ORIGINAL PAPER
Polish Journal of Microbiology
2025, Vol. 74, No 1, 60–70
https://doi.org/10.33073/pjm-2025-005

Hyperoside Alleviates *Helicobacter pylori*-Induced Gastric Epithelial Cell Injury by Regulating Nrf2/HO-1 Signaling

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Submitted 14 June 2024, accepted 8 January 2025, published online 25 March 2025

Abstract

Infection with *Helicobacter pylori* is the major causative factor of chronic gastritis, peptic ulcer, gastric cancer, and other diseases. Gastric mucosal epithelial injury characterized by abnormal apoptosis, oxidative stress, and inflammation is a crucial mechanism of *H. pylori* infection. Hyperoside (HYP) is a flavonol glycoside derived from many herbal plants, which exhibits potent anti-apoptotic, antioxidant, and anti-inflammatory properties. Our research explored whether it exerts protective effects on *H. pylori*-infected human gastric epithelial cells. GES-1 cells were first treated for 24 h with HYP (0, 10, 20, 40, 80, 100, or 120 μM) to determine the cytotoxicity of HYP. Subsequently, GES-1 cells were pre-treated for 4 h with HYP (80 μM), followed by exposure to *H. pylori* for 24 h. CCK-8 assay, flow cytometry assay, ELISA, RT-qPCR, DCFH-DA staining, the commercial assay kits, immunofluorescence staining, and western blotting were used to assess cell viability, cell apoptosis, pro-inflammatory cytokine levels, oxidative stress marker levels, and Nrf2/HO-1 signaling-related molecule levels. The Nrf2 inhibitor ML385 was employed to verify the beneficial role of Nrf2 activation in HYP-mediated GES-1 cell injury induced by *H. pylori*. The results showed that HYP pre-treatment reversed *H. pylori*-induced cell apoptosis, inflammation, and oxidative stress in GES-1 cells. Furthermore, HYP downregulated Nrf2, HO-1, and NQO1 protein levels in *H. pylori*-infected GES-1 cells. ML385 overturned the protective effects of HYP against *H. pylori*-induced GES-1 cell apoptosis, inflammation, and oxidative stress. In conclusion, HYP protects gastric epithelial cells against *H. pylori*-induced cell injury by activating the Nrf2/HO-1 pathway.

K e y w o r d s: Hyperoside; gastritis, *Helicobacter pylori*, human gastric epithelial cells, inflammation, oxidative stress; apoptosis

Introduction

Half of the worldwide population is infected with *Helicobacter pylori*, a Gram-negative, microaerophilic bacterium colonizing the gastric mucosa (Sun et al. 2023). Long-term infection of *H. pylori* can produce various virulence factors in the stomach, which causes chronic damage to the gastric mucosa and eventually evokes gastritis, gastroduodenal ulcer, MALT lymphoma, and gastric cancer (de Brito et al. 2019). Antibiotic-based combination therapy is the clinic's most effective treatment against *H. pylori* infections (Yang et al. 2014). However, the increased antibiotic resistance has greatly reduced the eradication rate of *H. pylori*, and long-term use of antibiotics might bring side effects, including allergic reactions, liver and kidney damage, gastrointestinal reactions, and neurological damage (Aumpan et al. 2023). Therefore, it is necessary to develop more effective non-antibiotic drugs with anti-*H. pylori* property for gastric disorders.

The epithelial cells on the surface of the gastric mucosa and the mucus secreted by them form the mucosal barrier, which has a protective effect on the gastric mucosa (Joh et al. 2003). *H. pylori* directly damages the gastric mucosa through inducing oxidative stress and inflammation in epithelial cells (Alzahrani et al. 2014). *H. pylori* activates NADPH oxidase to promote reactive oxygen species (ROS) accumulation in gastric epithelial cells, further upregulating pro-inflammatory cytokine expression and triggering cell apoptosis (Kim et al. 2022). Additionally, studies have revealed that the *H. pylori* infection load, the ROS production, and the pro-inflammatory cytokine levels are positively correlated to the extent of gastric mucosal injury (Hardbower et al. 2014). Therefore, inhibiting gastric mucosal epithelial cell oxidative stress, inflammation,

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and apoptosis can be used as functional characteristics to search for novel agents to treat *H. pylori* infections.

Hyperoside (HYP) is a flavonol glycoside that can be derived from many herbal plants, such as *Hypericum perforatum* L., *Artemisia capillaris* Thunb., and *Crataegus pinnatifida* Bunge (Yang et al. 2013). HYP possesses anti-cancer, antioxidant, anti-inflammatory, anti-fungal, anti-viral activities and protective effects against many organs, including the liver, joints, neurons, blood vessels, lungs, kidneys, heart, and brain (Xu et al. 2022). Previously, HYP was reported as one of the main compounds in an *Aristolochia olivieri* Colleg. ex Boiss. Leaves' methanolic extract, and this extract exhibited marked antimicrobial effects against *H. pylori* and potent anti-tumor effects against gastric cancer cells (Micucci et al. 2023).

Nevertheless, HYP's specific role and mechanism in gastric epithelial cell damage caused by *H. pylori* remain unknown. In addition, accumulating evidence has confirmed that *H. pylori* infection can reduce the activity and protein level of Nrf2, which plays a pivotal role in cellular antioxidant response and is essential for maintaining the integrity of gastric epithelium (Bacon et al. 2022). Some studies have confirmed that natural phytochemicals and drugs rich in antioxidants improve *H. pylori*-induced gastric inflammation and oxidative stress by activating the Nrf2 pathway. For example, Liu et al. (2023) demonstrated that rhein exhibits antioxidant and anti-inflammatory effects in chronic atrophic gastritis, at least in part by activating Nrf2 signaling. Activation of Nrf2 by α-lipoic acid contributes to the attenuation of oxidative stress and inflammation in *H. pylori*-infected gastric epithelial cells (Kyung et al. 2019). Importantly, numerous studies have revealed that HYP exerts its effective therapeutic role in experimental models of human diseases by activating the Nrf2/HO-1 pathway (Yang et al. 2017a; Ye et al. 2017).

