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Original Article

Protocatechuic acid, the main effective monomer in Wuqi Powder, can inhibit gastric ulcers induced by acetic acid and *Helicobacter pylori*

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Abstract: Objective: To explore the effective ingredients of Wuqi Powder and their mechanism of action, so as to provide a theoretical basis for clinical application. Methods: Enzyme-linked immunosorbent assay was used to determine interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α) levels. Rapid urease test and Giemsa staining were conducted to detect *Helicobacter pylori* (*H. pylori*) in gastric tissue. CCK-8, EdU and wound healing assay were used to measure the proliferation and migration of GES-1 cells. The number of intracellular and extracellular bacteria of GES-1 cells was counted to evaluate infection and adhesion of *H. pylori*. RT-qPCR was conducted to evaluate the level of alpA, alpB and cagA genes of *H. pylori*. Bioinformatics methods were used to predict the potential targets and signaling pathways of protocatechuic acid (PCA) in GES-1 cells. Then, RT-qPCR was used to detect the expression of target genes, and Western blot was conducted to detect the interaction of the target pathways. Results: PCA is the effective ingredient in Wuqi Powder, which alleviated the symptoms of gastric ulcers, reduced *H. pylori* in gastric tissue and IL-6, TNF-α in rat serum. In addition, PCA accelerated the proliferation and migration of GES-1 cells and inhibited the infection and adhesion of *H. pylori* to GES-1 cells. Furthermore, PCA inhibited the TNF and Smad pathways and activated the vascular endothelial growth factor A (VEGFA) pathway of GES-1 cells. Conclusion: PCA is the key component in treating gastric ulcers induced by acetic acid and *H. pylori*. It promotes gastric ulcer repair by inhibiting the Smad pathway, TNF pathway and activating the VEGFA pathway.

Keywords: Wuqi Power, protocatechuic acid, *Helicobacter pylori*, Smad, tumor necrosis factor, vascular endothelial growth factor A

Introduction

Gastric ulcer (GU) is a digestive disease caused by an imbalance between the gastric mucosa's protective and invasive factors. The main manifestations are partial defects, inflammation and necrosis of the mucosa. An ulcer defect is at least 0.5 cm in diameter and penetrates into the muscular mucosa [1]. Normally, the gastric mucosa has triple protection, including gastric mucus, gastric mucosal cells and the blood supply system in the stomach wall. GUs occur when these defense systems are damaged [2]. At present, the known factors that can lead to gastric mucosal barrier damage include excessive gastric acid, *Helicobacter pylori* (*H. pylori*) infection, use of non-steroidal anti-inflammatory drugs (NSAID) and excessive smoking and drinking. About 80%-90% of GUs are caused by *H. pylori* infection and NSAID use [3].

H. pylori, a gram-negative bacterium, has 4 to 7 sheathed flagella on its surface that penetrates the mucus layer covering gastric epithelial cells (GECs) and settles on the surface of the GECs [4]. In addition, the successful colonization of *H. pylori* in the stomach is due to its ability to secrete a variety of adhesion factors, such as adhesion lipoprotein A (AlpA), adhesion lipoprotein B (AlpB) and blood group antigen binding adhesion (BabA) [5]. They bind to specific receptors existing on the surface of GECs and firmly adhere to the GECs. In addition, *H. pylori* secretes large amounts of urease, which decomposes urea into ammonia and carbon dioxide. Ammonia is an alkaline substance that

allows *H. pylori* to tolerate low pH in the stomach [6]. Additionally, it is worth noting that *H. pylori* is the primary pathogen leading to GUs and a main inducements of gastric cancer [7]. Thus, *H. pylori* plays a role in GUs, and gastric carcinoma mainly relies on its various virulence factors, such as cytotoxin-associated gene A (CagA), which is encoded by genes on a cytotoxin-associated genes pathogenicity island (cagPAI) of *H. pylori.*

Furthermore, there is a pinhole-like structure called type IV secretion system (T4SS) on the surface of *H. pylori*, which is also encoded by the gene of cagPAI, and it can inject CagA into GECs. The injected CagA is then phosphorylated and involved in downstream signaling pathway transduction, leading to an inflammatory response and so on [8, 9]. In addition, *H. pylori* can produce vacuolating cytotoxin A (VacA), which is activated in a weakly acidic environment to cause vacuolar degeneration of GECs. This vacuolar degeneration not only harms the gastric mucosa but also delays the repair of GECs [10].

