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Original Research Article

Curculigoside attenuates *Helicobacter pylori*-induced inflammation and apoptosis of gastric mucosal epithelial cells via NF-κB pathway

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Abstract

Purpose: *To investigate the possible effects and mechanisms of action of curculigoside (Cur) on gastric ulcers.*
Methods: *Human gastric mucosal epithelial GES-1 cells were infected with Helicobacter pylori and then treated with Cur. Cell counting kit 8 (CCK-8), flow cytometry, and immunofluorescence assays were used to investigate the effect of Cur on cell viability and apoptosis after exposure to H. pylori. Inflammation status and reactive oxygen species (ROS) were assessed using enzyme-linked immunoassay (ELISA) and dichlorodihydrofluorescein (DCF) staining, respectively, while immunoblot and immunofluorescence assays were performed to determine the effect of Cur on the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway.*
Results: *Cur significantly increased H. pylori-induced cell viability and inhibited H. pylori-induced inflammatory cytokine production ($p < 0.01$). Furthermore, Cur significantly suppressed H. pylori-induced ROS production and apoptosis ($p < 0.01$). Through the NF-κB pathway, Cur attenuated H. pylori-induced inflammation and apoptosis of gastric mucosal epithelial cells.*
Conclusion: *In H. pylori infection, Cur treatment increases cell viability, reduces inflammatory cytokine production, suppresses ROS production, and inhibits apoptosis via NF-κB pathway. Further investigation using whole animal experiments would be needed to establish the role of Cur in the management of H. pylori-induced gastric ulcers.*

Keywords: *Gastric ulcers, Curculigoside, Inflammation, Apoptosis, NF-κB pathway*

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INTRODUCTION

Gastric ulcers are a common gastrointestinal clinical manifestation associated with oxidative stress and increased inflammation [1]. A variety of stressors cause pediatric gastric ulcers, the most common of which is increased gastric acid and pepsin secretion, which is closely related to *Helicobacter pylori* infection. *Helicobacter pylori* infection clusters within families, and gastric ulcers lead to serious complications if treatment is inadequate [2]. Although a variety of medications are used to treat gastric ulcers, these drugs produce adverse side effects.

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Furthermore, current treatment regimens are ineffective in controlling ulcer recurrence [3]. *H. pylori* colonizes human gastric epithelial cells and induces the release of pro-inflammatory factors and associated oxidative damage [4]. Pro-inflammatory factors trigger and activate neutrophils, which are major contributors to reactive oxygen species (ROS) production [5].

Nuclear factor kappa-light chain enhancer (NF-κB) is a transcription factor that activates B cells and is known to play a key role in a variety of biological processes such as inflammation, cell growth, and apoptosis [6]. The NF-κB induces the production of a large number of cytokines and growth factors, which further activates NF-κB, exacerbating the inflammatory response [7]. Inhibiting the NF-κB signaling pathway improves ethanol-induced gastric ulcers in rats [8]. Furthermore, NF-κB activation promotes inflammation and carcinogenesis of gastric mucosal epithelial cells during infection with *H. pylori*.

Curculigoside (Cur) is a phenolic glycoside antioxidant extracted from the plant *Curculigo orchioides* Gaertn, which has anti-inflammatory and anti-tumor properties [9]. Cur inhibits oxidative stress induced by hydrogen peroxide and further improve the viability of myocardial ischemia cells and reduces apoptosis [10]. Cur also suppresses disease activity index, histological damage, cell death, and iron death in mice with ulcerative colitis. Furthermore, oxidative stress and osteoclast formation are reduced by inhibiting the NF-κB pathway. This study investigates the effects of Cur treatment on *Helicobacter pylori*-induced inflammation and the mechanism of apoptosis of gastric mucosal epithelial cells.

EXPERIMENTAL

Cell culture

Human gastric mucosal epithelial GES-1 cell line was obtained from iCell Bioscience Inc (China). The GES-1 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10 % fetal bovine serum (FBS) and 100 μg/mL penicillin-streptomycin at 37 °C with 5 % CO_2, with the medium changed every 2 days. Cells were stimulated with Cur at concentrations of 5, 10, 15, and 20 μM. *H. pylori* (*H. pylori*, ATCC43504, CagA+, and VacA+) was cultured on Campylobacter agar plates containing 10 % sheep serum and incubated at 37 °C for 24 h. The GES-1 cells were cultured in the presence of *H. pylori* at different multiplicities of infection (MOIs).

