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Full paper

Oligo-peptide I-C-F-6 inhibits hepatic stellate cell activation and ameliorates CCl_4-induced liver fibrosis by suppressing NF-κB signaling and Wnt/β-catenin signaling

Haitao Sun [a, c], Guanxin Chen [a, c], Bin Wen [b], Jialing Sun [a], Haiyan An [a], Jie Pang [a], Wei Xu [a], Xuemei Yang [a], Songqi He [a, *]

[a] School of Traditional Chinese Medicine, Southern Medical University, Guangzhou 510515, China
[b] Traditional Chinese Medicine Department of No. 458 Hospital of PLA, Guangdong 510602, China

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ABSTRACT

Oligo-peptide I-C-F-6 is a *Carapax trionycis* extract component that has an effect on hepatic fibrosis, however, its mechanism of action is still unclear. This study investigated whether oligo-peptide I-C-F-6 could inhibit liver fibrosis by suppressing NF-κB and Wnt/β-catenin signaling, which are important in liver fibrosis. HSC-T6 cells were treated with oligo-peptide I-C-F-6, and rats were divided randomly into five groups: control (saline), CCl_4, CCl_4 plus oligo-peptide I-C-F-6 (0.12 and 0.24 mg/kg), and CCl_4 plus colchicine (0.11 mg/kg). Here, we demonstrated that oligo-peptide I-C-F-6 ameliorated liver injury, inflammation, and hepatic fibrogenesis induced by CCl_4. Oligo-peptide I-C-F-6 also inhibited the activation of hepatic stellate cells (HSCs) *in vivo* and *in vitro*, as evaluated by the expression of transforming growth factor-β1 (TGF-β1) and α-smooth muscle actin (α-SMA), which is a specific marker of HSC activation. Moreover, oligo-peptide I-C-F-6 significantly reduced the expression and distribution of β-catenin, P-AKT, phospho (P)-GSK-3β, nuclear factor κB (NF-κB) P65, phospho-P65, and IκB kinase α/β (IKK-α/β) levels; additionally, IκB-α level was elevated both *in vivo* and *in vitro*. Together, these results indicate that oligo-peptide I-C-F-6 has hepatoprotective and anti-fibrotic effects in animal models of liver fibrosis, the mechanism of which may be related to modulating NF-κB and Wnt/β-catenin signaling.

1. Introduction

Fibrosis is a pathophysiologic consequence of chronic liver injury, and the common underlying mechanism for most clinical complications of end-stage liver disease. Fibrosis is characterized by disordered synthesis, deposition, and degradation of hepatic extracellular matrix (ECM).[1–3] It is widely recognized that activated hepatic stellate cells (HSCs) are considered the primary cells responsible for the progression of hepatic fibrosis.[4,5] In addition, previous studies have confirmed that many signal conduction pathways show functional disorder in hepatic fibrosis, such as Nuclear factor-κB (NF-κB) and Wnt/β-catenin signaling.[6–8] Given these findings, we attempted to identify a suitable strategy to against hepatocyte damage and inhibit NF-κB and Wnt/β-catenin signaling for the treatment of liver fibrosis.

Carapax trionycis, which has a long history of usage in traditional Chinese medicine, is used for the treatment of hepatic fibrosis.[9,10] *C. trionycis* is a rich source of peptides; both the extract and pure peptides of *C. trionycis* display strong hepatoprotective effects.[11,12] Oligo-peptide I-C-F-6, with the amino acid sequence GAGPHGG and a molecular weight of 551.56 Da, is a component of the extract, and an important agent in influencing the effect of *C. trionycis* on hepatic fibrosis. Previous studies have shown that oligo-peptide I-C-F-6 is able to ameliorate CCl_4-induced liver fibrosis.[13]

In this study, we investigated the efficacy of oligo-peptide I-C-F-6 in hepatic fibrosis and explored the underlying mechanisms through the inhibition of the NF-κB and Wnt/β-catenin signaling pathway. Our results provide insights into the possible mechanisms of action of *C. trionycis*.

* Corresponding author.
E-mail address: hesongqijz@126.com (S. He).
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[c] Haitao Sun and Guanxin Chen equally contributed to this work (co-first author).

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2. Materials and methods

2.1. Chemicals and reagents

Oligo-peptide I-C-F-6, determined to be 98.2% pure by high-performance liquid chromatography, was obtained from Shanghai Science Peptide Biological Technology (Shanghai, China). Its chemical structure is shown in Fig. 1A. Colchicine tablets were purchased from Xishuangbanna Banna Pharmaceutical (Yunnan, China). CCl_4 was purchased from Beijing Chemical Works (Beijing, China). HSC-T6 cells were purchased from the Department of Infectious Disease, Nanfang Hospital (Guangzhou, China).

The UltraSensitive SAP (mouse/rabbit) immunohistochemistry and 3,3′-diaminobenzidine kits were purchased from Fuzhou Maixin Biotechnology (Fuzhou, China). β-Catenin, glycogen synthase kinase (GSK)-3β, phospho-GSK-3β (Ser9), AKT, phospho-AKT (Ser473), P65, phospho-P65 (Ser536), P50, IκB-α, IκB-β, and GAPDH antibodies were purchased from Cell Signaling Technologies (Danvers, MA, USA). The IKK-α/β, α-SAM and TGF-β1 antibodies were obtained from Abcam (Cambridge, MA, UK).

