, MA, USA). The homogenate was centrifuged at 1200 x g for 10 min at 4°C and protein concentrations were measured using a BCA assay kit. A total of 50 mg protein underwent 10% SDS-PAGE and was subsequently transferred onto nitrocellulose membranes (Merck KGaA). The membranes were blocked with 5% (w/v) non-fat milk powder in Tris-buffered saline containing 0.1% Tween-20 (TBST), followed by incubation at 4°C overnight with the appropriate primary antibody at the following dilutions: Anti-iNOS (sc-649; 1:2,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-COX-2 (sc-7951; 1:1,000; Santa Cruz Biotechnology, Inc.) and anti-phosphorylated (p)-ERK1/2 (sc-101760; 1:2,000, Santa Cruz Biotechnology, Inc.), with anti-β-actin (D110007; 1:5,000; Sangon Biotech, Co., Ltd., Shanghai, China) serving as the internal control. Following this, membranes were washed three times in TBST for 1 h and incubated with horseradish peroxidase (HPR)-conjugated anti-rabbit IgG secondary antibodies for 2 h at room temperature (sc-2004; 1:5,000; Santa Cruz Biotechnology, Inc.). Proteins were detected using a SuperSignal™ West Femto Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.) and calculated using Image-Pro Plus software version 3.0 (Media Cybernetics, Inc., Silver Spring, MD, USA).
Results

DAI and CMDI scores. In the UC model group, DAI (Fig. 2A) and CMDI (Fig. 2B) scores were significantly increased compared with the control group (P=0.0011 and 0.0000). Treatment with 50 or 100 mg/kg D-limonene significantly decreased these scores compared with untreated UC rats (P=0.0039 and 0.0021; P=0.0046 and 0.0016).

Inflammatory cytokines. Expression levels of the inflammatory cytokines NF-κB (Fig. 3A), TNF-α (Fig. 3B), IL-1β (Fig. 3C) and IL-6 (Fig. 3D) were significantly increased in UC rats, compared with the control group (P=0.0017, 0.0006, 0.0024 and 0.0035), whereas treatment with 50 or 100 mg/kg D-limonene significantly reduced the expression levels compared with untreated UC rats (P=0.0079 and 0.0051; P=0.0066 and 0.0049; P=0.0091 and 0.0063; P=0.0082 and 0.0059).

MMP-2 and -9 gene expression. mRNA expression levels of MMP-2 (Fig. 4A) and -9 (Fig. 4B) in the colonic mucosa of UC rats were markedly increased, compared with the control group (P=0.0007 and 0.0000). By contrast, MMP-2 and -9 mRNA expression levels were markedly reduced by treatment with 50 or 100 mg/kg D-limonene compared with untreated UC rats (P=0.0071 and 0.0042; P=0.00097 and 0.0031).

SOD and GSH activities. Activities of SOD (Fig. 5A) and GSH (Fig. 5B) in UC rats were reduced compared with control rats (P=0.0031 and 0.0023). Treatment with 50 or 100 mg/kg D-limonene markedly increased activities of the two antioxidants, SOD and GSH, compared with untreated UC rats (P=0.0082 and 0.0038; P=0.00090 and 0.0047).

iNOS protein expression levels. As presented in Fig. 6, there was a significant increase in iNOS protein expression levels in UC rats compared with the control group (P=0.0053). Treatment with 50 or 100 mg/kg D-limonene significantly reduced iNOS protein expression levels compared with untreated UC rats (P=0.0046 and 0.0016).

COX-2 protein expression levels. UC rats exhibited increased protein expression levels of COX-2 compared with control rats (P=0.0078; Fig. 7). Treatment with 50 or 100 mg/kg D-limonene significantly decreased COX-2 protein expression levels compared with untreated UC rats (P=0.0062 and 0.0029).

PGE2 production. The effect of D-limonene on PGE2 production was assessed in UC rats. There was a significant increase in PGE2 production in UC rats compared with the control group (P=0.0012; Fig. 8). Treatment with 50 or 100 mg/kg D-limonene significantly reduced PGE2 production compared with untreated UC rats (P=0.0071 and 0.0000). By contrast, MMP-2 and -9 gene expression.