Clinically, the accepted treatment for *H. pylori* infection is quadruple therapy, which are two antibiotics + proton-pump inhibitor (PPI) + bismuth [11]. Although the cure rate of this regimen is high, the resistance of *H. pylori* to commonly used antibiotics is also increased due to the abuse of antibiotics in clinical practice, limiting the clinical efficacy of the quadruple therapy [12]. Besides, the superimposed effect of the two antibiotics causes an increasing incidence of adverse reactions [13]. In addition, the use of the PPI leads to weakened digestive function, which may cause dyspepsia, abdominal distension and other adverse reactions [14]. Traditional Chinese medicine treatment pays attention to the overall concept, treating both the symptoms and root causes, and has a good effect on *H. pylori* infection and GUs [15-18]. Wuqi Powder is an empirical prescription created by Professor Zheng Liang based on his years of clinical treatment experience, it is composed of Schisandra Chinensis, Pseudoginseng, Bletilla striata, Aspongopus and Calcined cuttlebone [19]. Clinical studies have proved that Wuqi Powder significantly relieves the symptoms of GUs, such as stomachache, belching and loss of appetite, and reduces the level of inflammatory factors such as interleukin (IL)-1 and tumor necrosis factor (TNF)-α. Laboratory studies have confirmed that this prescription reduces inflammation and promotes gastric mucosa repair [20].

However, the actual active ingredients in Wuqi Powder and its molecular mechanism of promoting gastric mucosal repair are still unclear. Studies found that Schisandra Chinensis and Bletilla in Wuqi Powder showed a good effect on GUs [21]. Hence, in this study, we screened out protocatechuic acid (PCA), the standard effective monomer of Schisandra Chinensis and Bletilla. Recent study has reported the cytoprotective effect of PAC against gastric mucosa ulceration aggravated by potassium [22]. PCA was identified to effectively alleviate GUs in rats and inhibit *H. pylori* infection. In addition, PCA was verified to accelerate the proliferation and migration of GES-1 cells and inhibit the infection and adhesion of *H. pylori* to GES-1 cells. It was found that PCA may regulate the development of GES-1 through the Smad, vascular endothelial growth factor A (VEGFA) and TNF signaling pathways, thus promoting the repair of the damaged gastric mucosa. Our research aimed to investigate the internal molecular mechanism of Wuqi Powder in inhibiting *H. pylori* infection and alleviating GUs, so as to provide a theoretical basis for using Wuqi Powder to treat GUs.

Materials and methods

Screening of the effective monomer in Wuqi Powder

The chemical components of Schisandra Chinensis and Bletilla were acquired through the Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform (TCMSP, http://tcmspw.com/tcmsp.php). First, the screening was carried out according to oral bioavailability ≥30% and drug-likeness ≥0.18. Then the screened practical components of Schisandra Chinensis and Bletilla were intersected to obtain the standard practical components.

Culture of H. pylori

H. pylori SS1 strain was purchased from Bio vector (Beijing, China). It was cultured in broth added with 7% defibrinized horse blood at 37°C in a microaerobic environment with

3%-5% O_2 and 10% CO_2. The bacterial solution was diluted with sterile PBS before use.

Construction of GU rat model and the treatment

Male SPF healthy Wistar rats with a weight of (200±10) g were purchased from the Nanjing University of Chinese Medicine Experimental Animal Center. Routine care with free drinking and eating was carried out in the SPF laboratory of Nanjing University of Chinese Medicine at a relative humidity of 45%-55% and a temperature of 22°C-25°C. The experimental protocol was established according to the ethical guidelines of the Helsinki Declaration and was approved by the Medical Ethic Committee of The Second Affiliated Hospital of Nanjing University of Chinese Medicine (No. 2021-L098).