Our study aimed to explore the potentially therapeutic effects of HYP on *H. pylori*-induced gastric epithelial cell injury and to delineate the underlying mechanism. Based on the above literature, we hypothesized that HYP might alleviate *H. pylori*-induced damage to gastric epithelial cells by upregulating the Nrf2/HO-1 pathway.

Experimental

Materials and Methods

Cell culture. Human gastric epithelial GES-1 cells (#LZ-YX9760) were acquired from Rayzbio (China) and cultured in RPMI-1640 medium (#11875; Solarbio, China) added with 1% penicillin/streptomycin (#ZY90307; Zeye, China) and 10% fetal bovine serum (FBS; #LM87074C; LAMI Bio, China) in a 5% CO_2 humidified atmosphere at 37°C. Based on previous evidence, GES-1 cells were used to establish the *H. pylori* infection model to study gastric inflammation, oxidative stress, and apoptosis (Hu et al. 2022; Zhang et al. 2022).

Cell treatment and *H. pylori* infection. HYP (#HY-N0452; purity: 99.56%; MedChemExpress, China) was dissolved in DMSO. Standard strain *H. pylori* 43504™ ($cagA^+$, $vacA^+$) obtained from ATCC® (USA) was cultivated on rain-heart infusion plates containing 5% sterile and defibrated sheep blood under microaerobic conditions at 37°C for 24 h. *H. pylori* was then harvested in phosphate buffer solution (PBS) and added to the cells at a MOI of 100:1 for 24 h. GES-1 cells were treated for 24 h with HYP (0, 10, 20, 40, 80, 100, or 120 μM) to assess the cytotoxicity of HYP. In the subsequent assays, GES-1 cells were pre-treated with HYP (80 μM) for 4 h before infection with *H. pylori* for 24 h. To confirm the role of the Nrf2 signaling in the protective effects of HYP against *H. pylori*-induced cell injury, GES-1 cells were pre-treated with HYP (80 μM) and the Nrf2 inhibitor ML385 (5 μM; #CSGC19254; Chemstan, China) for 4 h, followed by stimulation by *H. pylori* for 24 h. The concentration of ML385 was selected according to the previous studies (Kim et al. 2022; Lv et al. 2023).

CCK-8 assay. To detect cell viability, about 1,000 GES-1 cells were seeded into each well of 96-well plates. After 24 h, cells were subjected to HYP pre-treatment and *H. pylori* infection and proceeded to grow 24 h. Then, ten microliters of CCK-8 reagent (#KTC011001; Reanta, China) were added into each well for 2 h at 37°C. The optical density (OD) value was measured at 450 nm using a microplate reader.

Flow cytometry assay. To assess cell apoptosis, GES-1 cells were seeded into 6-well plates (1×10^5 cells/well). After the indicated treatment, cells were collected, washed with ice-cold PBS, and resuspended in 400 μl of 1 × binding buffer. Thereafter, subsequently, 10 μl Annexin V-FITC (#C9212; Warbio, China) and 10 μl propidium iodide (PI) solution (#C9212; Warbio, China), was added to the cell suspension, followed by 15 min incubation away from light. Cell apoptotic proportions were analyzed on BD FACSCalibur™ Flow Cytometer (Becton, Dickinson and Company, USA).

Detection of intracellular ROS. Intracellular ROS levels were examined using the redox-sensitive dye, 2',7'-dihydrodichlorofluorescein diacetate (DCFH-DA), which can be hydrolyzed to 2',7'-dichlorodihydrofluorescein (DCFH) in the presence of intracellular esterases. It is a molecule that can be oxidized into highly fluorescent 2',7'-dichlorofluorescein (DCF). GES-1 cells were cultured in 6-well plates (1×10^5 cells/well) and then loaded with 10 μM DCFH-DA (#CS15204; Chemstan, China) diluted in serum-free medium for 20 min at 37°C. Finally, cells were washed thrice with

serum-free medium, and DCF fluorescence was quantified using a BD FACSCalibur™ Flow Cytometer.

Examination of oxidative stress markers. Following the indicated treatment, GES-1 cells were washed twice with ice-cold PBS, centrifugated for 4 min at 1,000 × *g*, and homogenized. The homogenates were centrifuged for 15 min at 4,000 × *g* to obtain the supernatants. MDA content, as well as SOD and CAT activities in the supernatants, were measured using an MDA assay kit (#abs580011; Absin, China), SOD assay kit (#abs580010; Absin, China), and CAT activity assay kit (#abs580060; Absin, China) following the manufacturer's instructions.

ELISA. GES-1 cells were seeded into 6-well plates (1×10^5 cells/well) followed by the indicated treatment. The culture medium was centrifuged at 15,000 rpm for 15 min at 4°C, and the supernatants were collected to quantify TNF-α (#XK6137S; XKBio, China), IL-6 (#XK6132S; XKBio, China), and IL-1β (#XK6129S; XKBio, China) levels using the corresponding ELISA kits.