Cell viability

The GES-1 cells were plated on 96-well plates at a density of 1 × 10^3 cells/well. Cell viability was assessed by adding CCK-8 solution. After treatment with Cur or *H. pylori*. Cells were washed with phosphate-buffered saline (PBS), and CCK-8 agent was added to cells. The absorbance (A) value in each well was quantified with a microplate reader at 450 nm. The absorbance in the experimental group was normalized to that of the control group.

Quantitative real time-polymerase chain reaction (qRT-PCR)

Total cellular RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. Total RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega Corporation). The resulting cDNA was amplified using the primers in Table 1.

Table 1: Quantitative PCR primer sequences used

Primer	Forward sequence (5′-3′)	Reverse sequence (5′-3′)
TNF-α	GGTGCCTATGTCTCAGCCTCTT	GCCATAGAACTGATGAGAGGGAG
IL-8	ACTGAGAGTGATTGAGAGTGGAC	AACCCTCTGCACCCAGTTTTC
IL-23	GAGCCTTCTCTGCTCCCTGATA	GACTGAGGCTTGGAATCTGCTG
MCP-1	AGAATCACCAGCAGCAAGTGTCC	TCCTGAACCCACTTCTGCTTGG
GAPDH	AGAAGGCTGGGGCTCATTTG	AGGGGCCATCCACAGTCTTC

Enzyme-linked immunosorbent assay (ELISA)

After stimulation as indicated, the levels of tumor necrosis factor α (TNF-α), interleukin-23 (IL-23), monocyte chemoattractant protein-1 (MCP-1), and IL-8 in cell supernatants were determined using ELISA assays according to the manufacturer's guidelines. The ELISA kits were obtained from Shanghai Xitang Biotechnology Co., Ltd (Shanghai, China).

Evaluation of ROS

Intracellular ROS levels were assayed using a 2',7'-dichlorofluorescein diacetate (DCFH-DA) fluorescent probe (Sigma-Aldrich). To this end, cells were fixed with formaldehyde, washed with PBS, and permeabilized with PBS containing 0.5 % Triton X-100. The cells were subsequently stained with DCFH-DA and analyzed with a fluorescent microscope.

Assessment of cell apoptosis

To determine the apoptotic cell number, Annexin V/propidium iodide (PI) apoptosis measurement was conducted using the cell apoptosis kit (Sigma-Aldrich, USA). Briefly, cells were digested into single cells and resuspended in reaction buffer containing Annexin V and PI for 5 min at room temperature while protected from light.

Immunoblot assay

Proteins were extracted with radioimmunoprecipitation assay (RIPA) buffer (Beyotime), and protein concentrations were analyzed using a BCA kit. Proteins were then separated using a 10 % sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5 % BSA in TBST buffer and incubated with primary antibodies targeting Bax, cleaved-caspase3, p-p65, p65, or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1000, Abcam, Cambridge, UK) for 1 h, at room temperatures. The blocked membranes were incubated with the horseradish peroxidase (HRP) labelled as secondary antibodies for 1 h, and the blots were analyzed using an enhanced luminol-based chemiluminescent (ECL) kit.

Immunofluorescence studies

Cells were fixed with formaldehyde, washed with PBS, and permeabilized with PBS containing 0.5 % Triton X-100. The permeabilized cells were stained with p65 primary antibody (Abcam, Cambridge, UK), rinsed in PBS, and incubated with fluorescent secondary antibody. The cells were then stained with 4',6-diamidino-2-phenylindole (DAPI), mounted with mounting media, and imaged using fluorescence microscopy.

Statistical analysis

GraphPad 7.0 was used for all statistical analysis. Data are presented as mean ± standard error of the mean (SEM), with $p < 0.05$ considered significant.

RESULTS

Cur restores cell viability in GES-1 cells infected with *H. pylori*

To evaluate the effect of Cur on GES-1 cell viability, CCK-8 assay was performed. GES-1 cells were incubated with the different doses of Cur (Figure 1 A). A low dose of Cur minimally affected cell viability, whereas a high dose of Cur (20 μM) significantly impaired cell viability (Figure 1 B). Next, cell viability was assessed after infection with *H. pylori* and treatment with Cur. GES-1 cell viability was reduced by *H. pylori* infection. However, Cur contributed to cell viability during *H. pylori* infection in a dose-dependent manner (Figure 1 C). These data reveal that Cur improved cell viability during *H. pylori* infection.