The GU modeled rats were induced by acetic acid (AA) and *H. pylori*. First, all rats were anesthetized, and the stomach tissue was exposed. Next, 20% AA (0.03 mL) was injected into the subserosa of glandular part with a micro-syringe, then the enterocoelia was closed. After that, the rats were fed with *H. pylori* (5×10^8 CFU/mL, 1 mL/each) [23]. The 60 rats were divided into 6 groups, control group, model group, model + 200 μg/mL PCA, model + 400 μg/mL PCA, model + 800 μg/mL PCA treatment group and positive drug treatment group (10 rats/group). The PCA and positive drug omeprazole were purchased from Sigma-Aldrich (Darmstadt, Germany). Omeprazole was given by gavage at 20 mg/kg [23]. Rats in the control group and model group were given 1 mL saline by gavage. The intragastric administration was performed once daily for a total of 14 days.

Detection of serum inflammatory factors by enzyme-linked immunosorbent assay (ELISA)

Blood from the tail vein of rats was collected and left standing at room temperature for 20 min, and then the upper serum was centrifuged at 2000 g for 10 min. Rat IL-6 and TNF-α ELISA kits (Beyotime, Shanghai, China) were used for detection, and the experiment was operated following the instructions. Briefly, the gradient dilution standard and serum to be tested were added to the plate precoated with antibodies and incubated for 2 h. After washing the plate, the biotinylated antibody was added for further incubation for 1 h. Then horseradish peroxidase-labeled streptavidin was added and incubated for 15 min in dark. Afterwards, TMB substrate was added and reacted for 15 min. Finally, the stop solution was injected, and the absorbance value at 450 nm was detected with a microplate meter (Thermo Fisher, MA, USA) immediately after mixing. After drawing the standard curve, the concentrations of IL-6 and TNF-α were calculated.

Determination of the gastric fluid index

After fasting for 24 h, the rats were anesthetized with isoflurane, and the pylorus was ligated surgically, and then the cardia was ligated 4 h later. The gastric contents were collected and centrifuged for 10 min. The supernatant was taken to measure the volume of gastric juice. The pH value of the supernatant was measured with a pH meter (Leici, Shanghai, China).

Evaluation of the degree of GUs

After sacrificing the rats, the stomach was taken out and rinsed with saline, then it was spread out on a piece of white paper and fixed, and images were taken to analyze the ulcer. After that, part of the gastric tissue was clipped and fixed with 4% paraformaldehyde. Then, the samples were subjected to conventional paraffin embedding and sectioning. Next, the sections were successively dewaxed, hydrated, stained with hematoxylin-eosin (Beyotime, Shanghai, China), dehydrated and sealed. Finally, the degree of GUs was observed with a microscope (Nikon, Tokyo, Japan).

Detection of the H. pylori in the stomach

Fresh gastric mucosal tissues of rats were taken, and the *H. pylori* was detected using the *H. pylori* rapid urease assay card (Saier, Shenzhen, China). In short, 1 mm^3 of gastric mucosa was placed in the culture area of the kit and cultured at room temperature for 10 min to observe the changes in color. The red in the culture well was positive, while the yellow was negative. In addition, the modified Giemsa staining method was applied to detect the existence of *H. pylori* on the gastric mucosa. The paraffin sections were first dewaxed, rehydrated and laid on a slide, then a piece of filter paper was placed on them, and a drop of

Giemsa dye (SolarBio, Beijing, China) was added. After 30 min, the filter paper was removed, the slides were rinsed with water for 2-3 min, then dried and sealed for observation.

Cell culture

Human gastric epithelial cell line GES-1 was obtained from American Type Culture Collection and cultured in DMEM high-glucose medium (Hyclone, Utah, USA) containing 100 mL/L fetal bovine serum (Hyclone, Utah, USA) for 2-3 days. The culture condition was 95% air and 5% CO_2 at 37°C. Cells could be passaged when they reached 80% confluence.

CCK-8 assay

Logarithmic phase GES-1 cells were inoculated into 96-well plates at a density of 1×10^4/well. Then the cells were divided into 5 groups: control group, 200 μg/mL, 400 μg/mL and 800 μg/mL PCA treatment groups and positive drug treatment group (200 μg/mL omeprazole). Six duplications were set in each group. After 48 h, 10 μL CCK-8 reagent (Beyotime, Nanjing, China) was added and incubated at 37°C for 2 h. A multifunctional microplate reader was used to determine the absorbance value at 450 nm (Thermo Fisher, MA, USA).