RT-qPCR. After the extraction of total RNA from GES-1 cells using TRIzol reagent (#15589226; Canspec Scientific, China), a First Strand cDNA Synthesis kit (#LM-63826; LAMI Bio, China) was used for total RNA (5 μg) reverse transcription. For real-time quantitative PCR, cDNA was analyzed using specific primers, 1 × LightCycler® 480 SYBR Green I Master (Roche, Germany), and Roche LightCycler® 480 software. The primer sequences were as follows: TNF-α, 5'-CCAGAACTCCAGGCGGTGTC-3' (F) and 5'-GGCTACGGGCTTGTCACTCG-3' (R); IL-6, 5'-ACTCACCTCTTCAGAACGAATTG-3' (F) and 5'-CCATCTTTGGAAGGTTCAGGTTG-3' (R); IL-1β, 5'-ATGCCTCGTGCTGTCTGAC-3' (F) and 5'-TCCCGACCATTGCTGTTTCC-3' (R); and GAPDH, 5'-GATCATCAGCAATGCCTCC-3' (F) and 5'-TCCACGATACCAAAGTTGTC-3' (R). GAPDH served as an endogenous control. The relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Western blotting. Cells were inoculated into 6-well plates (1×10^6 cells/well), followed by the indicated treatments. Then, GES-1 cells were lysed for 30 min in ice-cold RIPA lysis buffer (#AWB0136a; Abiowell, China). After the quantification of the protein samples using the BCA assay kit (#mlsw-3424; MLBio, China), the proteins (50 μg) were loaded onto a 12% SDS-PAGE before transferring to nitrocellulose membranes. The membrane was blocked for 1 h at RT in 5% non-fat milk dissolved in TBST, followed by the sequential culture with the primary antibodies (Affinity Biosciences, China) against Bax (#AF0120; 1:1000), Bcl-2 (#AF0769; 1:1000), Nrf2 (#AF7006; 1:1000), HO-1 (#DF6391; 1:1000), NQO1 (#DF6437; 1:1000) and GAPDH (#AF0911; 1:1000) overnight at 4°C and HRP-conjugated IgG secondary antibodies (#S0001; 1:3000; Affinity Biosciences, China) for 1 h at RT. The protein bands were visualized using the enhanced chemiluminescence (ECL) detection system. ImageJ software (NIH, USA) was employed for the densitometry analysis. Intensity values of the bands were normalized to GAPDH band intensity.

Immunofluorescence staining. GES-1 cells were cultured on glass coverslips in a 6-well plate (5×10^3/well). After the indicated treatments, cells were fixed with 4% paraformaldehyde, washed thrice in PBS, and permeabilized with 0.1% Triton X-100 in PBS. The cells were blocked for 1 h with 3% bovine serum albumin and rinsed thrice with PBS prior to incubation overnight at 4°C with the anti-Nrf2 primary antibody (#AF7006; 1:100; Affinity Biosciences, China). An Alexa Fluor 488 goat anti-rabbit IgG secondary antibody (#S0018; 1:100; Affinity Biosciences, China) was added for another 1 h before immunofluorescence detection. After 1 min nuclear staining with DAPI, images were captured under an Olympus immunofluorescence microscope.

Statistical analysis. IBM SPSS Statistics for Windows v22.0 (IBM Corp., USA) was adopted for data analysis. All data are expressed as the mean ± SD of at least three biological replicates. Student *t*-test and one-way ANOVA followed by Tukey's post-hoc test were respectively used when comparing two and multiple groups. Statistical significance was regarded to be $p < 0.05$.

Results

HYP reduces *H. pylori*-induced GES-1 cell apoptosis. First, GES-1 cells treated with HYP (0, 10, 20, 40, 80, 100, or 120 μM) for 24 h were analyzed by CCK-8 assay to determine the cytotoxicity of HYP. As revealed in Fig. 1A, treatment with HYP in the range of 0–80 μM caused no significant changes in GES-1 cell viability. At the same time, a marked decrement was observed in cell viability after treatment with HYP higher than 100 μM. Therefore, HYP (80 μM) was selected for the subsequent assays. To assess the protective effect of HYP against *H. pylori*-induced GES-1 cell injury, cells were pre-treated with HYP (80 μM) before exposure to *H. pylori*. CCK-8 assay showed that HYP pre-treatment rescued *H. pylor*i-induced decline in GES-1 cell viability (Fig. 1B). In the flow cytometry assay, the apoptosis level in *H. pylori*-induced GES-1 cells was prominently higher than in untreated cells, while HYP pre-treatment effectively attenuated *H. pylori*-triggered cellular apoptosis (Fig. 1C). Furthermore, pre-treatment with HYP reversed *H. pylor*i-induced increment in Bax protein level and decrement in Bcl-2 protein level (Fig. 1D–1E).

HYP mitigates *H. pylori*-induced GES-1 cell inflammation. The changes in the expression of several pro-inflammatory cytokines in response to HYP pre-treatment in *H. pylor*i-infected GES-1 cells were