TGF-β expression. The effect of D-limonene on TGF-β gene expression in UC rats is presented in Fig. 9. TGF-β mRNA expression levels were significantly increased in UC rats compared with the control group (P=0.0039). However, treatment with 50 or 100 mg/kg D-limonene significantly reduced TGF-β mRNA expression levels compared with untreated UC rats (P=0.0052 and 0.0016).

p-ERK1/2 protein expression levels. To assess the effects of D-limonene on the ERK1/2 signaling pathway, p-ERK1/2 protein expression levels were measured. Western blot analysis revealed that p-ERK1/2 protein expression levels were significantly reduced in UC rats compared with control rats (P=0.0058; Fig. 10). However, treatment with 50 or 100 mg/kg D-limonene significantly increased p-ERK1/2 protein expression levels compared with untreated UC rats (P=0.0028 and 0.0006).

Discussion

UC is a type of inflammatory bowel disease. It is hypothesized that the pathogenesis of UC involves the activation of the immune system by various microbial antigens, based on genetic material and environmental factors. This results in an imbalance of cytokines, which activates a variety of inflammatory cells and recruits these cells to the site of inflammation.

Table I. Primers used in the present study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5'-3')</th>
<th>Product size (bp)</th>
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<tbody>
<tr>
<td>MMP-2</td>
<td>F: ACCATCGGCCCCATCATCAAGT</td>
<td>348</td>
</tr>
<tr>
<td></td>
<td>R: CGAGCAAAGCATTACCCAC</td>
<td></td>
</tr>
<tr>
<td>MMP-9</td>
<td>F: CCGCTGGATTTCCACATTCA</td>
<td>600</td>
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<tr>
<td></td>
<td>R: ACCCCACCTCTTGTAGGCT</td>
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<tr>
<td>TGF-β</td>
<td>F: TGGTTCGCTCAAGAGAAGAC</td>
<td>284</td>
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<tr>
<td></td>
<td>R: TGGTTGTAGAGGCAAGGAC</td>
<td></td>
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<tr>
<td>β-actin</td>
<td>F: AAGGCTAAGGCAAACGTTGAA</td>
<td>241</td>
</tr>
<tr>
<td></td>
<td>R: TCAATGAGGTTGCTAGGTT</td>
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</tr>
</tbody>
</table>

MMP, matrix metalloproteinase; TGF, transforming growth factor-β; F, forward; R, reverse.

Statistical analysis. Data were analyzed by one-way analysis of variance, followed by Student-Newman-Keuls post hoc test, using SPSS version 22.0 (IBM SPSS, Armonk, NY, USA). Data are expressed as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Figure 1. The chemical structure of D-limonene.
inflammation, releasing further inflammatory cytokines and thus leading to chronic inflammation of the colon (20,21). The present study demonstrated that treatment with D-limonene significantly suppressed the DAI and CMDI, and inhibited TNF-α, IL-1β, IL-6 and NF-κB expression levels, in UC rats. Hirota et al (22) identified that D-limonene reduces allergic airway inflammation via inhibition of the expression levels of IL-5, IL-13, eotaxin, monocyte chemoattractant protein-1 and TGF-β1 in Dermatophagoides farinae-treated mice. Therefore, D-limonene may be a novel therapeutic agent for the treatment of UC.

During the process of oxidation, a variety of highly chemically reactive oxygen species may be generated, which leads to intestinal tissue damage and ulceration. The oxygen free
radical scavenging capacity of UC patients is decreased, therefore exacerbating disease (23). SOD is an important enzyme involved in the scavenging of oxygen free radicals and therefore preventing tissue damage; however, excessive levels of nitrous oxide reduces SOD levels, thus reducing its ability to scavenge oxygen free radicals (24,25). A build-up of free radicals induces a series of chain reactions, leading to biofilm lipid peroxidation, and thus continuously disrupts the normal structure and function of the enzyme (26). The present study demonstrated that D-limonene treatment markedly increased SOD and GSH activities in UC rats. Furthermore, Rizk et al (27) reported that D-limonene suppressed SOD and GSH activities in Schistosoma mansoni-infected mice. Thus, D-limonene may have antioxidative effects in UC rats.

