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Original Article

Protective role of fruits of *Rosa odorata* var. *gigantea* against WIRS-induced gastric mucosal injury in rats by modulating pathway related to inflammation, oxidative stress and apoptosis

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ABSTRACT

Objective: *Rosa odorata* var. *gigantea* is a popular medicinal plant. Some studies have demonstrated that ethanolic extract of the fruits of *R. odorata* var. *gigantea* (FOE) has gastroprotective properties. The aim of this study was to investigate the gastroprotective activity of FOE on water immersion restrained stress (WIRS)-induced gastric mucosal injury in a rat model and elucidate the possible molecular mechanisms involved.

Methods: A rat stress ulcer model was established in this study using WIRS. After rats were treated with FOE orally for 7 d, the effect of FOE treatment was analyzed by hematoxylin and eosin (H&E) staining, and the changes of inflammatory factors, oxidative stress factors, and gastric-specific regulatory factors and pepsin in the blood and gastric tissues of rats were examined by ELISA assay. Molecular mechanism of FOE was investigated by immunohistochemical assay and Western blot.

Results: Compared with the WIRS group, FOE could diminish both the macroscopic and microscopic pathological morphology of gastric mucosa. FOE significantly preserved the antioxidants glutathione peroxidase (GSH-PX), superoxide dismutase (SOD) and catalase (CAT) contents; anti-inflammatory cytokines interleukin-10 (IL-10) and prostaglandin E2 (PGE2) levels as well as regulatory factors tumor necrosis factor-α (TGF-α) and somatostatin (SS) contents, while decreasing malondialdehyde (MDA), nitric oxide synthase (iNOS), tumor necrosis factor (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), gastrin (GAS) and endothelin (ET) levels. Moreover, FOE distinctly upregulated the expression of Nrf2, HO-1, Bcl2 and proliferating cell nuclear antigen (PCNA). In addition, FOE activated the expression of p-EGFR and down-regulated the expression of NF-κB, Bax, Cleaved-caspase-3, Cyto-C and Cleaved-PARP1, thus promoting gastric mucosal cell survival.

Conclusion: The current work demonstrated that FOE exerted a gastroprotective activity against gastric mucosal injury induced by WIRS. The underlying mechanism might be associated with the improvement of anti-inflammatory, anti-oxidation and anti-apoptosis systems.

1. Introduction

Gastric mucosa injury is a common pathological state of multifactorial gastrointestinal disease worldwide, affecting the quality of life of millions of patients (Pereira Junior et al., 2021). According to the survey, 20–60 people out of every 100 000 population suffer from gastric mucosa injury, accounting for 5%–10% of the world's mortality (Kim et al., 2021). The main causes of gastric mucosal damage are infection by *Helicobacter pylori*, the administration of steroidal and nonsteroidal anti-inflammatory drugs (NSAIDs), stress, smoking, alcohol consumption, and nutritional deficiencies (Xia et al., 2021). Many people are exposed to these risk factors, making them vulnerable to associated diseases such as gastritis, which can progress to gastric ulcers. If gastritis and gastric ulcers are not properly treated, they may gradually worsen and lead to

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unexpected complications, such as bleeding or perforation (Park et al., 2021). A gastric ulcer occurs as a result of the imbalance between the aggressive factors in the gastric system, including gastric acids or pepsin, and the protective factors, such as mucus secretion, prostaglandins, sulfhydryl compounds, nitric oxide, and antioxidants (Fu et al., 2021). Typical treatments for gastric ulcers are acid suppressant drugs, such as type-2 histamine receptor antagonists and proton pump inhibitors, but they have some adverse effects. Long-term use of acid suppressants can lead to gynecomastia, impotence, osteoporotic bone fracture, and deficiencies of iron and magnesium, as well as vitamin B_{12} hypergastrinemia after discontinuation (Du, Gao, & Zhang, 2021; Li et al., 2021). Therefore, the development of more effective mucosal protective agents with fewer side effects for protecting gastric mucosa from injury has become crucial.

Many Chinese herbal medicines have been traditionally used for the treatment of gastritis and ulceration, mainly because of their antioxidant and cytoprotective properties. *Rosa odorata* Sweet var. *gigantea* (Coll. et Hemsl.) Rehd. et Wils, an evergreen or semi-evergreen climbing shrub of *Rosa* genus, mainly distributed in Yunnan Province, China (Wang et al., 2022). The fruits of *R. odorata* var. *gigantea* appears black, sweet-tasting and nutritious after becoming mature. Mature fruits contains 0.834% protein, 33.0 mg/100 g total amino acid and rich mineral elements, which can be used as fresh fruit supplementing K^+ and Ca^{2+}. Long-term consumption has a superb healthcare effect, which can reduce the occurrence of cancer, prevent metabolic disorders and arrhythmia, promote normal development of the pancreas as well as prevent anemia and skeletal lesions. Moreover, fruits of *R. odorata* var. *gigantea* is also rich in superoxide dismutase (SOD), which is the primary substance for scavenging free radicals in organisms (Li et al., 2020). As a consequence, more functions of fruits of *R. odorata* var. *gigantea* are expected to be explored.

The current study aimed to investigate the gastroprotective and antiulcerogenic activities of FOE along with the exploration of the underlying mechanisms using water immersion-restraint stress (WIRS)-induced gastric ulcer rat model.

2. Materials and methods

2.1. Plant materials

The fruits of *R. odorata* were collected from Qujing City of Yunnan Province and authenticated by Dr. Yu Chen, Kunming Institute of Botany, Chinese Academy of Sciences. Specimens (20201019F-YN) were stored in Tianjin Modern Innovative TCM Technology Co., Ltd. The fresh fruits were dried (37.14% dry to wet weight ratio) and 100 g were cut into small pieces and extracted with ethanol (600 mL × 2 times). The pooled extracts were evaporated under reduced pressure at 40 °C until complete dryness to give 23.9 g of loose and superfine light brownish substance (extraction rate 23.9%). The research group previously identified 21 components from FOE based on UPLC/Q-TOF-MS, including phenylalanine and its isomer, catechins-4′-*O*-glucoside, taxifolin-3-*O*-glucoside and 3,4-dihydroxy-5-methoxy benzenebutanoic glucoside ester etc (Liu et al., 2022).

2.2. Chemicals and reagents

Omeprazole enteric capsules (OME) were purchased from Youcare Pharmaceutical Group Co., Ltd. (Beijing, China). CMC-Na was produced by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Detection kits of malondialdehyde (MDA) and SOD were supplied by Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Detection kits of catalase (CAT), glutathione peroxidase (GSH-PX), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), interleukin-10 (IL-10), prostaglandin (PGE2), somatostatin (SS), gastrin (GAS), endothelin (ET) and pepsin were obtained from Tianjin YITE Life-science R&D Co., Ltd (Tianjin, China). ELISA detection kits of inducible nitric oxide sythase (iNOS) as well as RIPA lysis buffer containing the inhibitors of protease and phosphatase were purchased from Shanghai Beyotime Co., Ltd (Shanghai, China). BCA protein assay kit was obtained from Beijing Solarbio Science & Technology Co., Ltd (Beijing, China). Goat Anti-Rabbit IgG (H + L) HRP and Keap1, Nrf2, HO-1, NF-κB, IKKα/β, Bax, Bcl-2, Proliferating Cell Nuclear Antigen (PCNA) antibodies were provided by Affinity Biosciences (Jiangsu, China). The Caspase-3 antibody was produced by Cell Signaling Technology (Boston, USA). All other experimental supplies were purchased from commercial sources.