EdU cell proliferation assay

The proliferation of GES-1 cells was evaluated by EdU Cell Proliferation Kits (Sangon, Shanghai, China). GES-1 cells were inoculated in a 24-well plate, treated with different drugs and incubated for 48 h. Afterwards, 10 μM EdU reagent was added to the medium and incubated for 2 h. After rinsing with PBS, 150 μL fixative was added to fix the cells for 30 min, and 150 μL Glycine (2 mg/mL) was added. After being rinsed with PBS for 3 times, 300 μL 0.5% Triton X-100 was added for penetration. Then 100 μL detection solution was injected and incubated for 30 min in dark. After rinsing, Hoechst dye was used for staining in the dark for another 30 min. Immediately after rinsing, a laser confocal microscope (Nikon, Tokyo, Japan) was used for observation.

Wound healing assay

GES-1 cells were cultured in a 24-well plate and were divided into 5 groups, then a straight line was drawn on the monolayer cells with a sterile needle. Afterwards, 200/400/800 μg/mL PCA and 200 μg/mL omeprazole diluted in the culture medium were added, while the control group was added with the same amount of sterile PBS. After 48 h of culture, the healing of the scratch in each group was observed and accorded by the microscope.

In vitro inhibition of H. pylori

To study the inhibition rate of PCA on *H. pylori* cultured *in vitro*, the *H. pylori* solution was first diluted with PBS to 1×10^6 CFU/mL, and then 200/400/800 μg/mL PCA and 200 μg/mL omeprazole were added, respectively. The value of $OD_{590\ nm}$ was determined by an ultraviolet spectrophotometer (Shimadzu, Tokyo, Japan) after the cells were cultured in a shaker at 37°C for 24 h.

In order to study the effect of PCA on the infection ability of *H. pylori*, GES-1 cells were treated with PCA (200, 400, 800 μg/mL) and omeprazole (200 μg/mL) for 48 h. Then *H. pylori* was introduced into GES-1 cells with a multiplicity of infection (MOI) of 100 and incubated for 6 h. GES-1 cells were rinsed with PBS several times and lysed. The cell lysates and cell culture supernatant were diluted and coated on the agar plate, then incubated overnight at 37°C. The number of colonies was counted and converted into log10 CFU/mL according to the dilution factor.

Real-time quantitative PCR (RT-qPCR)

Bacterial total RNA extraction kit and Trizol reagent (Sangon, Shanghai, China) were used to extract RNA from *H. pylori* and GES-1 cells. First, cDNA Synthesis System (Merck, Darmstadt, Germany) was used to obtain the cDNA. Then, the TaqMan Fast Advanced Master Mix (Merck, Darmstadt, Germany) was used to construct the PCR reaction system. The PCR reaction procedure was as follows: denaturing (95°C, 10 s), annealing (56°C, 15 s), and extension (72°C, 30 s) for 35 cycles. 16S rRNA was used as an internal reference for *H. pylori,* and β-actin was selected as an internal reference for GES-1 cells. Finally, $2^{-\Delta\Delta CT}$ method was applied to analyze the relative expression levels of genes. The primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd., and the sequences are shown in **Table 1**.