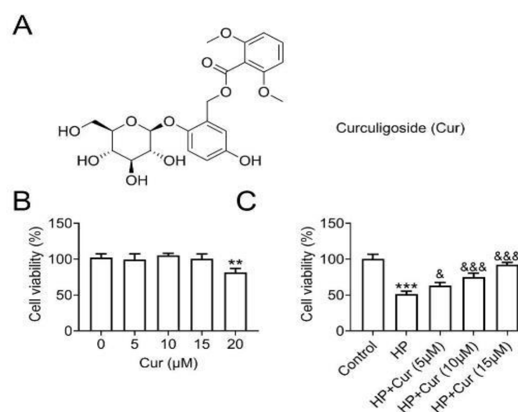

Figure 1: Cur enhanced cell viability in GES-1 cells infected with *H. pylori*. (A) The structure of Cur. (B) GES-1 cell viability at increasing Cur dose. (C) GES-1 cell viability in response to *H. pylori* infection and elevated Cur levels. $^{**}P < 0.01$, $^{***}p < 0.001$ vs control, $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ vs *H. pylori*

Cur reduced pro-inflammatory cytokine levels

The GES-1 cell inflammation response was assessed by measuring the mRNA and protein levels of inflammatory cytokines. *H. pylori* stimulation enhanced GES-1 cell inflammation, as was revealed by increased mRNA and protein levels of TNF-α, IL-8, MCP-1, and IL-23 (Figure 2 A and B). On the other hand, Cur treatment during *H. pylori* infection of GES-1 cells attenuated mRNA and protein levels of TNF-α, IL-8, MCP-1, and IL-23. Based on the data, Cur reduced *H. pylori*-induced pro-inflammatory cytokine levels in GES-1 cells.

Cur suppressed oxidative stress

Because *H. pylori* induced the release of pro-inflammatory factors and associated oxidative damage upon colonization of GES-1 cells, ROS levels were measured in different treatment groups. *H. pylori* induction significantly increased ROS levels in GES-1 cells, but Cur treatment attenuated *H. pylori*-induced ROS production (Figure 3). These results suggest that Cur reduces oxidative stress in GES-1 cells.

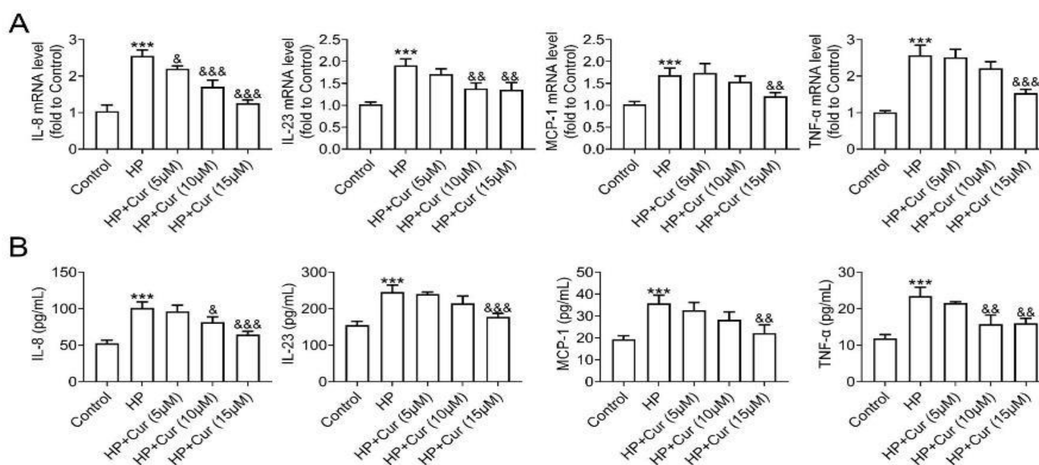

Figure 2: Cur inhibited pro-inflammatory cytokine levels in GES-1 cells. (A, B) The mRNA and protein levels of TNF-α, IL-8, MCP-1, and IL-23 as measured using RT-qPCR and ELISA assays, respectively. $^{***}P < 0.001$ vs control, $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ vs *H. pylori*

