Jinhua Weikang capsule protects against Helicobacter pylori-induced inflammatory responses via the nuclear factor-kappa B signaling pathway

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OBJECTIVE: To investigate the inhibitory effect of Jinhua Weikang capsule (JWC) on gastric inflammation induced by Helicobacter pylori (H. pylori) via the nuclear factor-kappa B (NF-κB) signaling pathway in Kunming mice.

METHODS: We investigated the anti-inflammation potential of JWC extract in vivo in a H. pylori-induced gastritis mouse model. The expression of inflammation-related molecules was evaluated by Western blotting, and the concentrations of in vivo inflammatory markers were measured by enzyme-linked immunosorbent assay. Inflammatory cell infiltration was evaluated by histopathological examination, and mRNA levels of related genes were evaluated by quantitative reverse transcription polymerase chain reaction.

RESULTS: JWC had a dose-dependent protective effect against H. pylori-induced gastritis by protecting gastric epithelial cells and inhibiting inflammatory cell infiltration. Mechanistically, JWC decreased the protein levels of phosphorylated IκBα and NF-κB p65, mRNA levels of NF-κB pathway molecules, and plasma levels of tumor necrosis factor-α and interleukin 1 beta.

CONCLUSION: An important finding of our study is that JWC attenuated gastrointestinal inflammation and ulceration and exerted a protective effect against gastric injury via inhibition of inflammation reactions and regulating the canonical NF-κB signaling pathway in vivo.

Keywords: Helicobacter pylori; NF-kappa B; Signal transduction; Jinhua Weikang capsule

INTRODUCTION

Helicobacter pylori (H. pylori) is a microaerophilic, Gram-negative flagellate bacterial pathogen associated with gastric cancer development. Although the precise mechanisms and pathogenic processes leading to H. pylori-elicited diseases remain poorly understood, some evidence suggests that such disorders are mediated by activated immune responses and influenced by environmental factors and host genetic factors. H. pylori is considered to be a major pathogen involved in peptic ulcers, gastritis, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue lymphoma, and...
plays an important role in the preliminary stages of gastric cancer. Chronic inflammation is believed to be a key factor in the processes of these gastrointestinal diseases. H. pylori and other bacterial infections activate signaling pathways that result in overexpression of pro-inflammatory cytokines and many other genes related to ongastricpathology. Therefore, genetic polymorphisms of pro-inflammatory cytokines involved in inflammatory responses may influence persistent H. pylori infection and clinical outcomes of H. pylori-induced diseases. Many genes activated by gastric pathology after bacterial infection are modulated by nuclear factor-kappa B (NF-κB). The canonical activation pathway of NF-κB has been identified as crucial for the initiation and maintenance of many sporadic and inflammation-associated gastrointestinal diseases.

Jinghua Weikang capsule (JWC) is a patented medicine approved by the SFDA in China (Z10970067) for gastric ulcers, duodenal ulcers, chronic gastritis, and inhibition of H. pylori-induced diseases, which has proven efficacy in clinical use. In this study, we investigated whether JWC affects inflammation in H. pylori-induced gastritis via the NF-κB signaling pathway in vivo and examined genes involved in the inflammatory and immune responses, which may be beneficial for the design of more effective strategies.

MATERIALS AND METHODS

JWC

JWC was kindly provided by Tasly Pharmaceutical Co., Ltd., (Batch No. 1510001, Tianjin, China) in the form of volatile oil. JWC consists of Chenopodium Ambrosioides L. and Adina pilulifera with the quality confirmed by GC/MS analysis. The major constituents of JWC included α-terpinene, 4-isopropyltoluene, and terpinolene, which is consistent with previous reports.

H. pylori strains

H. pylori Sydney strain 1 (SS1, cagA+, vacA s2/m2) was kindly provided by the Department of Gastroenterology of Peking University First Hospital and stored at −80 °C. The strain was grown in Brucella broth blood agar in a microaerophilic environment.