2.3. Animals

A total of 42 8-week-old male Sprague-Dawley rats (180–200 g) were acquired from SPF Biotechnology Co., Ltd. (Beijing, China). License number: SCXK (Beijing) 2019–0010. The animals were housed in the Institute of Radiation Medicine Chinese Academy of Medical Sciences and allowed to acclimatize for one week before starting the experiment and exposed to standard laboratory conditions (controlled room temperature, 20–25 °C; relative humidity, 40%−70%; and 12 h light/dark cycle) with free access to food (It is mainly composed of protein, fat, carbohydrates, fiber and vitamins, etc. It is purchased from Beijing Auli Feed Co., Ltd. and tap water. The experimental protocols were approved by the Institute of Radiation Medicine Chinese Academy of Medical Sciences and conducted following the "Principles of Laboratory Animal Care and Use in Research" (State Council of China, 1988). Affidavit of Approval of Animal Ethical and Welfare Approval Number: IRM-DWLL-2021159.

2.4. Experimental design and WIRS-induced gastric injury

A total of 42 rats were randomly allocated into six groups with seven rats for each. Animals of groups I and II served as the normal control and model control (WIRS) group received a daily intragastric oral dose (1 mL) of distilled water using gavage for 7 d. Animals of group III served as the positive group and received gavage in a dosage of aqueous solutions of OME (20 mg/kg b.wt, dissolved in 0.5% CMC-Na); The rats in groups IV, V, VI were received gavage in a dosage of FOE dissolved in double distilled water (62.5, 125 and 250 mg/kg b.wt, respectively). On the seventh day of study, after each rat receiving gavage of the corresponding solution for the last time, gastric lesions were induced in groups II to VI according to the method of Takagi (Takagi & Okabe, 1968). Rats were deprived of food but had free access to water 24 h prior to the induction of gastric injury. The animals were immobilized in perforated polymethyl methacrylate tubes (length × width × height = 16 cm × 4 cm × 4 cm), which was vertically immersed in a bath with water at 16–18 °C for 6 h up to the level of the animal xiphoid process. After 6 h, each rat was anesthetized with chloral hydrate (4%), and fresh blood obtained by aorta abdominalis centrifuged (3 000 r/min) for 10 min at 4 °C. Serum was stored at −80 °C for subsequent measurement. The gastric tissues were immediately excised, opened along the greater curvature, washed with normal saline solution, and examined for gastric lesions in a manner of calculating the ulcer index (UI) following the macroscopic scoring system previously presented by Guth (Guth, Aures, & Paulsen, 1979) as follows: no lesion (score 0), epithelial lesion or the lesion < 1 mm (score 1), 1 mm ≤ lesion < 2 mm (score 2), 2 mm ≤ lesion < 3 mm (score 3), 3 mm ≤ lesion < 4 mm (score 4), 4 mm ≤ lesion (score segmentally), and twice for

width > 1 mm. The scores were relative values, and the average UI of each group was obtained by dividing the total scores by the number of animals. The inhibition effect (%) of each protective material was calculated by using the following formula:

$$\text{Ulcer inhibition}(\%) = (\text{UI}_{\text{in model}} - \text{UI}_{\text{in test}}) \times 100/\text{UI}_{\text{in model}}$$

Specimens of glandular gastric tissues were divided into two portions; one fixed in 4% paraformaldehyde for histopathological examination and immuno-histochemical assessment, while in the other portion, gastric tissues were weighed and stored separately at −80 °C to be used for the evaluation of inflammatory biomarkers, antioxidants, and protein expression analysis.

2.5. Histopathological evaluation

Gastric tissues were excised and fixed in 4% paraformaldehyde for more than 48 h. After dehydrating in gradient alcohol and embedding in paraffin, three or four paraffin-embedded sections (4–5 μm thick) were prepared and stained with hematoxylin and eosin (H&E) for histological evaluation. Then the pathological changes in the gastric tissues were observed under a Leica microscope (DMi1 Leica, Germany).

2.6. Assessment of gastric juice acidity and ELISA

Assessment of gastric juice acidity: The gastric contents were collected into centrifuge tubes and successively centrifuged at 4 000 r/min for 10 min, and pH value of the supernatant was measured by acidometer (ATAGO PAL-pH, Japan).

Measurement of inflammatory mediators and oxidative stress biomarkers: Blood samples were collected and centrifuged at 3 000 rpm for 10 min, and then the serum samples were stored at −80 °C until analysis. Gastric tissues were homogenized with cold saline and centrifuged at 12 000 r/min at 4 °C for 10 min, and the supernatant of the homogenate was collected and stored at −80 °C. Measurement of inflammation-related factors (iNOS, IL-10, PGE2, TNF-α, IL-6, and IL-1β) and oxidative stress-related factors (MDA, SOD, CAT, and GSH-PX) according to the ELISA kit instructions.

The determination of gastric-specific regulatory factors involved estimating the TGF-α content using a rat ELISA kit to indicate the extent of endogenous gastric mucosal barrier protective factor. Furthermore, SS and GAS, markers for the ability of gastric tissues to secrete gastric acid and pepsinogen, were determined using specific kits with solid-phase sandwich ELISA. The measurement of the endogenous injury factor ET was performed using a corresponding ELISA kit, following the manufacturer's instructions.

Finally, the detection of pepsin activity in gastric juice involved using a specific ELISA kit according to its instructions, as pepsinogen is secreted by gastric fundus chief cells and activated into pepsin under pH 1.5–5.0.

2.7. Western blot analysis

Rat gastric tissues (50 mg) were homogenized and lysed in ice-cold RIPA lysis buffer containing 1% phenylmethylsulfonyl fluoride (PMSF), phosphatase inhibitor and protease inhibitor cocktail. Subsequently, the samples were centrifuged at 10 000 g and 4 °C for 10 min. After centrifugation, the supernatant was collected and subjected to a BCA Protein Assay kit to measure protein concentration. The samples were denatured with reducing SDS sample buffer and boiled in water at 95 °C for 10 min. Subsequently, Western blot analysis was applied to the examination of Keap1, Nrf2, HO-1, NF-κB p65, p-IKKα/β, Bcl2, Bax, Caspase3 and PCNA protein expression. The same amount of protein from all lysates of each sample was separated by 10%−15% SDS-PAGE, and then transferred onto the PVDF membranes, which was incubated overnight with the corresponding primary antibody at 4 °C, followed by the incubation of a secondary antibody. Thereafter, the proteins were measured with Omni-ECL™ Pico light chemiluminescence detection reagents (Epizyme Biomedical Technology Co., Ltd., Shanghai, China) and Amersham Imager 680 system (General Electric, USA). All Western blot studies were repeated three times.

2.8. Immunohistochemical analysis

Immunohistochemistry was used to detect the expression level of p-EGFR, p-Src, Cyto-C and Cleaved-PARP1 protein in the cell nucleus of gastric tissue. Gastric tissue sections were successively dewaxed, rehydrated and incubated in bovine serum albumin (BSA, 5%) for 30 min. Then, the tissue sections were washed three times with Tris-buffered saline, and incubated overnight with the following primary antibodies at 4 °C: p-EGFR, p-Src, Cyto-C and Cleaved-PARP1 in a certain proportion. After being washed triply with phosphate buffer saline (PBS), the immunostaining was amplified and completed by the Horseradish peroxidase complex. Sections were developed and visualized using DAB chromogen. The substrate system gave a brown color at the site of the target antigen. Sections were counterstained with hematoxylin and coverslipped for microscopical examination. Finally, each section was observed under the Leica microscope. The quantification of proteins was done by measuring the area% expression from positive fields per slide using image analysis software (Image J, NIH, USA).

2.9. Statistical analysis

The experimental data were expressed as mean ± standard deviation (SD), and analyzed with the IBM SPSS Statistics for Windows, version 21.0 (IBM Corp., Armonk, N.Y., USA). Differences between groups were evaluated by ANOVA. Statistical analysis was performed using the GraphPad Prism Software (Version 8.0.1, USA). A value of $P < 0.05$ was considered statistically significant, and a value of $P < 0.01$ was considered highly significant.