Table 1. Primer sequences for RT-qPCR

Primer		Sequence
alpA	F	5'-TCAATTCGGAAGAGCTAGGAC-3'
	R	5'-GACCATTGATACCAATTGACTGA-3'
alpB	F	5'-TTACAGGACTAAACTTAGAA-3'
	R	5'-ATACCGTAACGAATCAAGTAC-3'
cagA	F	5'-CAGATCTCGATCATGCAACT-3'
	R	5'-TACATGATGTCGTACAGTAAG-3'
16s rRNA	F	5'-GATGCACAAGTAGTAGAGTAC-3'
	R	5'-TGCATCAAGCTACGCACATG-3'
VEGFA	F	5'-AGTTCAGTAACGTTCATAGTTAC-3'
	R	5'-TCATCACAAGCAATTGCTGAG-3'
TNF	F	5'-CTGCTGAATGTAACAGCACGAT-3'
	R	5'-ACTACAGTAGCAGTAGAGCAC-3'
smad7	F	5'-GTACATTAGCATTAGCAGCTA-3'
	R	5'-ACAGCATGACCAGGTAGATT-3'
smad3	F	5'-CAAGTCTAGCAGCATACATC-3'
	R	5'-ATCGTCAAGCATCTGACCAGA-3'
smad2	F	5'-GCAATGCACTCGAATCACGC-3'
	R	5'-TGAACGTAGTCGAACAGTTAG-3'
smad4	F	5'-GACTGACGTTCCATGCGACA-3'
	R	5'-CTCCTTAATGTCACGCACGAT-3'
β-actin	F	5'-ATTGATGACTGTCAACTCAG-3'
	R	5'-TAACCTCTGACGTACATCTG-3'

F: Forward; R: Reverse.

Bioinformatics analysis

The targets of PCA were identified by the CTD database (http://ctdbase.org/), and the target genes related to GU were screened using the keyword "gastric ulcer". The intersecting gene of PCA and GU was obtained using the Venn diagram. The interaction network analysis of the selected target pathway proteins was performed with the use of the String database (https://string-db.org/).

Cell transfection

GES-1 cells were inoculated in 24-well plates, and cell transfection was carried out when the confluence reached 80%. The Turbofect Transfection Reagent (Thermo Fisher, MA, USA) was used for cell transfection. Sh-VEGFA, sh-TNF and sh-NC used in this research were synthesized by Sangon (Shanghai, China), and the sequences of these sh-RNA were as follows: sh-VEGFA: 5'-UAAGACGUGCUUAGACAUC-3', sh-TNF: 5'-UUCGCAAUAGCCUAAUGCU-3', sh-NC: 5'-CCCCUUUUUAAAAAGGCGG-3'. The subsequent experiments were performed 48 h after the transfection.

Western blot

The transfected cells were collected and lysed, and the concentration of the extracted protein was determined by BCA protein detection kits (Abcam, Cambridge, UK). The samples were mixed with loading buffer and heated at 100°C for 10 min. After electrophoresis, the separated protein was transferred onto the polyvinylidene fluoride (PVDF) membrane. Subsequently, the PVDF membrane was blocked with skimmed milk for 1 h at room temperature. Next, the membrane was incubated with different primary antibodies, including anti-Smad7 (ab216428, 1:1000), anti-Smad3 (ab204462, 1:5000), anti-Smad2 (ab228765, 1:2000), anti-Smad4 (ab236321, 1:1000), and anti-β-actin (ab179467, 1:5000) overnight at 4°C. The next morning, the membrane was rinsed several times with PBST and then incubated with horseradish peroxidase conjugated goat anti-rabbit IgG (ab205718, 1:10000) at room temperature for 1 h. All antibodies were purchased from Abcam (Cambridge, UK). Finally, the ECL reagent (SolarBio, Beijing, China) was used to develop the bands, and detection software (Bio-Rad, California, USA) was used to analyze the intensity of bands.

Statistical analysis

All experiments were conducted independently at least three times. The software SPSS 23.0 was applied for statistical analyses. All data were presented as $\bar{x} \pm sd$. When the data met a normal distribution, the differences between two groups were tested by the Student's t-test. For the data that did not meet the normal distribution, the differences between two groups were tested by Mann-Whitney test. One-way ANOVA follow by Dunnett test were used to test the differences among multi-groups. $P<0.05$ means the difference was statistically significant.

Results

PCA is an effective monomer in Wuqi Powder

Wuqi Powder is comprised of Schisandra Chinensis powder, Notoginseng powder, Bletilla stri-