Animal treatments and tissue sampling

Male KM mice (6 weeks old, weight: 18-22 g, Certification No. SYXK 2014-0010, Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) were bred in a specific pathogen-free animal room at the animal facility of Peking University Health Science Center and divided randomly into six groups (n = 10 per group): sham group, control group, LCM group (12.33 mg/kg lansoprazole, 205.54 mg/kg clarithromycin, and 164.40 mg/kg metronidazole, 1 week, intragastric), and JWC groups treated with three doses (25, 50, and 100 mg/kg daily for 4 weeks, intragastric). Except for the sham group, mice were infected oro-gastrically with five doses (during a 10 d period) of 1 × 10⁸ colony-forming units of H. pylori strain SS1. Based on our previous study, this method provides a high probability of primary colonization. As reported previously, infected mice developed inflammation with higher H. pylori histological scores by increases in hyperplasia and dysplasia, and infiltration of inflammatory cells compared with uninfected mice. Infected mice were either treated with low dose JWC (25 mg/kg), intermediate dose JWC (50 mg/kg), and high dose JWC (100 mg/kg) per day for 4 weeks oro-gastrically or positive control LCM (12.33 mg/kg lansoprazole, 205.54 mg/kg clarithromycin, and 164.40 mg/kg metronidazole) per day for 1 week oro-gastrically. All test components were diluted in Milli-Q (MQ) water. All experimental protocols were approved by the Institutional Animal Ethics Committee of Peking University Health Science Center in accordance with the guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Animals were sacrificed under anesthesia at 4 weeks after administration. Serum and gastric tissue specimens were obtained at the time of necropsy. The serum was obtained by centrifugation at 3000 g for 10 min and stored at −80 °C for biochemical analysis. One-centimeter tissue sections were obtained and fixed in 4% paraformaldehyde for embedding in paraffin. The remaining tissue was used for protein and mRNA extractions.

Enzyme-linked immunosorbent assays (ELISA)

Levels of TNF-α and IL-1β were quantified using commercial ELISA kits (Biotechnology Co., Ltd., EK-Bio-science, Shanghai, China). The results were obtained using a microplate reader (infinite M200PRO, Tecan, Männedorf, Switzerland). All assays were performed according to the manufacturer’s instructions. Optical density measurements were taken at 450 nm. The absolute concentrations of TNF-α and IL-1β in culture medium were calculated from standard curves.

Western blot analysis

Biopsy specimens of mouse stomach were snap-frozen in liquid nitrogen and stored at −80 °C. The specimens were homogenized on ice using lysis buffer containing protease inhibitors (Applygen Technologies Inc., Beijing, China) and centrifuged at 16 000 g for 15 min for nuclear and total protein extractions using a Nuclear and Cyttoplasmic Protein Extraction Kit (key-GEN bioTECH, China) and DNA/RNA/Protein Isolation Kit (keyGEN bioTECH, Jiangsu, China). The protein concentration was determined with a BCA protein assay kit (Beyotime Biotechnology Company, Shanghai, China). Protein samples (30 µg) were boiled for 5 min and separated by 10% SDS-PAGE at 100 V
for 2 h. Wet transfer of proteins from the gel to a methanol-activated polyvinylidene difluoride membrane was conducted at 300 mA for 1.5 h. The membrane was blocked in Tris-buffered saline with 0.1% Tween-20 (TBST) containing 5% BSA (Amresco, Ohio, USA) and then incubated with anti-phospho-NF-κB p65 [S536, Cell Signaling Technology (CST), MA, USA], anti-NF-κB p65 [D14E12, Cell Signaling Technology (CST), MA, USA], anti-IκKα [3G12, Cell Signaling Technology (CST), MA, USA], anti-IκKB [D30C6, Cell Signaling Technology (CST), MA, USA], anti-phospho-IκBα [14D4, Cell Signaling Technology (CST), MA, USA], and anti-β-actin [13E5, Cell Signaling Technology (CST), MA, USA] antibodies at 4 °C overnight. After washing in TBST three times, the membranes were incubated with the corresponding HRP-conjugated secondary antibodies [7074P, goat anti-rabbit IgG, HRP-linked antibody, Cell Signaling Technology (CST), MA, USA] at room temperature for 1 h and then visualized using Immunoblot Western Chemiluminescent HRP Substrate (ECL, Millipore, MA, USA). The protein abundance was determined by the protein band intensity using Image Lab software (Version 5.1, Bio-rad, CA, USA) and normalized to β-actin (loading control).