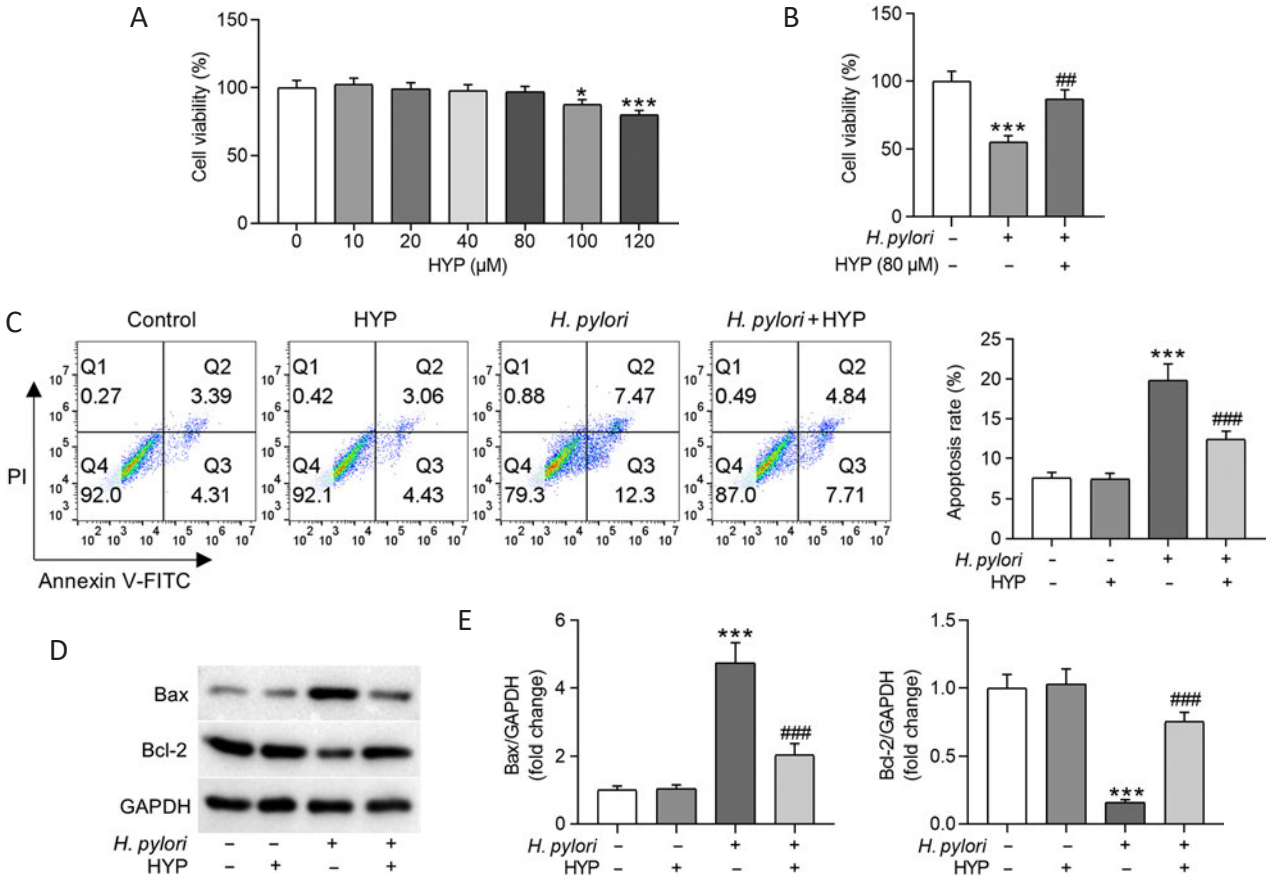

Fig. 1. HYP reduces *Helicobacter pylori*-induced GES-1 cell apoptosis.

A) Assessment of GES-1 cell viability after 24 h treatment with HYP (0, 10, 20, 40, 80, 100, or 120 μM) by CCK-8 assay;
B) evaluation of the viability of *H. pylori*-infected GES-1 cells pre-treated with or without HYP (80 μM) by CCK-8 assay;
C) analysis of GES-1 cell apoptosis following HYP pre-treatment and *H. pylori* stimulation through flow cytometry assay;
D–E) measurement of Bax and Bcl-2 protein levels in *H. pylori*-infected GES-1 cells with or without HYP pre-treatment via western blotting.
$^{*}p < 0.05$, $^{***}p < 0.001$ vs. control; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. *H. pylori*.

detected to evaluate whether HYP prevents *H. pylori*-induced inflammation. ELISA illustrated that *H. pylori* infection resulted in a remarkable elevation in TNF-α, IL-6, and IL-1β levels in cell supernatants of GES-1 cells, which, however, was overturned by HYP pre-treatment (Fig. 2A–2C). Consistently, *H. pylori*-induced enhancement in TNF-α, IL-6, and IL-1β mRNA levels was antagonized by HYP pre-treatment (Fig. 2D–2F).

HYP ameliorates *H. pylori*-induced oxidative stress in GES-1 cells. The influence of HYP on the intracellular ROS production in *H. pylori*-infected GES-1 cells was estimated by observing the fluorescence intensity of DCF derived from the oxidation of the fluorogenic probe DCFH-DA. Flow cytometric analysis revealed that HYP pre-treatment considerably suppresses *H. pylori*-induced noticeable escalation in ROS production (Fig. 3A–3B). MDA content increased while SOD and CAT activities decreased upon *H. pylori* infection in GES-1 cells. In contrast, HYP pre-treatment impaired MDA production and enhanced antioxidant SOD and CAT activities in *H. pylori*-exposed GES-1 cells (Fig. 3C–3E).

HYP activates the Nrf2/HO-1 signaling in *H. pylori*-infected GES-1 cells. Next, the molecular mechanisms through which HYP protects against *H. pylori*-induced GES-1 cell injury were investigated. Through western blotting, we discovered that Nrf2, HO-1, and NQO1 protein levels were markedly lower in *H. pylori*-exposed GES-1 cells than in untreated control cells. However, HYP pre-treatment increased Nrf2, HO-1, and NQO1 levels in *H. pylori*-infected GES-1 cells (Fig. 4A–4D). Immunofluorescence staining of Nrf2 in GES-1 cells displayed the same results as western blotting. As observed in Fig. 4E, the inhibition of *H. pylori* on Nrf2 nuclear translocation was abrogated by pre-treatment with HYP (Fig. 4E). Notably, the Nrf2 inhibitor ML385 debilitated HYP-induced increase in Nrf2, HO-1, and NQO1 levels in *H. pylori*-exposed GES-1 cells (Fig. 4F–4I).

The Nrf2 inhibitor ML385 reverses the protective effects of HYP against *H. pylori*-induced GES-1 cell injury. Finally, we verified whether ML385 affects the amelioration of HYP on *H. pylori*-induced GES-1 cell apoptosis, inflammation, and oxidative stress. As mani-