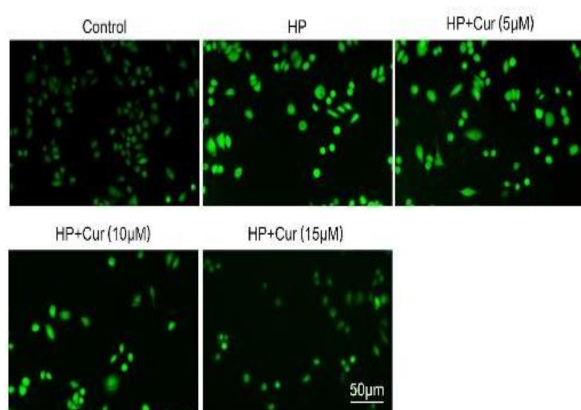

Figure 3: Cur repressed oxidative stress induced in GES-1 cells. DCF staining of GES-1 cells in response to *H. pylori* and elevated levels of Cur

Cur relieved cell apoptosis

The effect of Cur on cell apoptosis was evaluated using flow cytometry. Cell apoptosis increased in *H. pylori*-stimulated GES-1 cells, whereas Cur treatment during *H. pylori* stimulation decreased GES-1 cell apoptosis (Figure 4 A). Furthermore, *H. pylori* infection significantly induced the expressions of Bax and cleaved-caspase-3, but the addition of Cur reduced these observed expressions (Figure 4 B). These data confirmed that Cur reversed cell apoptosis induced by *H. pylori*.

Cur attenuated *H. pylori*-induced inflammation and apoptosis

To reveal the underlying mechanisms involved in the effect of Cur in inflammation and apoptosis of gastric mucosal epithelial cells, the NF-κB signaling pathway was investigated. *H. pylori* infection led to elevated levels of p-NF-κB in GES-1 cells (Figure 5 A), with Cur treatment inhibiting the observed p-NF-κB elevation. Moreover, *H. pylori* infection led to p65 accumulation in the nucleus, but Cur treatment inhibited the nuclear accumulation of p65 (Figure 5 B). These results indicate that Cur attenuate *H. pylori*-induced inflammation and apoptosis of gastric mucosal epithelial cells through the NF-κB pathway.

DISCUSSION

Stomach ulcers are very common digestive disorders, which develop when the protective lining of the stomach is damaged [11,12]. Globally, about 10 % of people will develop stomach ulcers in their lifetime [13]. Therefore, it is imperative to determine the pathogenesis of stomach ulcer and improve current therapeutics [1]. Studies have increasingly shown that inflammation, ROS, and apoptosis are closely linked to the development of gastric ulcers [14]. In this study, a phenolic glycoside antioxidant, Cur, showed potential as a promising therapeutic agent for stomach ulcers.

Through a series of *in vitro* assays, the results confirm that Cur attenuates *H. pylori*-induced inflammation and apoptosis of gastric mucosal epithelial cells. In this study, human gastric mucosal epithelial cells were infected with *H. pylori* to establish an *in vitro* gastric ulcer model. As determined with CCK-8 and ELISA assays, Cur increased the viability and suppressed the production of inflammatory factors. Further investigation revealed that Cur suppressed *H. pylori*-induced ROS production and apoptosis.

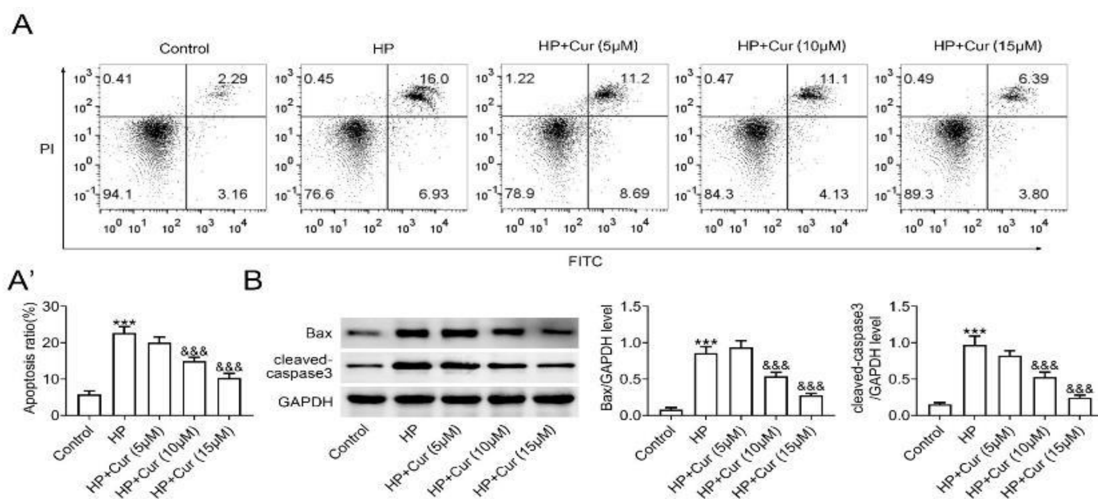

Figure 4: Cur relieved cell apoptosis in GES-1 cells infected by *H. pylori*. (A) GES-1 cell apoptosis in response to *H. pylori* and elevated levels of Cur were detected by flow cytometry. (B) The expression of Bax and cleaved-caspase-3 in response to *H. pylori* infection and elevated Cur levels. $^{***}P < 0.001$ vs control, $^{\&\&\&}p < 0.001$ vs *H. pylori*