3. Results

3.1. Macroscopic score measurement of gastric mucosa

The gastric mucosa tissues of rats were smooth with complete wrinkled walls and regular trend. No hyperemia, edema, or mucosal damage was observed in the normal control group, as shown in Fig. 1A. The macroscopic examination of gastric mucosa revealed tissue damage evidenced by severe gastric mucosal damage appearing as glandular area hyperemia, mucosal edema accompanied by a dot and linear hemorrhage necrosis, indicating that the rat model of stress gastric mucosal injury was successfully established (Fig. 1B). The FOE groups and positive group showed alleviative gastric injury manifested as decreased hemorrhage as well as smooth and glossy mucosa to varying degrees, FOE-H group and positive group of which showed overly gastroprotective action compared with the model group (Fig. 1C−F). In order to reflect the protective effect of gastric mucosa more directly, Guth standard was used to quantitatively assess the gastric lesions and calculate ulcer inhibition rate. As shown in Table 1, the model group indicated by an average UI of 24.71 ± 1.38 ($P < 0.01$). However, FOE at doses of 62.5, 125 and 250 mg/kg produced a significant reduction in the percentage of UI (by 25.73%, 54.25%, and 77.92%, respectively) compared with the model group ($P < 0.01$).

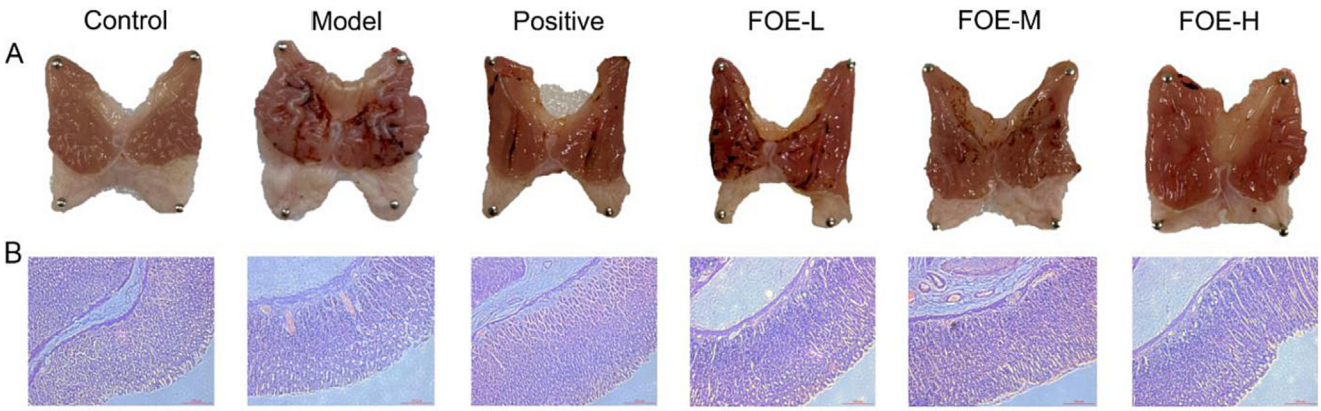

Fig. 1. Macroscopic representative images (A) and histopathological representative images (B) of gastric mucosa of rats in different groups. Positive group (20 mg/kg omeprazole, OMZ); FOE-L (62.5 mg/kg FOE); FOE-M (125 mg/kg FOE); FOE-H (250 mg/kg FOE).

Table 1
Integral evaluation of WIRS-induced gastric mucosal injury in rats (mean ± SD, n = 7).

Groups	Number	Ulcer index (UI)	Ulcer inhibition (%)
Control	7	–	–
Model	7	24.71 ± 1.38$^{\#\#}$	–
Positive	7	9.86 ± 1.07**	60.41
FOE-L	7	18.43 ± 2.23**	25.73
FOE-M	7	11.29 ± 1.60**	54.25
FOE-H	7	5.43 ± 2.57**	77.92

Note: Positive group (20 mg/kg omeprazole); FOE-L (62.5 mg/kg FOE); FOE-M (125 mg/kg FOE); FOE-H (250 mg/kg FOE). $^{\#\#}P < 0.01$ *vs* control group, $^{**}P < 0.01$ *vs* model group.

Table 2
Results of pH value determination in gastric juice of rats (mean ± SD, n = 7).

Groups	Number	pH value
Control	7	3.22 ± 0.12
Model	7	1.62 ± 3.38$^{\#\#}$
Positive	7	3.39 ± 0.16**
FOE-L	7	2.50 ± 4.27**
FOE-M	7	2.79 ± 5.24**
FOE-H	7	2.84 ± 0.13**

Note: Positive group (20 mg/kg omeprazole); FOE-L (62.5 mg/kg FOE); FOE-M (125 mg/kg FOE); FOE-H (250 mg/kg FOE). $^{\#\#}P < 0.01$ *vs* control group, $^{**}P < 0.01$ *vs* model group.

3.2. Histopathological evaluation of gastric mucosa

To estimate the protective effect of FOE on gastric tissues in microscopic conditions, we conducted a histopathological analysis. Fig. 1B showed the histopathological alterations in gastric specimens of different experimental groups. The gastric tissue structure of rats in the control group was complete accompanied by the glands in the lamina propria arranging neatly as well as no observation of gastric mucosal epithelial cells shedding, edema and hyperemia. In the model group induced by WIRS, gastric mucosa tissues were necrotic, with the phenomena of epithelial cells separating and shedding, glands arranging irregularly, submucosa edema, cell nuclear pyknosis or even dissolution, capillary dilation and compression, as well as red blood cell extravasation. Moreover, inflammatory cell infiltration could be observed partly in the model group. In FOE groups, it was clear that the gastric mucosa and submucosa appeared with normal lamina epithelia, a few desquamated epithelial cells, normal gastric gland, and few inflammatory cells' infiltration, indicating gastric mucosa injury was obviously attenuated with FOE demonstrated by protecting the mucous structure. Notably, FOE-H had the most gastroprotection in terms of histopathological evaluation, which was almost the same as that of OME.

3.3. Effects of FOE on gastric juice acidity

The pH value of gastric juice in gastric mucosal injury rats were presented in Table 2. Gastric juice pH value of the model group was significantly lower than that in the control group ($P < 0.01$), indicating increased gastric juice secretion in the model group. Promeprazole is a proton pump inhibitor that can effectively inhibit gastric acid secretion. FOE restrained gastric acid secretion and protected gastric mucosa from WIRS-induced injury with the gastric juice pH value increasing of all rats in FOE groups such that the pH value of FOE-H group was the closest to that of OME, a proton pump inhibitor that effectively inhibits gastric acid secretion.

3.4. Effects of FOE on inflammatory mediators

The variation of inflammatory mediators (iNOS, TNF-α, IL-6, IL-1β, IL-10 and PGE2) in gastric tissues and serum were assessed aiming at exploring the blocking-up of FOE on inflammation. As shown in Fig. 2, WIRS induced a significant increase in the content of gastric iNOS, TNF-α, IL-6 and IL-1β, while IL-10 and PGE2 concentrations were significantly decreased in comparison with the control group. FOE prevented WIRS-induced effects on inflammatory biomarkers iNOS, TNF-α, IL-6, IL-1β, IL-10 and PGE2, restoring their values to near that of the control and positive groups (Fig. 2A–F). The content change of inflammatory mediators in the serum of rats showed the accordant trend as those in gastric tissues (Fig. 2G–L). The results suggested that FOE could reduce the occurrence of inflammatory response via regulating the expression of inflammatory mediators, thus realizing the protective effect on WIRS-induced gastric mucosa injury.