COX-2 is expressed at low levels in healthy mucosa and during UC remission; however, its expression levels are significantly increased in active UC. It is primarily expressed in epithelial, endothelial and inflammatory cells (28). The enhanced expression levels of COX-2 are a protective response in the recovery process, which improves the protection of intestinal mucosal cells, promotes the hyperplasia of intestinal epithelial cells and intestinal blood flow, and promotes the repair of epithelial cells. COX-2 may inhibit the apoptosis of epithelial cells by reducing arachidonic acid (AA) and regulating
D-limonene may suppress MMP-2 and -9. Rehman et al (13) identified that D-limonene inhibits doxorubicin-induced oxidative stress and inflammation via COX-2 and iNOS signaling pathways in the kidneys of Wistar rats.

PGE2, a type of PG, is a metabolite of the 20-carbon unsaturated fatty acid AA (31). AA exists in the cell membrane phospholipid bilayer, and upon exposure to external stimuli, is hydrolyzed by activated phospholipase A2 and C, and is synthesized to PG. This process is mediated by COX and a series of synthetases (31). PGE2 is an important inflammatory factor and a previous study (32) demonstrated that it may increase vascular permeability and cause edema, inducing leukocyte chemotaxis, leading to inflammatory cell infiltration, and thus resulting in colonic mucosal inflammation, tissue damage and ulceration. An additional study (33) demonstrated that mucosal PGE2 content in UC patients is significantly increased, and is associated with the degree of mucosal inflammation. The present study revealed that treatment with D-limonene significantly decreased PGE2 production in UC rats. Yoon et al (16) suggested that D-limonene reduces lipopolysaccharide-induced production of iNOS and PGE2 in RAW 334.7 macrophages.

TGF-β is a cytokine with a variety of physiological functions. Mothers against decapentaplegic (SMAD) proteins are signaling molecules within cells that may be activated by the compound generated by TGF-β and its receptor, which additionally transmits the signals into the nucleus (34). The TGF-β1/SMAD3 signaling pathway contributes to the regulation of the immune response, induces the synthesis of the extracellular matrix components, collagen and mucin, inhibits the release of extracellular collagen proteolytic enzymes, promotes fibrosis, and facilitates repair of damaged tissue. A previous study (35) demonstrated that compared with healthy individuals, the expression levels of TGF-β1 and β2 protein and mRNA in active or non-active UC patients were significantly increased. In the present study, D-limonene significantly inhibited TGF-β mRNA expression levels in UC rats.

ERK is an important member of the mitogen-activated protein kinase (MAPK) system, which serves important roles in the mediation of inflammatory responses and the regulation of inflammatory cytokine production, the promotion of epithelial cell proliferation and differentiation, and the inhibition of intestinal epithelium apoptosis (36). p-ERK1/2 translocates from the cytoplasm to the nucleus and is thus involved in a variety of cellular biological reactions (37).
The MAPK signaling pathway is important for the biological effect of TGF-β1 (38,39). It has been reported (38) that the relative protein expression levels of p-ERK1/2 and p-MAPK kinase 1/2 in the colonic mucosa of UC rats are increased compared with healthy rats. Ruffino et al (40) demonstrated that D-limonene may have anti-inflammatory, antitoxic and anti-inflammatory effects in a cell model of osteoarthritis via increasing ERK1/2 activation. The results of the present study revealed that D-limonene significantly activated the ERK1/2 signaling pathway in UC rats.

In conclusion, the present study demonstrated that D-limonene suppresses MMP-2 and -9 mRNA expression levels via regulation of the iNOS, COX-2, PGE2, TGF-β and ERK1/2 signaling pathways in a UC rat model, indicating its potential antioxidant and anti-inflammatory properties. The current study indicates that D-limonene may be a novel potential target for the therapeutic effects of UC.

References