RNA extraction and qRT-PCR
Total RNA was extracted from the gastric tissue with TRIzol reagent (Invitrogen, CA, USA). RNA (1 μg) was reverse transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, CA, USA). The transcripts were amplified in one tube containing 1 μL cDNA, 1 μL of each primer, and 10 μL Power SYBR Green PCR Master Mix (Applied Biosystems, CA, USA) in a total volume of 20 μL. PCR amplification was performed at 95 °C for 10 min, followed by 40 cycles at 95 °C for 30 s, 54 °C for 30 s, 72 °C for 30 s, and a final extension at 95 °C for 30 s, 55 °C for 30 s, 95 °C for 30 s. Real-time PCR was conducted using an ABI 7500 Fast real-time PCR system (Applied Biosystems). Three replicates were run for each assay. The primer sequences were designed using Primer3 and obtained from Tianyi Huiyuan Biological Technology Co., Ltd., Beijing, China. Primer sequences for qPCR of NF-κB p65, IκBα, IκKα, IκKB, and GAPDH mRNAs are shown in Table 1. The mRNA expression levels of NF-κB p65, IκBα, IκKα, IκKB, and GAPDH were quantitatively analyzed by the 2^{-ΔΔCt} method and normalized to GAPDH levels.17

Histology, scanning electron microscopy, and immunohistochemistry of gastric lesions
Gastric tissues were embedded in paraffin, and 5 μm-thick sections were prepared after fixing in 4% paraformaldehyde and then stained with hematoxylin-eosin (HE) and Warthin-Starry (WS) or subjected to immunohistochemistry to analyze H. pylori and in-flammation. A light microscope (Leica ICC50 HD, Wetzlar, Germany) was used for histopathological examination. The avidin-biotin complex immunoperoxidase procedure was carried out according to the manufacturer’s instructions (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The sections were incubated with a rabbit anti-H. pylori polyclonal antibody [D14E12, Cell Signaling Technology (CST), Massachusetts, USA]. Then, the sections were counterstained with Harris’s hematoxylin. Electron microscopy specimens were prefixed in a solution of 2.5% glutaraldehyde for 2 h and after washing with a cacodylate buffer solution and phosphate-buffered saline. The samples were post-fixed in 2% osmium tetroxide for 2 h and dehydrated with ethanol. All specimens were sputter coated with gold palladium. The tissues were observed under an electron microscope (S-3400N, Hitachi, Japan) at 5 KV.

Statistical analysis
SPSS 22.0 (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY, USA) was used. The Student’s t-test was conducted to analyze data. All data are presented as the mean ± standard deviation ( x̄ ± s) of three independent experiments. For data with heterogeneity of variance, the Kruskal-Wallis H test was used, and the Nemenyi test was applied for comparisons between groups. P < 0.05 (two-tailed) was considered to be statistically significant.

RESULTS

JWC protects gastric tissue in KM mice
H. pylori infection was confirmed by HE, WS, and immunohistochemical staining as well as scanning electron micrographs of the gastric epithelium. H. pylori infection and inflammatory cell infiltration in gastric tissue were evaluated by HE staining. HE staining showed an orderly stomach cell structure in the sham infection and inflammatory cell infiltration in gastric tissue were evaluated by HE staining. HE staining showed an orderly stomach cell structure in the sham infection and inflammatory cell infiltration in gastric tissue were evaluated by HE staining. HE staining showed an orderly stomach cell structure in the sham.
Serum concentrations of inflammatory cytokines
The production of proinflammatory cytokines IL-1β and TNF-α in all groups was measured by ELISA (Figure 3). JWC down-regulated serum concentrations of TNF-α and IL-1β in Kunming mice, and IL-1β and TNF-α levels in the Control group were significantly increased (P < 0.001) compared with those in the Sham group, validating inflammation in the KM models due to microbial infection. After treatment with JWC and LCM, the levels of IL-1β and TNF-α were decreased significantly compared with those in the control group.

JWC suppresses activity of the IKKα/β-IκB-NF-κB signaling pathway in gastric tissue
After treatment with JWC, the expression of p-NF-κB, p-IκBα, IKKα, and IKKβ was decreased at both protein and mRNA levels and showed a dose-dependent tendency to a certain extent, suggesting that JWC may exert an anti-inflammatory effect through inhibition of the NF-κB signaling pathway at an intermediate dose (Figures 4, 5). The relationship between mRNA level decreases and protein level increases needs to be investigated further.