3.5. Effects of FOE on oxidative stress biomarkers

The effect of oxidative stress on WIRS-induced gastric mucosal injury was explored by monitoring changes in antioxidants and lipid peroxidation levels. Results of the oxidative stress biomarkers (MDA, SOD, CAT and GSH-PX) in gastric tissues and serum were exhibited in Fig. 3. In the gastric tissues, exposure to WIRS presented a high level of lipid peroxidation, which was indicated by the increase of MDA compared with the control group. In addition, WIRS significantly inhibited the activity of the antioxidant element SOD, CAT and GSH-PX. In comparison with the corresponding

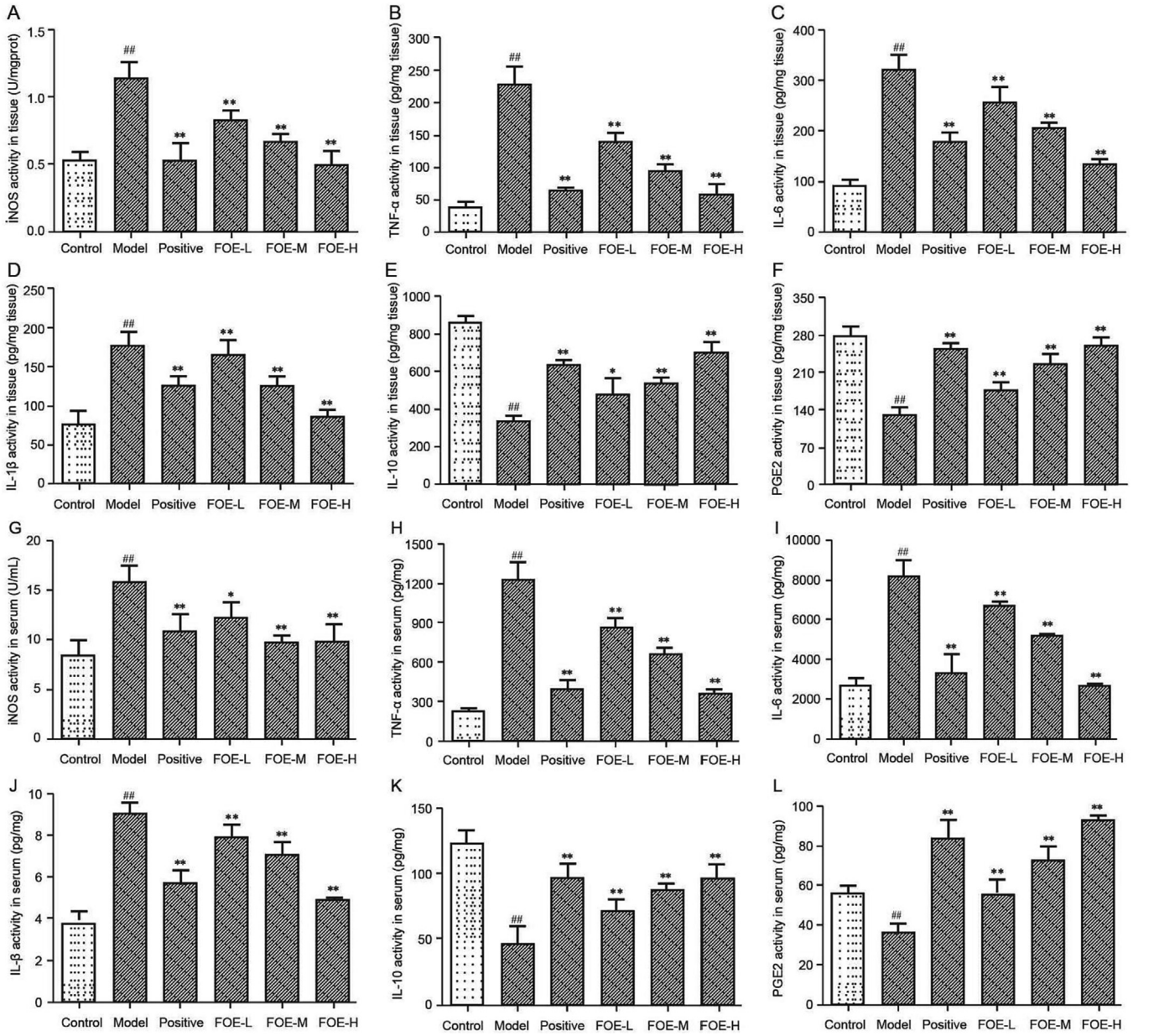

Fig. 2. Effects of FOE on inflammatory mediators (iNOS, TNF-α, IL-6, IL-1β, IL-10 and PGE2) levels in gastric tissues (A–F) and serum (G–L) induced by WIRS (mean ± SD, $n = 7$). Positive group (20 mg/kg of omeprazole); FOE-L (62.5 mg/kg FOE); FOE-M (125 mg/kg FOE); FOE-H (250 mg/kg FOE). $^{\#\#}P < 0.01$ *vs* control group, $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs* model group.

biomarkers in the model group, FOE obviously offsets these oxidation abnormalities as proved by the reversal of the levels of these factors. Especially, the MDA, SOD, CAT and GSH-PX levels of the FOE-H group were comparable with those of the positive group. The results indicated that FOE can regulate oxidative stress index, enhance the activity of the antioxidant system to realize the protective effect on WIRS-induced gastric mucosa injury.

3.6. Effects of FOE on gastric-specific regulatory factors

The level of gastric-specific regulatory factors (TGF-α, SS, GAS and ET) in gastric tissues and serum were exhibited in Fig. 4. It was clearly that the expression of TGF-α and SS were higher in the gastric tissues of the control group, while the expression of GAS and ET was lower. The levels of TGF-α and SS of the model group were remarkably decreased, whereas GAS and ET presented an upward trend compared with the control group. FOE regulated the ascending expression of TGF-α and SS, but decreased the content of GAS and ET. Notably the gastric-specific regulatory factors were apparently altered in FOE-H and positive groups up to near normal values in gastric tissues (Fig. 4A–D). The changes in TGF-α, SS, GAS and ET levels in the serum of rats were consistent with those in gastric tissues (Fig. 4E–H).

3.7. Effects of FOE on pepsin activity in gastric juice of WIRS-induced rats

The results of pepsin activity in the gastric juice of rats with gastric mucosal injury were shown in Fig. 5. The pepsin activity in gastric juice of the model group was significantly higher than

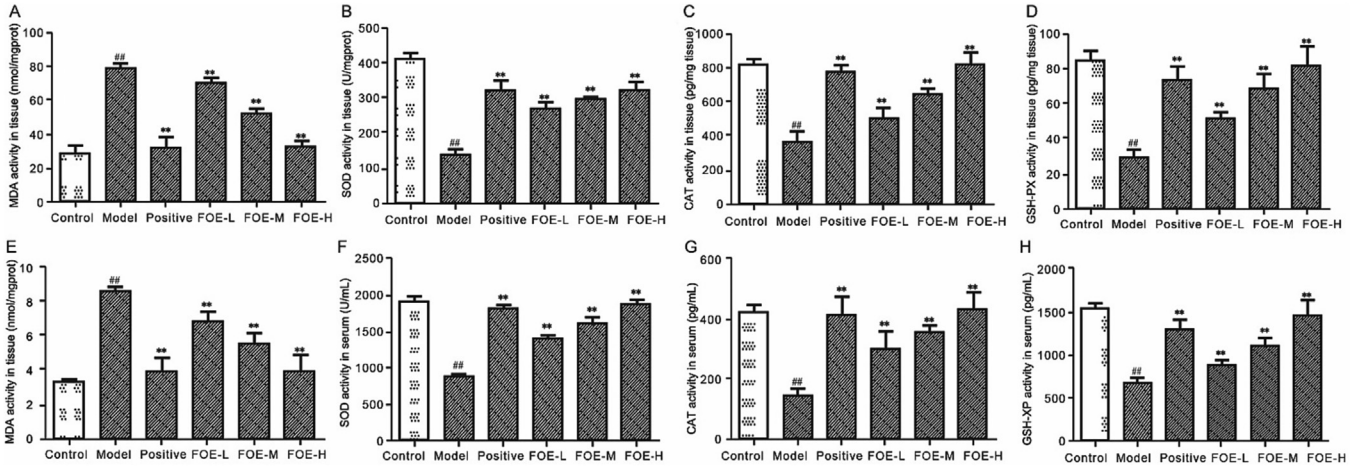

Fig. 3. Effects of FOE on oxidative stress biomarkers (MDA, SOD, CAT and GSH-PX) levels in gastric tissues (A–D) and serum (E–H) induced by WIRS (mean ± SD, n = 7). Positive group (20 mg/kg omeprazole); FOE-L (62.5 mg/kg FOE); FOE-M (125 mg/kg FOE); FOE-H (250 mg/kg FOE). $^{\#\#}P < 0.01$ *vs* control group, $^{**}P < 0.01$ *vs* model group.