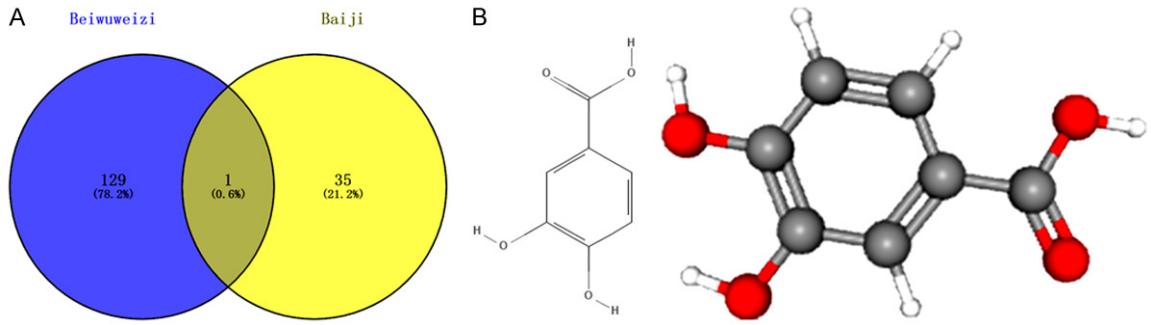

Figure 1. Screening the effective monomer of Wuqi Powder. A: TCMSP database was applied to screen out the standard active ingredient PCA of Schisandra Chinensis and Bletilla striata; B: The chemical structure of PCA was obtained from PubChem. TCMSP: Traditional Chinese Medicine Systems Pharmacology Database and Analysis Platform; PCA: Protocatechuic Acid.

ata powder, Aspongopus powder and Calcined cuttlebone powder. Schisandra Chinensis and Bletilla striata are considered to have a good effect on GUs. Therefore, we screened the active components in Schisandra Chinensis and Bletilla striata with the help of the TCMSP database and found that the common active component of both was PCA (**Figure 1A**). The molecular structure diagram of PCA (**Figure 1B**) was obtained from the PubChem (http://pubchem.ncbi.nlm.nih.gov/) database, and the molecular formula of PCA was $C_7H_6O_4$.

PCA alleviated GUs in rats

In order to investigate whether PCA is beneficial to the treatment of GUs, we established a rat model of GU and treated them with different doses of PCA. The gastric mucosal intima of rats in the model group suffered from severe bleeding and large ulcers. The surface of the ulcers had become black due to the acidic environment in the stomach. The ulcers of rats treated with PCA were alleviated in a dose-dependent manner. Moreover, the healing of ulcers in the omeprazole treatment group was slightly better than that in model + 800 μg/mL PCA group (**Figure 2A**). In addition, H&E staining results showed severe bleeding, submucosal edema, deficiency of epithelial cells and inflammatory cell infiltration in the gastric mucosa of rats in model group. However, the above symptoms were alleviated to different degrees in the PCA and omeprazole treatment groups (**Figure 2B**). In addition, we detected the volume and the pH value of gastric juice in each group. As the dose of PCA increased, the volume of gastric juice in rats gradually decreased, and the pH value gradually increased to the average level (**Figure 2C**). These findings indicate that PCA has the effect of alleviating GUs in rats.

PCA inhibited H. pylori infection in vivo

We attempted to investigate whether PCA could inhibit the *H. pylori* infection in gastric mucosa. First, we detected the levels of IL-6 and TNF-α in the serum of rats in each group. We found that the levels of inflammatory cytokines in the model + 400/800 μg/mL PCA groups and omeprazole treatment groups decreased markedly compared with the model group (**Figure 3A**). In addition, the rapid urease test results showed that after PCA treatment, the amount of *H. pylori* on the gastric mucosa was significantly reduced (**Figure 3B**). Modified Giemsa staining also demonstrated that PCA inhibited *H. pylori* infection in the gastric mucosa in a dose dependent manner (**Figure 3C**).

PCA promoted the proliferation and migration of GECs

Although we found that PCA promoted the recovery of GUs, the mechanism of this effect remains to be explored. Therefore, we studied the effect of PCA on GECs. By treating GES-1 cells with different concentrations of PCA, we found that PCA effectively accelerated the proliferation of GES-1 cells with the increase of dose (**Figure 4A** and **4B**). At the same time, we confirmed that PCA significantly promoted the migration of GES-1 cells *in vitro* (**Figure 4C**), which is conducive to the repair of the damaged gastric mucosa.