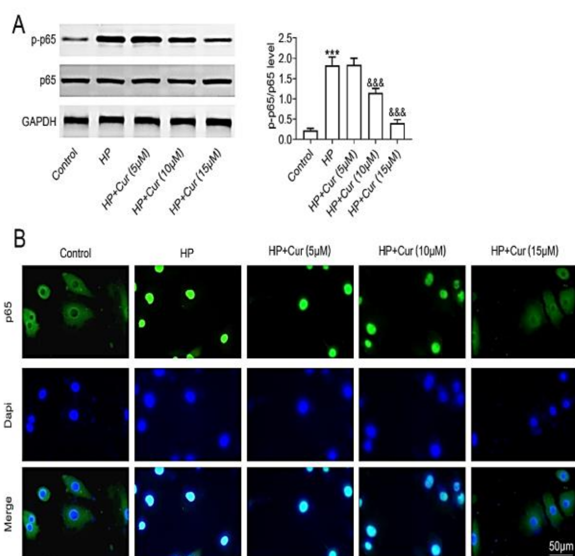

Figure 5: Cur attenuates *H. pylori*-induced inflammation and apoptosis of gastric mucosal epithelial cells through the NF-κB pathway. (A) Immunoblot depicting p-NF-κB expression in *H. pylori* and Cur-treated GES-1 cells. (B) Immunofluorescent staining of p65 in *H. pylori* and Cur-treated GES-1 cells. $^{***}P < 0.001$ vs control group, $^{\&\&\&}p < 0.001$ vs *H. pylori* group

Interestingly, several studies of Cur have confirmed multiple biological activities, such as anti-inflammatory and anti-tumor effects [15]. Cur has been reported to mitigate hepatic ischemia/reperfusion-induced (HI-RI-induced) oxidative stress, inflammation, and apoptosis by activating the nuclear factor erythroid 2–related factor 2/heme oxygenase-1 (Nrf-2/HO-1) pathway [16]. Similarly, this study revealed the effects of Cur on the oxidative stress, inflammation, and apoptosis of gastric epithelial cells. Cur has also been reported to ameliorate bone loss by affecting mesenchymal stem cell fate in aging mice [17]. Furthermore, Cur promotes osteogenic differentiation, preventing ovariectomized-induced osteoporosis. Curcumin also protects against titanium particle-induced osteolysis by enhancing osteoblast differentiation as well as reducing osteoclast formation.

Notably, *H. pylori* colonizes human gastric epithelial cells and induces the release of pro-inflammatory factors. Therefore, ROS production and inflammation are two critical components of this disease. Gastric ulcers are peptic ulcers caused by long-term inflammation. Drugs that inhibit gastric acid secretion and protect gastric mucosa should be used to treat serious gastric ulcer inflammation. However, the precise mechanism requires further investigation.

NF-κB plays a key role in a variety of biological processes, including inflammation, immune responses, cell growth, and apoptosis. Inhibition of the NF-κB signaling pathway alleviated ethanol-induced gastric ulcers in rats [18]. Furthermore, NF-κB activation promotes inflammation and carcinogenesis in gastric mucosal epithelial cells during *H. pylori* infection. In this study, Cur attenuated *H. pylori*-induced inflammation and apoptosis of gastric mucosal epithelial cells through the NF-κB pathway, suggesting that NF-κB pathway may serve as a promising target to treat gastric ulcers.

CONCLUSION

In *H. pylori* infection, Cur treatment increases cell viability, reduces inflammatory cytokine

production, suppresses ROS production, and inhibits apoptosis. Furthermore, Cur attenuates *H. pylori*-induced inflammation and apoptosis of gastric mucosal epithelial cells via NF-κB pathway.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Yongjian Li and Xiaoyan Su designed and carried out the experiments. Yongjian Li analyzed and interpreted the data. Xiaoyan Su prepared the manuscript.

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