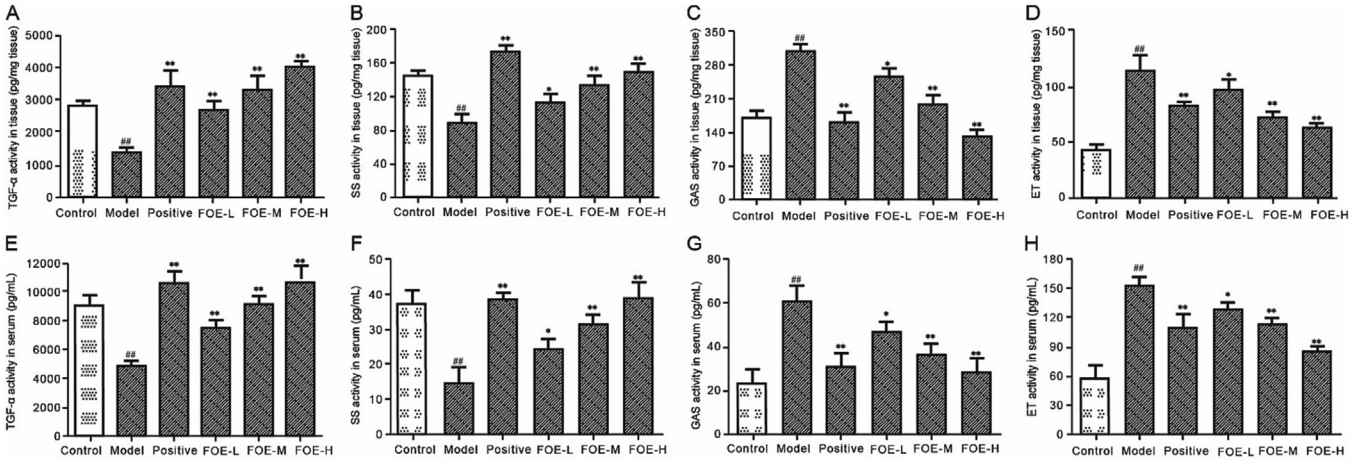

Fig. 4. Effects of FOE on gastric-specific regulatory factors (TGF-α, SS, GAS and ET) levels in gastric tissues (A–D) and serum (E–H) induced by WIRS (mean ± SD, n = 7). Positive group (20 mg/kg omeprazole); FOE-L (62.5 mg/kg FOE); FOE-M (125 mg/kg FOE); FOE-H (250 mg/kg FOE). $^{\#\#}P < 0.01$ *vs* control group. $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs* model group.

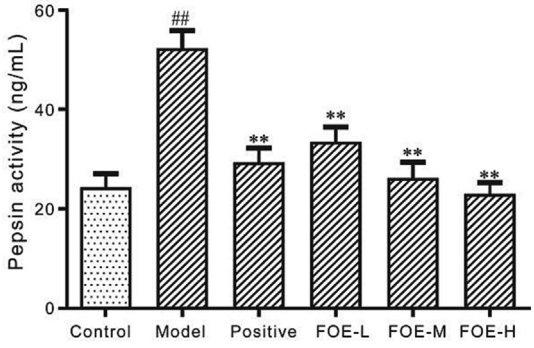

Fig. 5. Effects of FOE on pepsin activity in gastric juice of WIRS-induced rats (mean ± SD, n = 7). Values are expressed as mean ± SD (n = 7). Positive group (20 mg/kg omeprazole); FOE-L (62.5 mg/kg FOE); FOE-M (125 mg/kg FOE); FOE-H (250 mg/kg FOE). $^{\#\#}P < 0.01$ *vs* control group, $^{**}P < 0.01$ *vs* model group.

that in the control group, indicating that WIRS caused the gastric mucosa digestion by gastric acid and pepsin, so as to result in gastric mucosa injury. In comparison with the model group, FOE decreased the pepsin activity in gastric juice, and the pepsin activity in FOE-M and FOE-H groups was lower than that in the positive group.

3.8. Effect of FOE on expression of NF-κB pathway-related proteins

In order to further verify whether the anti-inflammatory activity of FOE contributes to gastroprotection, the expression of NF-κB pathway-related proteins were also assayed by Western blot. As shown in Fig. 6, WIRS-induced strikingly increase expression of NF-κB p65 and p-IKKα/β with comprison to the control group. Nevertheless, FOE significantly decreased the level of NF-κB p65 and p-IKKα/β in a dose-dependent manner.

3.9. Effect of FOE on expression of Nrf2 pathway-related proteins

The expression levels of oxidative stress-related proteins (Keap1, Nrf2 and HO-1) were determined in the present work by Western blot as presented in Fig. 7. Western blot results showed that WIRS induced Keap1, Nrf2 and HO-1 protein expression levels up-regulating slightly but significantly compared with the control group. After different doses of FOE intervention, the expression of

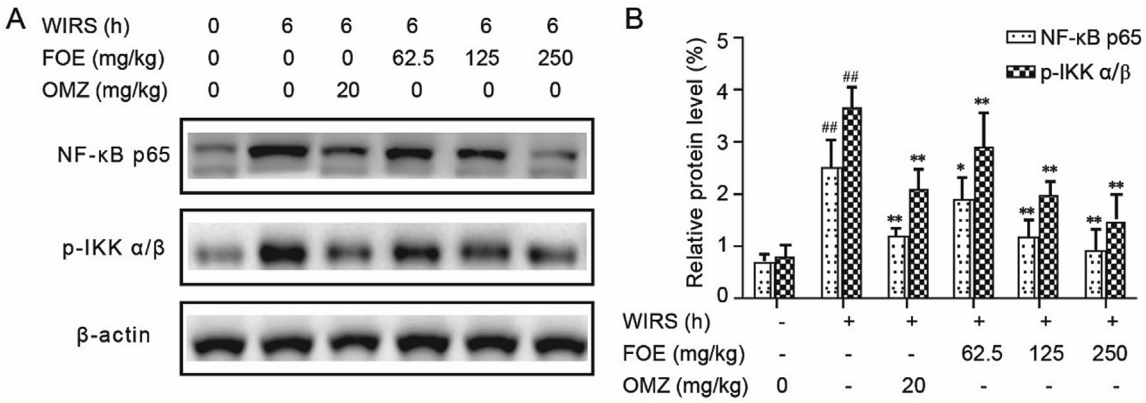

Fig. 6. Effect of FOE on expression of NF-κB pathway-related proteins in WIRS-stimulated gastric tissues (mean ± SD, n = 7). Protein expression of NF-κB p65 and p-IKKα/β were measured by Western blot analysis (A). Statistical analysis of NF-κB p65 and p-IKKα/β were conducted by Image J software (B). $^{\#\#}P < 0.01$ *vs* control group, $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs* model group. OMZ: omeprazole.