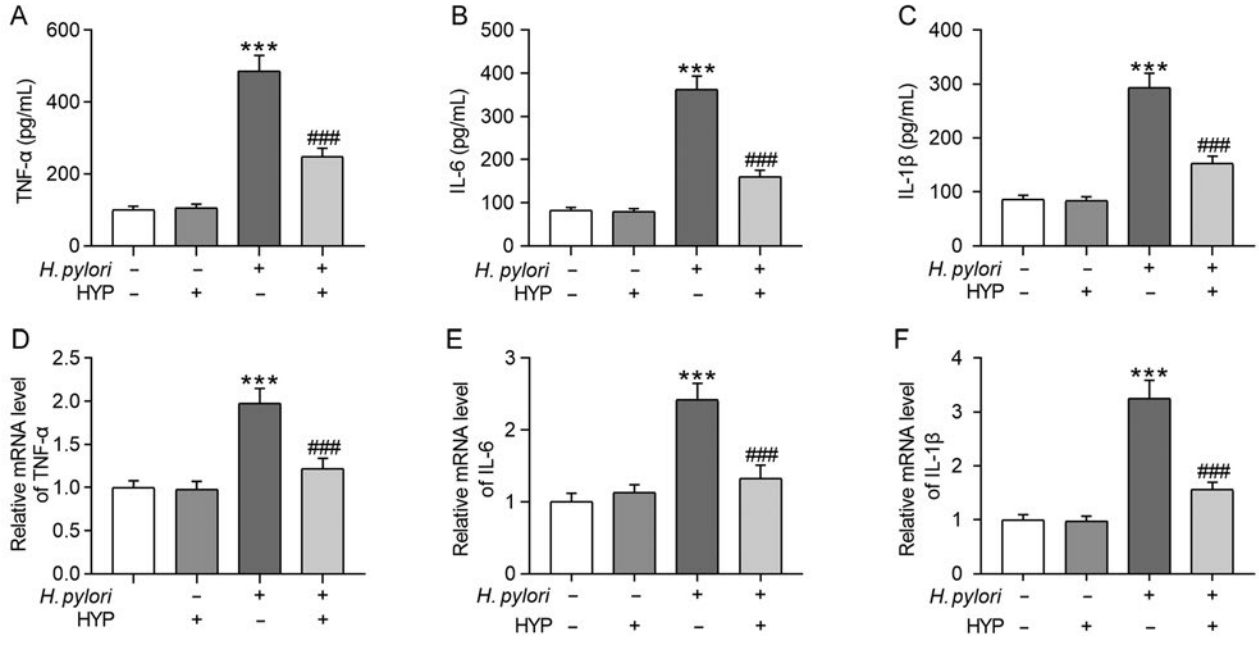

Fig. 2. HYP mitigates *Helicobacter pylori*-induced GES-1 cell inflammation.

GES-1 cells were pre-treated with HYP for 4 h (80 μM), followed by *H. pylori* infection for 24 h.
A–C) Detection of TNF-α, IL-6, and IL-1β levels in GES-1 cell supernatants by ELISA;
D–F) analysis of TNF-α, IL-6, and IL-1β mRNA levels in GES-1 cells through RT-qPCR.
$^{***}p < 0.001$ vs. control; $^{\#\#\#}p < 0.001$ vs. *H. pylori*.

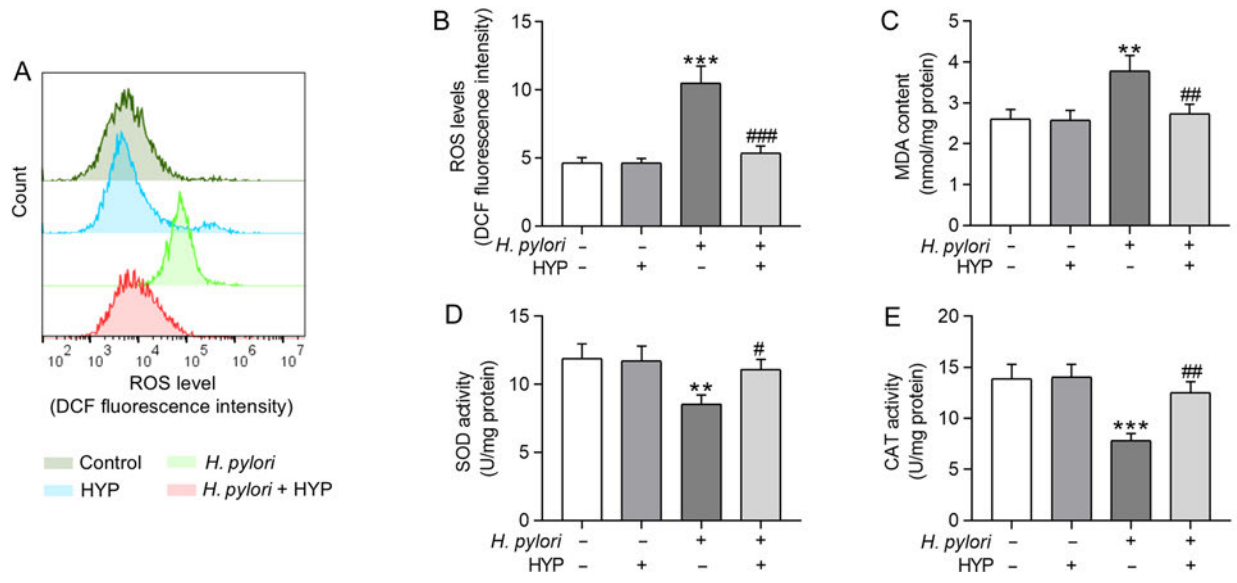

Fig. 3. HYP ameliorates *Helicobacter pylori*-induced oxidative stress in GES-1 cells.

GES-1 cells were pre-treated with HYP for 4 h (80 μM), followed by *H. pylori* infection for 24 h.
A) Measurement of DCF fluorescence intensity in GES-1 cells using flow cytometry;
B) quantification of intracellular ROS production by measuring DCF fluorescence intensity;
C–E) examination of MDA content and SOD and CAT activities in GES-1 cells using the corresponding assay kits.
$^{**}p < 0.01$, $^{***}p < 0.001$ vs. control; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. *H. pylori*.

fested by flow cytometry assay, HYP-induced reduction in the apoptosis of *H. pylori*-exposed cells was overturned by ML385 treatment (Fig. 5A). The elevation in Bcl-2 protein levels and reduction in Bax protein level caused by HYP treatment in *H. pylori*-stimulated GES-1 cells were inhibited by treatment with ML385 (Fig. 5B–5C). In the presence of ML385, the suppressive effects of HYP on TNF-α, IL-6, and IL-1β mRNA levels and intracellular ROS production weakened (Fig. 5D–5H), suggesting that HYP plays an anti-inflammatory and antioxidant role in *H. pylori*-exposed GES-1 cells through promoting the Nrf2 signaling.