2.2. Animals

Six-week-old male Wistar rats, with an average body weight of 200 ± 20 g, were obtained from the Animal Experiment Center of Southern Medical University (Guangzhou, China). The animal certificate number was SCXK2011-0015. Animals were kept under standard conditions of humidity (50%–55%), temperature (22 ± 1 °C), and a 12-h dark/light cycle, with free access to standard laboratory chow and water. The study design and all animal experiments/procedures were approved by the ethics committee for the experimental use of animals at Southern Medical University, Guangzhou, China (L2016038).

2.3. Experimental design

After an acclimation period of five days, rats were divided randomly into five groups (eight animals per group): control, CCl_4, CCl_4 plus oligo-peptide I-C-F-6 (two doses: 0.12 and 0.24 mg/kg), and CCl_4 plus colchicine (positive control; 0.11 mg/kg). CCl_4 was dissolved in 60% peanut oil for administration. All rats, except those in the control group, were injected intraperitoneally with CCl_4 at a dose of 3 mL/kg body weight for the first dose. This was followed by twice-weekly injections of 2 mL/kg for 8 weeks with daily hypodermic injection of oligo-peptide I-C-F-6 for the treated groups and intragastric administration of colchicine for the positive control group. For the control group, peanut oil and saline were injected. Twenty-four hours after the last intervention, blood samples were collected from the abdominal aorta after anaesthetization. After removal, the liver was immediately cut into small pieces (0.5–2.0 cm) and frozen in liquid nitrogen for histopathological study. For blood samples, serum was separated by centrifugation at 3000 × *g* for 15 min and kept at −80 °C before use.

2.4. Cell culture and treatments

HSC-T6 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 1% (v/v) penicillin-streptomycin and 10% (v/v) fetal bovine serum at 37 °C in an incubator with 5% CO_2. Cells were incubated overnight and exposed to oligo-peptide I-C-F-6 dissolved in complete medium at different concentrations for 24 h.

2.5. Growth inhibition assay

HSC-T6 cells were plated at 5×10^3 cells/well in 96-well plates. Cell Counting Kit-8 was obtained from Dojindo Molecular Technologies (Kumamoto, Japan). Cells were incubated in 10% Cell Counting Kit-8 solution at 37 °C. Proliferation was detected at 12, 24, and 48 h by measuring the absorbance at 450 nm.

2.6. Liver biological activities

Serum samples were used to evaluate liver status by testing the activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and measuring total protein (TP), albumin (ALB), and globin (G) levels at the TCM-Integrated Cancer Center of Southern Medical University (Guangzhou, China). We also analyzed the levels of collagen type IV (COL4), hyaluronic acid (HA), laminin (LN), procollagen III (PCIII), matrix metalloproteinase-2, -9 (MMP-2,-9), tissue inhibitor of metalloproteinase-1 (TIPM-1), connective tissue growth factor (CTGF), tumor necrosis factor alpha (TNF-α), and vascular endothelial growth factor (VEGF) using ELISA assay. The COL4, HA, PCIII and LN ELISA kits were obtained from Wuhan ColorfulGene Biological Technology (Wuhan, China). The MMP-2, MMP-9, TIPM-1, CTGF, TGF-β1, TNF-α, and VEGF ELISA kits were purchased from Cusabio Biotech (Wuhan, China).

2.7. Superoxide dismutase and lipid peroxidation measurements

Serum was analyzed for its level of malondialdehyde (MDA) and superoxide dismutase (SOD) using kits obtained from Nanjing Jian Cheng Bioengineering Institute (Nanjing, China). The experimental procedures were performed according to the manufacturer's protocol.

2.8. Histopathological study and immunohistochemical staining

Liver samples were fixed in 10% formalin. After dehydration, clearing in xylene, and paraffin embedding, 5-μm-thick sections were cut and stained with H&E and Masson's trichrome, which was purchased from Beijing Leagene Biotechnology (Beijing, China). The expression and localization of β-Catenin, P65, α-SMA, and TGF-β1 (all at 1:200) in the liver were detected by immunohistochemical staining. Endogenous peroxidase activity was blocked by hydrogen peroxide and the sections were respectively incubated with corresponding antibody, and then stained with the SAP kit and the DAB kit. Finally, slides were restained with hematoxylin, mounted and observed by light microscopy and examined in a blind fashion. Positive areas within the fibrotic region were then observed. Sections were observed with an optical microscope (Ni-U, Nikon Corporation, Tokyo, Japan), and images were analyzed with IPP software.

2.9. Western blot analyses

Protein samples were prepared using radioimmunoprecipitation assay buffer. The bicinchoninic acid assay kit was used to determine protein concentrations. Equal amounts of proteins (50 μg) were loaded, separated via 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and then transferred to PVDF membranes. After blocking with 5% skimmed milk/TBST for 1 h at room temperature, the PVDF membranes were incubated with primary antibodies against β-catenin, GSK-3β, P-GSK-3β, AKT, P-AKT, α-SMA, or TGF-β1, P65, phospho-P65, P50, IKK-α/β, IκB-α, and IκB-β, as well as the internal control GAPDH antibody (all at 1:1000), overnight at 4 °C. The protein band intensities were detected accordingly.

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