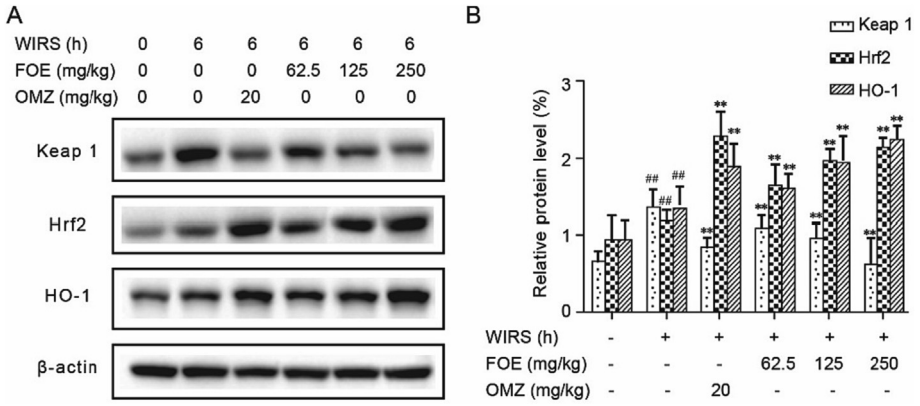

Fig. 7. Effect of FOE on expression of Nrf2 pathway-related proteins in WIRS-stimulated gastric tissues (mean ± SD, n = 7). Protein expression of Keap1, Nrf2 and HO-1 were measured by Western blot analysis (A). Statistical analysis of Keap1, Nrf2 and HO-1 were conducted by Image J software (B). $^{\#\#}P < 0.01$ *vs* control group, $^{**}P < 0.01$ *vs* model group. OMZ: omeprazole.

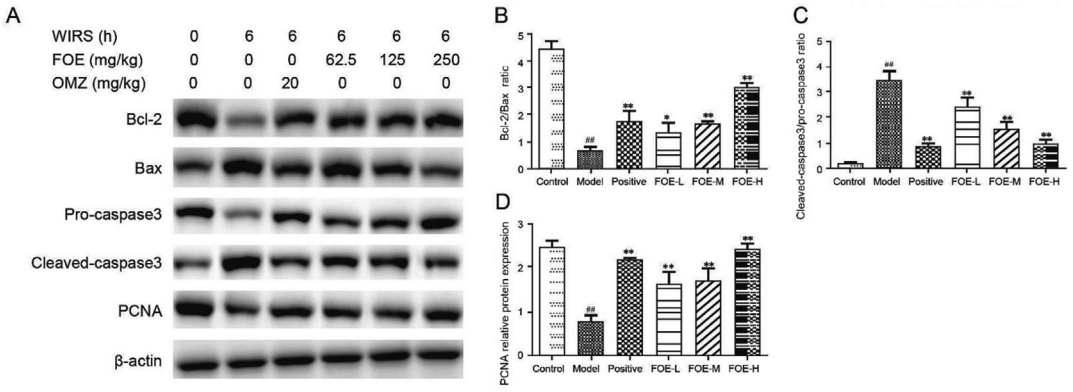

Fig. 8. Effect of FOE on expression of apoptosis pathway-related proteins in WIRS-stimulated gastric tissues (mean ± SD, n = 7). Protein expression of Bcl-2/Bax, Cleaved-caspase3/Pro-caspase3 and PCNA were measured by Western blot analysis (A). Statistical analysis of Bcl-2/Bax, Cleaved-caspase3/Pro-caspase3 and PCNA were conducted by Image J software (B–D). $^{\#\#}P < 0.01$ *vs* control group, $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs* model group. Positive group (20 mg/kg omeprazole, OMZ); FOE-L (62.5 mg/kgFOE); FOE-M (125 mg/kg FOE); FOE-H (250 mg/kg FOE).

Keap1 protein in the gastric tissue of rats was significantly decreased, while the expression of Nrf2 and HO-1 protein were enhanced to varying degrees in a significant dose-dependent manner, suggesting that FOE regulated the balance of oxidative stress response and alleviates WIRS-induced gastric mucosal injury in rats based on Nrf2-related antioxidant pathway response.

3.10. Effect of FOE on expression of proteins involved in apoptosis pathway

Protein expression of apoptotic pathway-related proteins was detected by Western blot throughout gastric mucosal injury. As indicated in Fig. 8, the PCNA protein expression and Bcl-2/Bax ratio

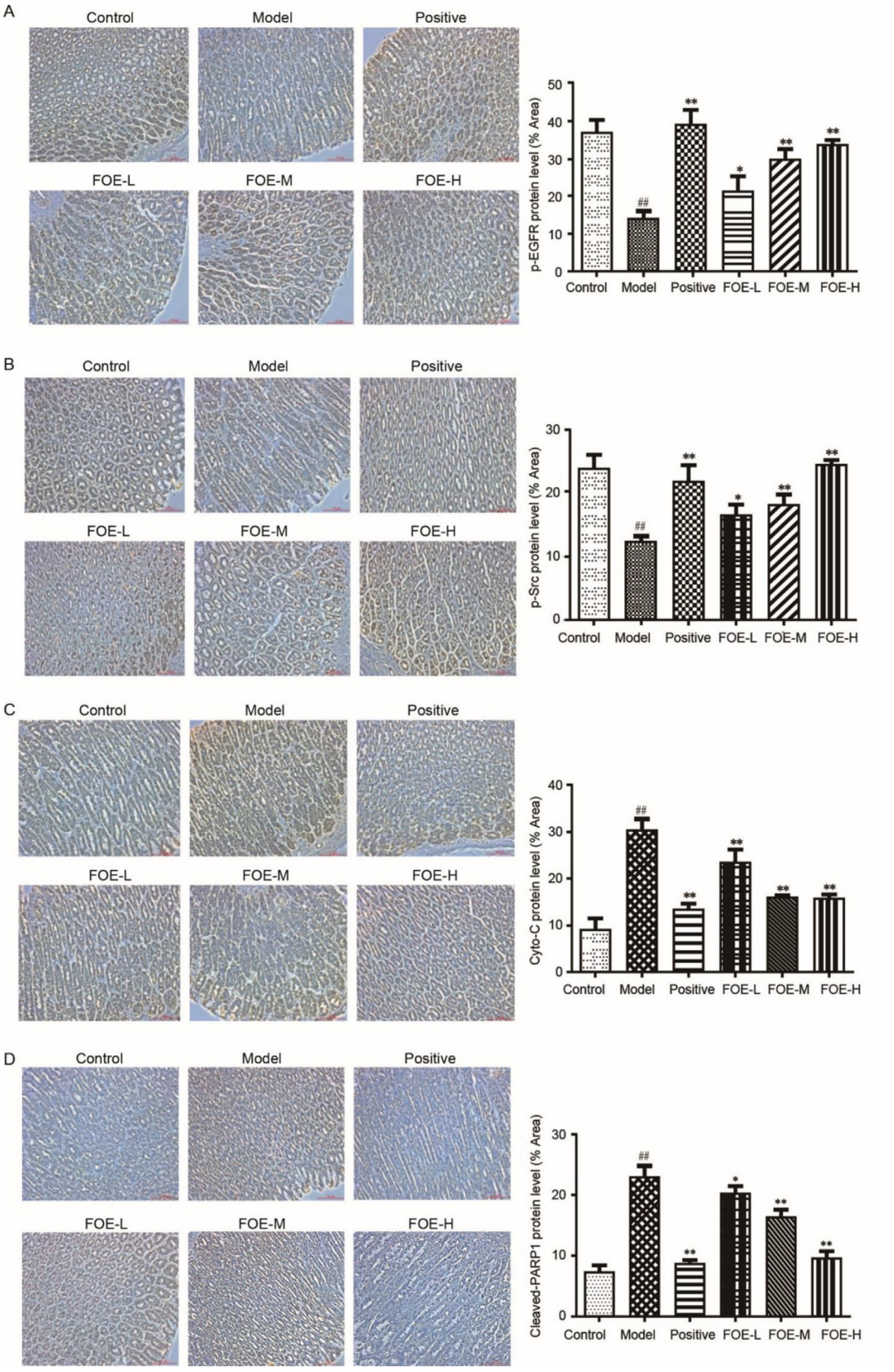

Fig. 9. Immunohistochemistry of p-EGFR (A), p-Src (B), Cyto-C (C) and Cleaved-PARP1 (D) proteins expression in WIRS-stimulated gastric tissues (mean ± SD, $n = 7$). Positive group (20 mg/kg omeprazole); FOE-L (62.5 mg/kg FOE); FOE-M (125 mg/kg FOE); FOE-H (250 mg/kg FOE). $^{\#\#}P < 0.01$ *vs* control group, $^{*}P < 0.05$ and $^{**}P < 0.01$ *vs* model group.