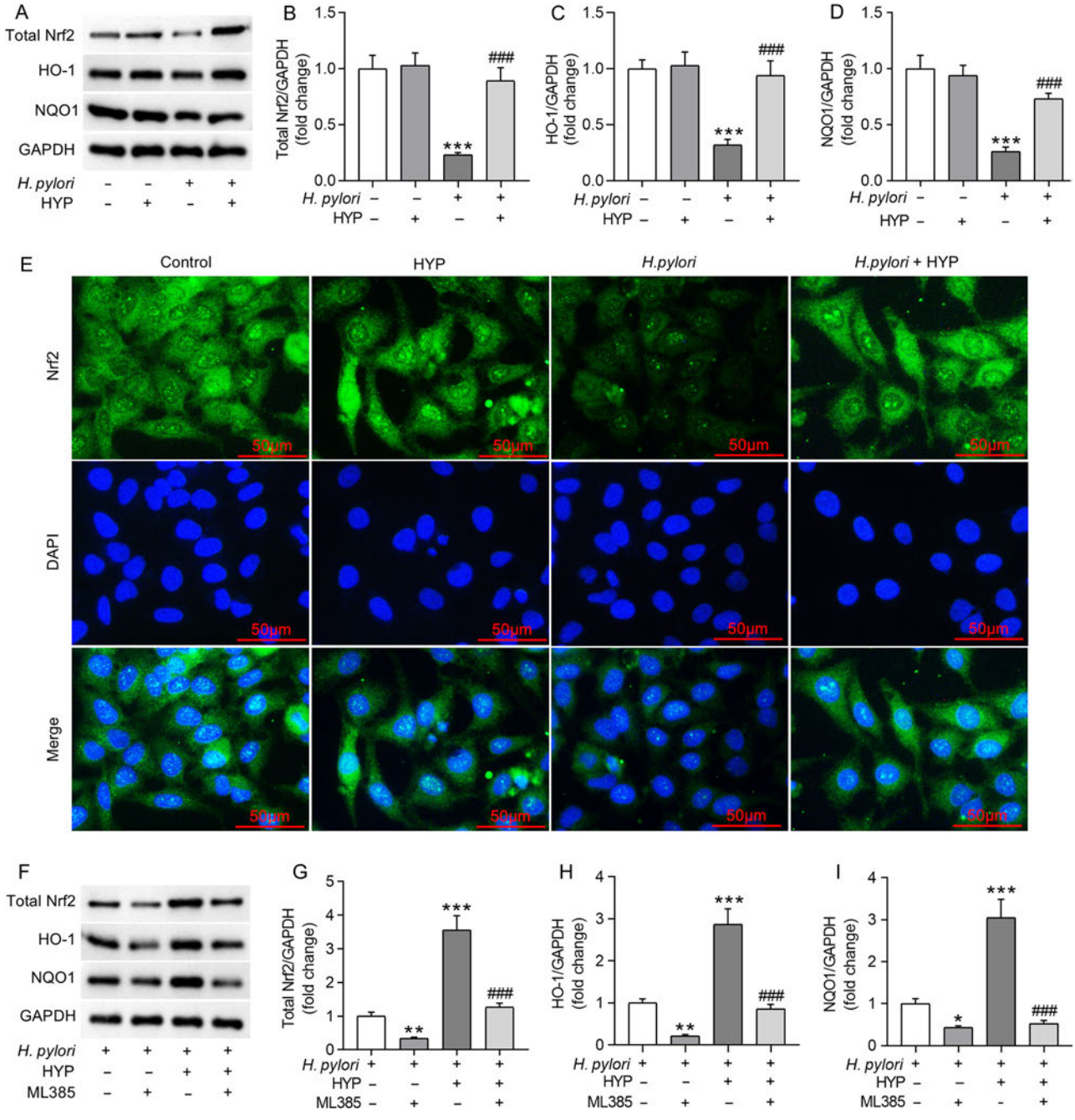

Fig. 4. HYP activates the Nrf2/HO-1 signaling in *Helicobacter pylori*-infected GES-1 cells. GES-1 cells were pre-treated with HYP (80 μM) for 4 h, followed by *H. pylori* infection for 24 h.

A–D) Determination of Nrf2, HO-1, and NQO1 protein levels in GES-1 cells by western blotting.
$^{***}p < 0.001$ versus control; $^{\#\#\#}p < 0.001$ versus *H. pylori*;
E) representative immunofluorescence staining images showing Nrf2 expression (green) in GES-1 cells. Nuclei were stained with DAPI (blue);
F–I) estimation of Nrf2, HO-1, and NQO1 protein levels in GES-1 cells that were pre-treated with HYP (80 μM) and ML385 (5 μM) for 4 h before infection with *H. pylori* through western blotting.
$^{**}p < 0.01$, $^{***}p < 0.001$ vs. *H. pylori*; $^{\#\#\#}p < 0.001$ vs. *H. pylori* + HYP.

Discussion

H. pylori is a common bacterium parasitizing the gastrointestinal tract and is the primary causative factor of gastritis, peptic ulcer, gastric cancer, and other diseases (Baj et al. 2020). Combined antibiotic therapy, although effective, has limited therapeutic effects due to increased antibiotic resistance (Medakina et al. 2023). In recent years, increasing plant-derived natural chemicals with anti-inflammatory and antioxidant properties, such as apple peel polyphenol, garlic extract, tea product, carotenoids-rich algae, apigenin, and querce-