DISCUSSION
There have been a number of studies illustrating the clinical effects of JWC in various diseases, and evidence suggests that JWC may regulate multiple targets and pathways for synergistic positive benefits. However, the mechanisms and specific pathways have not been elucidated. In this study, we revealed a critical role of the NF-κB pathway in H. pylori-induced inflammation, and that JWC protects gastric tissue from H. pylori-induced injury and inhibits inflammation via the NF-κB signaling pathway, which provides a possible mechanistic link between inflammation and cancer. H. pylori infection induces an array of inflammatory cytokines, leading to gastritis and peptic ulcers. In vivo, cytokine production changes coincide with the process of gastritis towards severity. The production of IL-1β and TNF-α in gastric mucosa of H. pylori-infected gastritis mice, which are involved in the activation of macrophages and neutrophils at infectious sites, suggests the involvement of H. pylori in various diseases. In addition, exacerbated production of inflammatory cytokines may be crucial for a poor prognosis of H. pylori-induced diseases. Many signaling pathways mediated by IL-1β and TNF-α participate in inflammation and apoptosis regulation, such as PI3k-AKT/mTOR, JAK-STAT,
cies difference, although mouse models are widely ap-

plied in research of this disease. H. pylori does not nor-

MAPK, and TGF-β/SMAD. NF-κB p65 is an essen-
tial positive regulator when specifically activated in re-
sponse to inflammation, providing many amenable phar-

macological targets for inflammation diagnosis. Based
on the data in our study, we speculate that sup-

pression of NF-κB may be responsible for the anti-in-

flammatory and stomach-protective effects of JWC. It
is a popular Chinese patented medicine and provides
active ingredients in one prescription, which work to-

gether to attain better therapeutic benefits and on nu-

merous targets in treating complex diseases. Based on
the obtained results of inflammation protective effects
of JWC, further investigation needs to be performed
with a focus on the possible mechanisms involved in
the anti-inflammatory effects of JWC.

Previous studies have reported that the mechanisms un-
derlying the effects of JWC are based on its antifungal,
insecticidal, and anti-H. pylori effects. JWC pro-
tects gastric mucosa, cures gastric ulcers, and boosts
multiplication, differentiation, migration, and repair of
endothelial cells. It is possible that the observed effects
are associated not only the elimination of H. pylori,
but also anti-inflammatory and anti-adhesion ef-
fects. Further investigation should be performed
with more focus on connections between different
mechanisms induced by the multiple active compo-
nents. Moreover, a limitation of our study is that the
mouse infection model cannot accurately represent H.
pylori-induced gastritis in humans because of the spe-
cies difference, although mouse models are widely ap-
plied in research of this disease. H. pylori does not nor-
mally colonize mice. The mouse-adapted strains that
colonize the murine stomach are different strains from
those in humans with the important virulence factors
Cag A and Vac A lost during adapted colonization.

JWC has been used to treat patients with gastritis for
decades with the advantages of invigorating vital ener-

gy, clearing heat toxicity, and moistening the stomach.
Our previous study demonstrated anti-inflammatory
activities of JWC in vivo and in vitro. However, the
specific mechanisms of the anti-inflammatory effects
of JWC were not elucidated. In this study, we demon-
strated that JWC decreased the protein levels of phospho-
ylated NF-κB p65, IκBα, and IKKα/β, mRNA levels of
IKKα/β, IκBα, and NF-κB, and plasma levels of
TNF-α and IL-1β. These data were consistent and

clearly indicated that JWC suppressed H. pylori-in-
duced gastritis dose-dependently.

In conclusion, our findings demonstrate a JWC syn-
ergetic effect of anti-inflammatory activity via the
NF-κB signaling pathway by down-regulating NF-κB
p65, IκBα, IKKα, and IKKβ expression and provides
insights into the mechanism underlying the JWC ac-
tion in treating H. pylori infection and its related gas-
tric diseases.
Figure 5 mRNA levels of the IKKa/β-IkB-NF-κB signaling pathway
A: nuclear factor-κB (NF-κB) mRNA expression in each group; B: inhibitor of κB α (IκBα) mRNA expression in each group; C: IκB kinase α (IKKα) mRNA expression in each group; D: IκB kinase β (IKKβ) mRNA expression in each group. 1: sham group; 2: control group; 3: LCM group; 4: 25 mg/kg JWC; 5: 50 mg/kg JWC; 6: 100 mg/kg JWC. Sham: mouse without intervention; control: mouse model with H. pylori infection; LCM: mouse model treated with 12.33 mg/kg lansoprazole, 205.54 mg/kg clarithromycin, and 164.40 mg/kg metronidazole, 1 week, intragastric; JWC: mouse model treated with three doses of Jinghua Weikang capsule: 25, 50, and 100 mg/kg daily for 4 weeks, intragastric. *P < 0.05 vs control group, **P < 0.05 vs sham group.

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