in the model group were dramatically decreased compared with the control group. Nonetheless, the downward trends were to be remarkably reversed by FOE compared with the model group. Besides, the proportion of Cleaved-caspase3/Pro-caspase3 expression was definitely increased compared with the control group. After rats were pre-disposed with FOE, the abnormal expression of Cleaved-caspase3/Pro-caspase3 ratio were evidently reversed compared with the model group.

3.11. Immunohistochemical analyses of p-EGFR, p-Src, Cyto-C and Cleaved-PARP1

Immunohistochemistry was conducted to investigate the effect of FOE on the expression of apoptosis initiation and induction factors including p-EGFR, p-Src, Cyto-C and Cleaved-PARP1 (Fig. 9). The control group showed very strong p-EGFR (Fig. 9A) and p-Src (Fig. 9B) protein expression in epithelial cells and the gastric glands. The model group revealed a significant descent of p-EGFR and p-Src protein level and Cleaved-PARP1 (Fig. 9D) as evidenced by the intense brown color distributed throughout the antigen sites on the gastric tissues, while in FOE and positive groups, less detectable immunoreactivity was observed when compared with the model group, indicating that FOE suppressed the process of apoptosis by affecting apoptosis initiation and induction factors, so as to realize the protective effect on stress gastric mucosal injury.

4. Discussion

Gastric mucosal injury is a multifactorial gastrointestinal disease, of which psychological stress is more and more recognized by the public (Li et al., 2018). In our previous study, due to the functional and anatomical similarity to the human stomach, the rats were chosen as the model and administered ethanol intragastrically to simulate human gastric mucosal injury caused by excessive drinking. FOE has exhibited antioxidant, anti-inflammatory, and cytoprotective activity on ethanol-induced gastric mucosal injury. However, the mechanism of WIRS damage to gastric mucosa is not completely clear. To the best of our knowledge, this study is the first to evaluate the gastroprotective potential of FOE against WIRS-induced gastric injury in rats. Previous studies have shown that it is related to the direct damage of gastric epithelial cells and mucus layer, or the indirect damage such as the influence of gastric mucosal hemodynamics, infiltration of leukocytes and ensued inflammatory and oxidative stress and apoptosis distortion (Miyoshi et al., 2003; Nur Azlina, Qodriyah, Chua, & Kamisah, 2017; Xu et al., 2020). Thus, this study focused on the forthputting of FOE to guard against WIRS-induced gastric injury through anti-inflammation, anti-oxidation and anti-apoptosis. The main findings in our study were that FOE could dose-dependently improve the WIRS-induced: (1) acute gastric mucosal injury (macroscopic and microscopic lesion score and histological improvement); (2) inflammatory response (reduction of iNOS, TNF-α, IL-6 and IL-1β levels, increased IL-10 and PGE2 content, inhibited NF-κB p65 and p-IKKα/β expression, and reduced inflammatory cells infiltration in the gastric mucosa); (3) oxidative stress (increased SOD, CAT and GSH-PX activity, reduced MDA content, and enhanced Nrf2 and HO-1 expression); (4) apoptotic damage (upregulated p-EGFR, p-Src, Bcl-2/Bax and PCNA expression, and downregulated Cyto-C, Cleaved-caspase3/Pro-caspase3 and Cleaved-PARP1 expression). Moreover, the gastroprotective effects of FOE (250 mg/kg b.wt) in all the investigated parameters were comparable to that of omeprazole, a widely used standard drug. In this research, WIRS-induced gastric mucosal injury model was established. WIRS has become the first choice for the induction of the experimental stress-mucosal injury model due to its higher success rate and accessibility. WIRS causes gastrointestinal injuries via several mechanisms including the inhibition of PGE2 synthesis, reduction of bicarbonate release, which disrupts the mucus barrier, and the induction of cytotoxicity. These cytotoxic effects contribute to the recruitment of reactive oxygen species (ROS)-releasing leukocytes and inflammatory cytokines with subsequently reduced gastric blood flow, all of which may contribute to gastric cell apoptosis (Izgut-Uysal et al., 2014; Ohta et al., 2005; Wei et al., 2018).

In terms of our results, WIRS could trigger severe inflammation in gastric tissues, accompanied by activation of the NF-κB pathway and up-regulation of its downstream signals, such as TNF-a, IL-1β and IL-6. These findings are in line with previous researches. In fact, NF-κB is expressed in almost all cells and performs a nonnegligible role in the pathogenesis of gastric mucosal injury. Four transcript variants encoding different isoforms have been found including NF-κB p65/p105/p50/p52 (Yeo, Hwang, Song, & Lee, 2021). In this article, we detected NF-κB p65 due to its contribution to inflammation. Many pro-inflammatory stimuli and ROS can lead to the activation of NF-κB through the phosphorylation of inhibitors of κB (IκBs) by the IκB kinase (IKK) complex. Afterwards, free NF-κB translocates into the nucleus and consequently results in the transcriptional activation of a variety of pro-inflammatory mediators, such as TNF-α, IL-1β and IL-6(Arab, Saad, El-Sahar, & Al-Shorbagy, 2020). Interestingly, FOE mitigated the gastric damage by inhibiting the NF-κB pathway, and then suppressing downstream proinflammatory elements (Duran et al., 2020). These correlates were consistent with mitigation of leukocyte infiltration. It is undoubtedly an efficacious strategy for the protection of gastric mucosal injury to ameliorate the aberration of these inflammatory pathological factors (Yu et al., 2020).

Considerable evidence highlights the implication of oxidative stress in the pathophysiology of gastric mucosal injury (Oshimo et al., 2021). Oxidative stress arises due to an imbalance between ROS production and the cellular antioxidant defense system. In the ulcerated tissues, the infiltrating neutrophils excessively produce superoxide radical anions (O^{2-}), which react with lipids, causing lipid peroxidation. Lipid peroxidation is the consequence of ROS reaction against cell membrane and produces significant levels of MDA, which leads to oxidative gastric damage (Korbut, Brzozowski, & Magierowski, 2020; Xue et al., 2021). The present findings indicated that the application of WIRS indulged MDA and significantly inhibited the production of the Nrf2. These changes prevented transcription factor Nrf2 from serving as a sensor to regulate the expression of antioxidant enzymes HO-1 driven by antioxidant response elements (ARE) in response to oxidative stimulation (He et al., 2022). The observations were consistent with previous researches. Interestingly, our results showed that FOE remarkably reverses the redox induced by WIRS. A good explanation for inhibiting oxidative stress was the observed remission of neutrophil infiltration and the decrease of membrane lipid peroxidation level. Ample evidence demonstrated that antioxidants play a central role in process of gastric mucosal injury (El-Shiekh et al., 2021). Our data validated the restoration of Nrf2 along with the prototypical Nrf2 target genes (*HO-1* and *Nqo1*) and replenish of crucial antioxidation elements (SOD, CAT and GSH-PX) in the wake of FOE gastroprotection. All of the data suggested the potential prospects of FOE in protecting gastric mucosal injury through anti-oxidation.