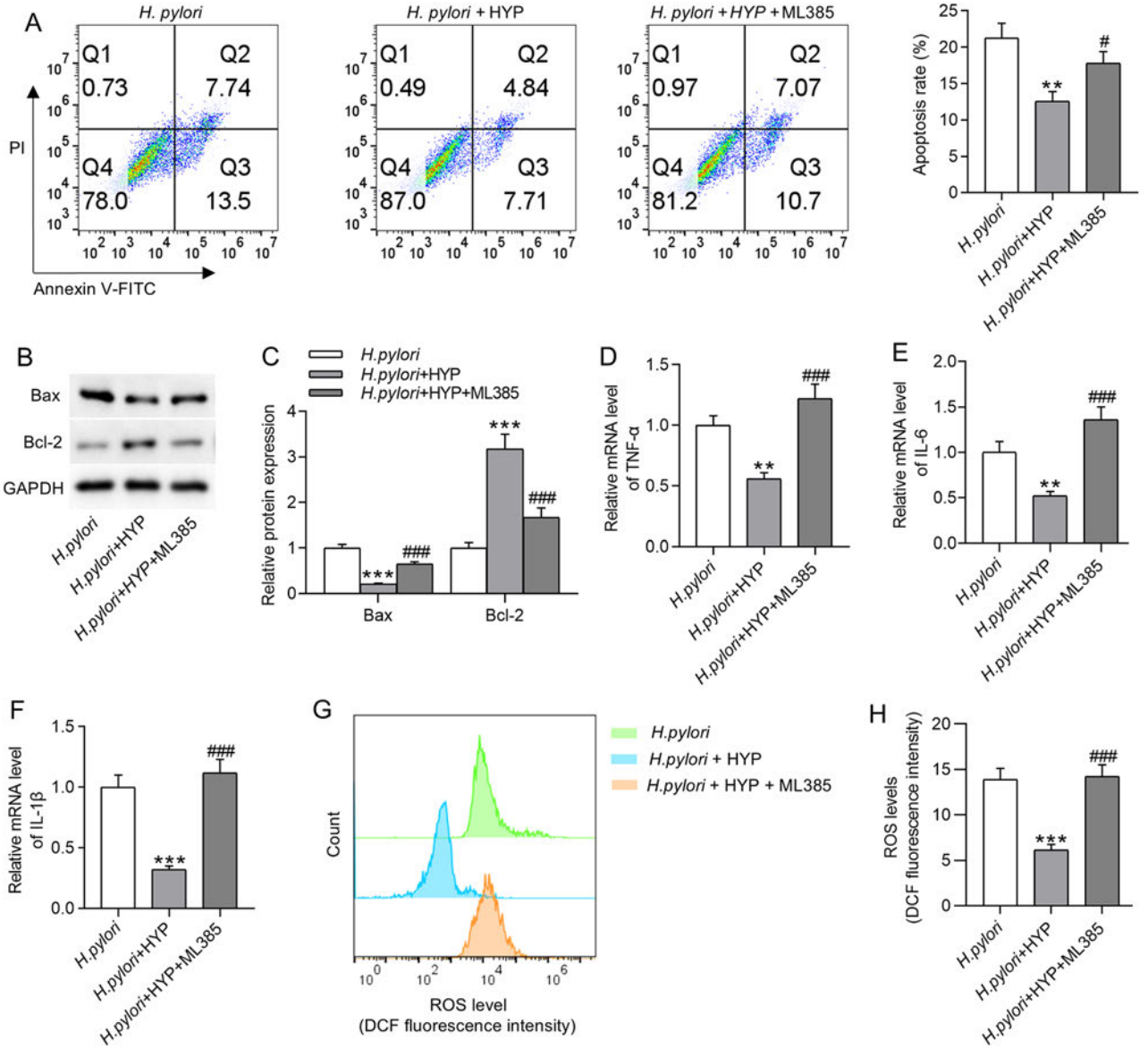

Fig. 5. The Nrf2 inhibitor ML385 reverses *Helicobacter pylori*-induced GES-1 cell injury. GES-1 cells were pre-treated with HYP (80 μM) and ML385 (5 μM) for 4 h before infection with *H. pylori*.

A) Evaluation of GES-1 cell apoptosis using flow cytometry assay; B)–C) examination of Bax and Bcl-2 protein levels in GES-1 cells through western blotting;

D)–F) analysis of TNF-α, IL-6, and IL-1β mRNA levels in GES-1 cells via RT-qPCR; G) assessment of DCF fluorescence intensity in GES-1 cells using flow cytometry; H) quantification of intracellular ROS production by measuring DCF fluorescence intensity. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. *H. pylori*; $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ versus *H. pylori* + HYP.

tin have been validated to exert beneficial therapeutic effects against *H. pylori* infection (Wang 2014). Our study investigated the possible cytoprotective effects of HYP on *H. pylori*-induced damage to human gastric epithelial cells and its mechanism. Our findings revealed that HYP treatment exhibited anti-apoptotic, antioxidant, and anti-inflammatory activities in *H. pylori*-exposed GES-1 cells by promoting the Nrf2/HO-1 signaling.

H. pylori-induced gastric mucosal injury is pathophysiologically hallmarked by the apoptosis of gastric epithelial cells (Wu et al. 2014). Several enzymes and cytotoxic proteins are secreted upon *H. pylori* infection, which induces gastric epithelial cell apoptosis and damages the gastric mucosa (Yang et al. 2017b). DNA damage leads to increased epithelial cell apoptosis, stimulating cell proliferation to maintain tissue homeostasis. Neoplasia can occur if hyperproliferation caused by dysregulated apoptosis is uncontrolled (Lee et al. 2020). It has been revealed that infection of *H. pylori* can trigger EGFR phosphorylation and activation, which thereby increases Bcl-2 level and decreases Bax level (Yan et al. 2009). Thus, inhibiting abnormal epithelial cell apoptosis prevents gastric mucosa injury.

Previously, the anti-apoptotic role of HYP has been clarified in many reports. For instance, HYP mitigates high glucose-induced human renal tubular epithelial HK-2 cell apoptosis by modulating the miR-499a-5p/NRIP1 axis (Zhou et al. 2021). HYP alleviates doxorubicin-induced mouse cardiomyocyte HL-1 cell apoptosis by suppressing the ASK1/p38 pathway (Chen et al. 2023b). Herein, our results showed that HYP treatment significantly counteracted *H. pylori*-induced elevation in cell apoptotic rate and Bax level and reduction in Bcl-2 level in GES-1 cells, suggesting the anti-apoptotic role of HYP.

Neutrophils activated by *H. pylori* can produce oxygen free radicals (hydrogen peroxide and superoxide ions), which may damage the gastric mucosa (Butcher et al. 2017). ROS production has been found to be positively correlated with *H. pylori*-associated infection and histologic injury (Han et al. 2022). Excessive ROS accumulation during oxidative stress damages DNA and gastric epithelial cell apoptosis (Ding et al. 2007). Due to the oxidation of unsaturated fatty acids in cell membranes, the formation of ROS leads to the secretion of MDA, a lipid peroxidation product (Lian et al. 2018). ROS-scavenging enzymes such as GSH-Px, CAT, and SOD are activated to protect cells against ROS-induced oxidative stress (Inupakutika et al. 2016).

Furthermore, chloramine (NH3Cl) is a toxic oxidant produced by *H. pylori* after its invasion into the gastric mucosa. Cells exposed to NH3Cl present the activation of MTP and caspase 3, which cause the release of cytochrome c, initiate the caspase cascade, and ultimately give rise to the apoptotic process (Olivares et al. 2005). Emerging evidences have suggested that oxidative stress can enhance inflammatory cytokine expression, aggravate inflammatory infiltration of the gastric mucosa, and increase the risk of gastritis carcinogenesis (Chen et al. 2023a). Reducing oxidative stress is considered a promising strategy for preventing the development of *H. pylori*-associated gastric diseases (Zhang et al. 2022). Previously, HYP was reported to protect human hepatocyte L02 cells against H_2O_2-induced oxidative damage by activating the Nrf2-AREsignaling via promoting GSK-3β inhibitory phosphorylation and inhibiting Keap1 (Xing et al. 2015). Tian et al. (2023) disclosed that HYP prominently attenuated oxidative stress response and improved renal pathological and functional damage in rat renal calculi models by promoting the AMPK/Nrf2 signaling pathway, as demonstrated by the downregulated MDA and ROS levels and upregulated SOD activity. Our research showed that HYP effectively reduced ROS production and MDA content and elevated SOD and CAT activities in *H. pylori*-stimulated GES-1 cells, showing its antioxidant effects.

ROS produced by *H. pylori* infection can further regulate various pathways, including PI3K/Akt, MAPK, and NF-κB, thereby directly and indirectly activating the inflammatory signaling and facilitating the release of cytokines such as TNF-α, IL-6, and IL-1β (Wu et al. 2023). TNF-α is a well-known cytokine that promotes leukocyte recruitment, stimulates monocyte-macrophages and other cell types to produce cytokines and chemokines, triggers inflammatory and immune responses, induces gastric epithelial cell apoptosis, and disrupts the gastric mucosal barrier (Chen et al. 2015). IL-1β has been reported to be highly expressed in *H. pylori*-infected gastric mucosa (Zhang et al. 2006). IL-6 is considered a key element in the progression of gastritis (Liu et al. 2022). Inhibiting *H. pylori*-induced inflammatory reactions contributes to the inhibition of the progression of gastritis and other gastric diseases (Zhang et al. 2015). Huang et al. (2021) once demonstrated that HYP activated sonic hedgehog and Wnt/β-catenin pathways through upregulating SIRT1, thereby reducing pro-inflammatory cytokine expression and alleviating neuroinflammation in lipopolysaccharide-stimulated mouse hippocampal neuronal HT22 cells. Mai et al. (2023) suggested that HYP attenuated pro-inflammatory cytokine levels in both mouse models of sepsis-induced acute lung injury and lipopolysaccharide-treated human lung microvascular endothelial cells. Several reports have indicated that *H. pylori* infection induces the production of proinflammatory cytokines in GES-1 cells, including TNF-α, IL-6, and IL-1β (Li et al. 2022; Wu et al. 2024). Consistent results were observed in the present study. Moreover, we found that *H. pylori*-caused increments in TNF-α, IL-6, and IL-1β levels in GES-1 cells were overturned by HYP treatment. Additionally, HYP is a derivative of quercetin and has a higher bioactivity than quercetin. Previous reports have shown that quercetin can reduce *H. pylori*-induced gastric inflammation and apoptosis (Zhang et al. 2017; Wang et al. 2024), supporting our findings in this study.

Nrf2 acts as a critical transcriptional factor in cellular defense against oxidant-related damage through its regulation of several antioxidant enzymes, including SOD and HO-1 (He et al. 2020). Under normal conditions, Nrf2 anchors to the redox sensor Keap1 and is suppressed in the cytoplasm. However, the interaction between Nrf2 and Keap1 is disturbed under inflammatory stimuli and oxidative stress, resulting in Nrf2 nuclear translocation. Nrf2 binds to Maf proteins in the nucleus to stimulate AREs and induce antioxidant enzyme expressions (Dodson et al. 2019). Activation of Nrf2 protects cells against oxidative injury and inflammation through several downstream cytoprotective pathways (Yanaka 2018). HO-1 can produce bilirubin and carbon monoxide, which have antioxidant properties, by promoting the breakdown of hemoglobin. Bilirubin neutralizes excess free radicals and reduces the

extent of oxidative damage to cells. Carbon monoxide enhances the antioxidant capacity of cells by activating intracellular antioxidant enzymes (Ma et al. 2022). In addition, HO-1 can regulate the extent of inflammation by inhibiting inflammatory cytokine release and regulating the expression of inflammation-related genes, and it has a role in reducing tissue damage (Ryter 2021). Previous studies have reported that *H. pylori* can lead to downregulating Nrf2/HO-1 signaling in gastric epithelial cells, which explains the exaggerated inflammation and oxidative stress (Kyung et al. 2019; Zhang et al. 2021). Previously, HYP was revealed to inhibit neuronal death, excessive ROS accumulation, and oxidative damage caused by 6-hydroxydopamine-induced neurotoxicity by inducing Nrf2-dependent HO-1 activation (Kwon et al. 2019). HYP mitigates colonic tissue apoptosis and inflammation by activating the Nrf2 signaling pathway, thereby improving acute colitis in mice (Yang et al. 2017a). In this work, HYP treatment antagonized the decrements in Nrf2, HO-1, and NQO1 protein levels caused by *H. pylori* infection in GES-1 cells, indicating its activation on the Nrf2/HO-1 pathway. Importantly, the Nrf2 inhibitor ML385 reverses the protection of HYP against *H. pylori*-triggered GES-1 cell damage.

Our research innovatively demonstrated that HYP alleviated *H. pylori*-induced apoptosis, inflammation, and oxidative stress in gastric epithelial cells by inducing the Nrf2/HO-1 pathway. Accordingly, supplementation with HYP may be beneficial in preventing *H. pylori*-induced gastric epithelial injury and the associated gastric diseases.

Availability of data and material

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

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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