Numerous studies demonstrated that apoptosis also goes hand in hand with the occurrence of gastric mucosal injury, and the continuous excessive production of apoptosis will destroy the integrity of gastric mucosa and eventually induce gastric mucosa dysfunction (Wang et al., 2021). Mechanistically, our data indicated WIRS

intake made the anti-apoptosis gene *Bcl-2* down-regulated markedly driven by ROS and pro-inflammatory signals. In this circumstance, the restriction to the apoptotic protein Bax was limited, which in turn led to the mitochondrial escape of cytochrome C and subsequently activated Caspase-3, which is consistent with previous studies (El Badawy, Ogaly, Abd-Elsalam, & Azouz, 2021; Hu et al., 2021). After receiving FOE, it could be clearly observed that the anti-apoptosis capacity of gastric mucosa was enhanced, manifested in the recovery of Bcl-2 level and the control of Bax and Caspase-3. In fact, previous studies have demonstrated that FOE has potential anti-apoptotic activity. And our data strongly indicated that FOE could partly participate in the palliation of gastric mucosal injury caused by WIRS though anti-apoptosis.

The present findings also revealed that the p-EGFR and p-Src protein expressions were obviously suppressed in WIRS-induced rat gastric tissues. The p-EGFR mediated by TFF1 triggers Src, PLC/PKC and so on signal cascades, transmits signals sequentially by phosphorylation of amino acid residues, activates Rho GTPase, promotes the migration, proliferation and differentiation of epithelial cells in adjacent ulcer tissue, reconstructs the mucosal barrier and then accelerates the healing of damaged gastric mucosa (Baus-Loncar & Giraud, 2005; Tarnawski & Ahluwalia, 2012). Furthermore, PARP-1 is a major functional gene in the poly ADP-ribose polymerase (PARP) family and DNA damage sensor. When cell DNA is damaged, PARP-1 can quickly be activated, and then formed poly-ADP ribose complex (PAR) to act in a repair role. However, the over-activation of PARP-1 exhausts endogenous NAD and ATP, which leads to energy depletion and cell death (Rao et al., 2001). Hence, PARP-1 plays a pivotal role in DNA repair, transcription and chromatin modulation. Simultaneously, PARP-1 also acts a key role in the mitochondria to nuclear shuttling mechanism, which triggers Caspase-independent apoptotic cell death through apoptosis-inducing factor (AIF) (Aihara, Engevik, & Montrose, 2017). Apoptotic stimuli of excessive reactive oxygen species, excessive hydrochloric acid and other apoptotic signals result in PARP-1 activation in response to excessive DNA damage triggering the AIF release from mitochondria, thereby accelerating PARP1-dependent apoptosis or cell death (Strzalka & Ziemienowicz, 2011). In this study, we noticed that the cleaved PARP-1 protein expression was obviously enhanced in the WIRS-damaged rat gastric tissues. PCNA is directly involved in cell proliferation as a helper protein of DNA polymerase, which often decreases in all kind of damaged tissues and increases during the recovery period (Viana et al., 2019). In our present experiment, the PCNA protein expression in the WIRS-damaged rat gastric tissues showed an obvious decreasing trend, and FOE obviously boosted its expression. The data suggested that the increased cell proliferation mediated by PCNA was probably one of the mechanisms involved in FOE's protective effect.

5. Conclusion

In summary, inflammation, oxidative stress and apoptosis are closely related to the occurrence of gastric mucosal injury. Our findings clearly substantiated the potent gastroprotective effect of FOE against WIRS-induced gastric injury with comparable efficacy to omeprazole. FOE gastroprotection is mediated, at least in part, through its antioxidant, anti-inflammatory, and anti-apoptotic mechanisms. The potential mechanism seems to be related to the induction of antioxidant and anti-apoptosis enzymes synthesis by activating Nrf2/HO-1 and Bcl-2 through EGFR/Src-dependent pathway, and to the inhibition of NF-κB inflammation signaling cascades. A schematic diagram of the potential molecular mechanism of FOE-mediated gastroprotective effect on WIRS-induced gastric injury was presented as Fig. 10. We verified that FOE acts through multiple pathways, but the linkages between the pathways need to be further investigated, and the main active substances of FOE need to be followed up.

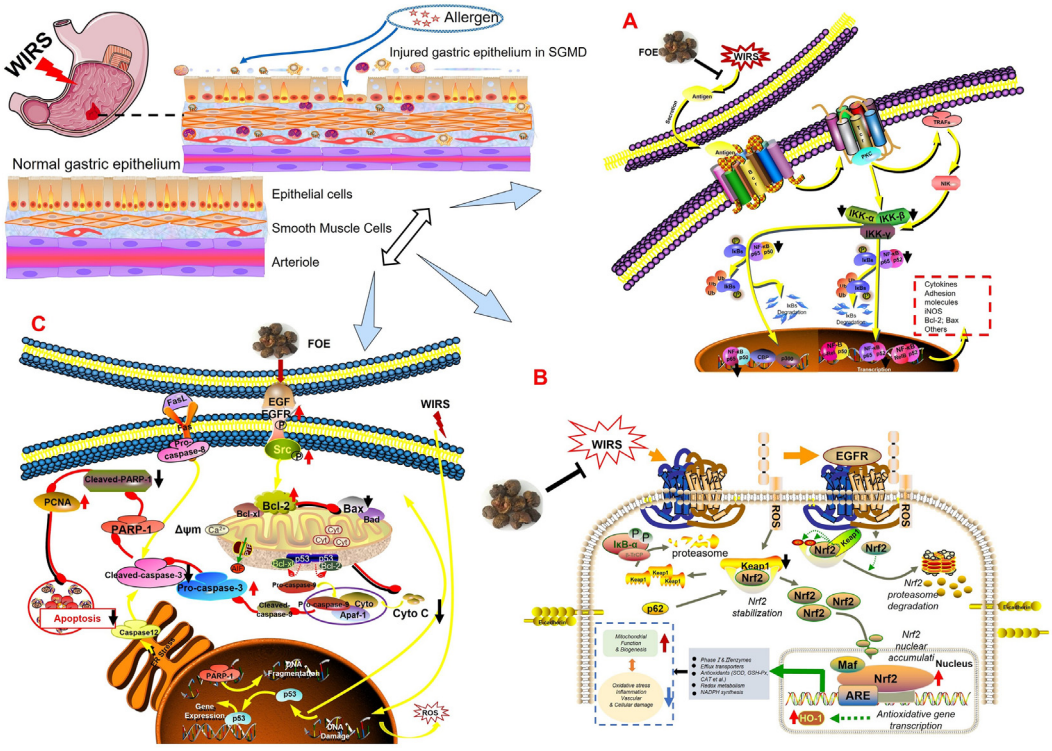

Fig. 10. Potential molecular mechanism of FOE-mediated gastroprotective effect on WIRS-induced gastric injury. NF-κB inflammation signaling cascades (A); Nrf2/HO-1 oxidative stress signaling cascades (B); EGFR/Src-dependent signaling cascades (C).

CRediT authorship contribution statement

Xinnan Liu: Writing – original draft, Investigation, Project administration. **Zhen Yuan:** Writing – review & editing, Conceptualization, Data curation. **Lifei Luo:** Conceptualization, Methodology. **Teng Wang:** Investigation, Visualization. **Feng Zhao:** Supervision, Writing – review & editing. **Jingze Zhang:** Project administration, Validation. **Dailin Liu:** Writing – review & editing, Visualization, Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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