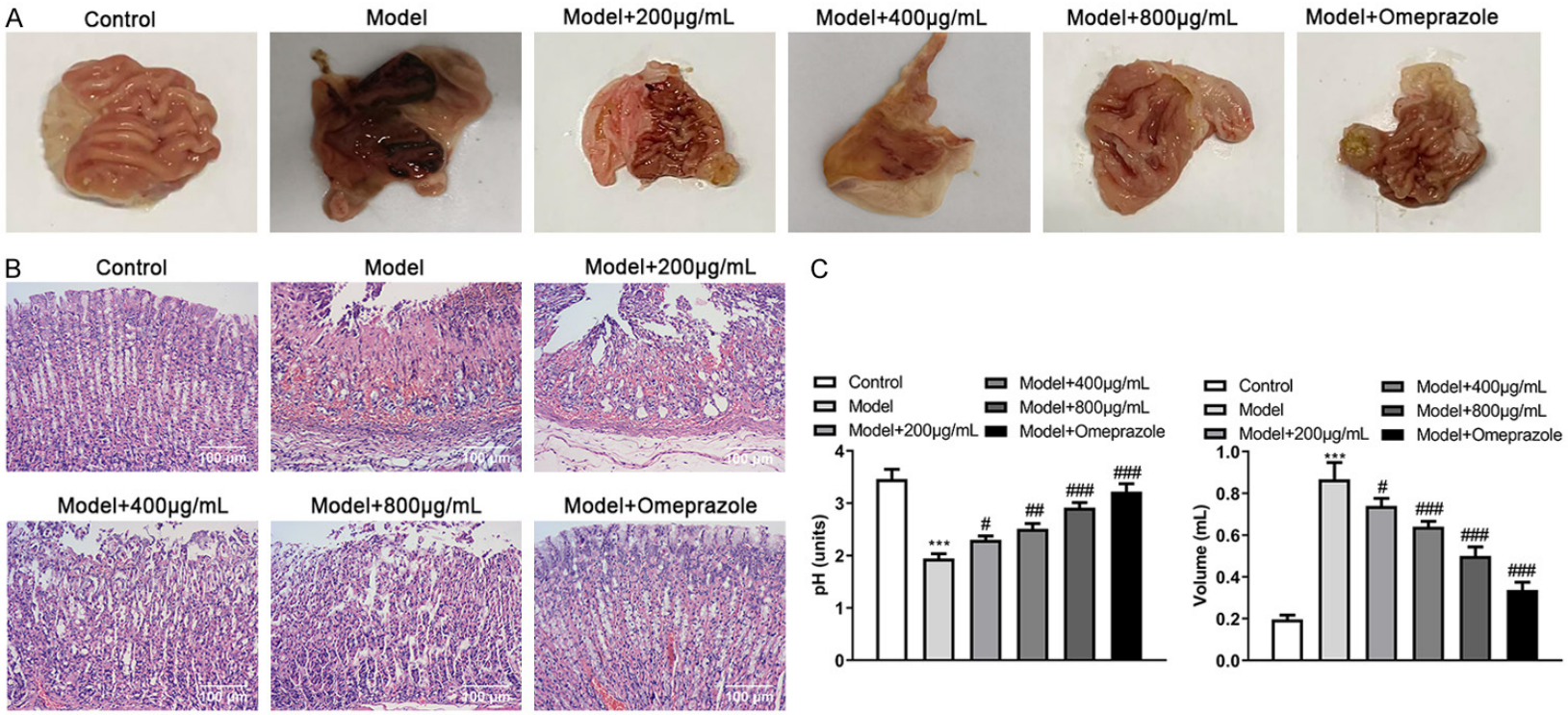

Figure 2. PCA alleviates GUs in rats. A: Anatomical images of the gastric mucosa of rats in control group, model group, model + 200 μg/mL PCA, model + 400 μg/mL PCA, model + 800 μg/mL PCA treatment groups and model + omeprazole treatment group (n = 10/group); B: H&E staining of the gastric mucosa in each group (n = 3/group). Scale bar = 100 μm, magnification × 20; C: Gastric fluid indexes (pH and volume) of rats in each group (n = 10/group). Compared with the control group, ***P<0.001; compared with the model group, $^{\#}$P<0.05, $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001. PCA: Protocatechuic Acid; GU: Gastric Ulcer.

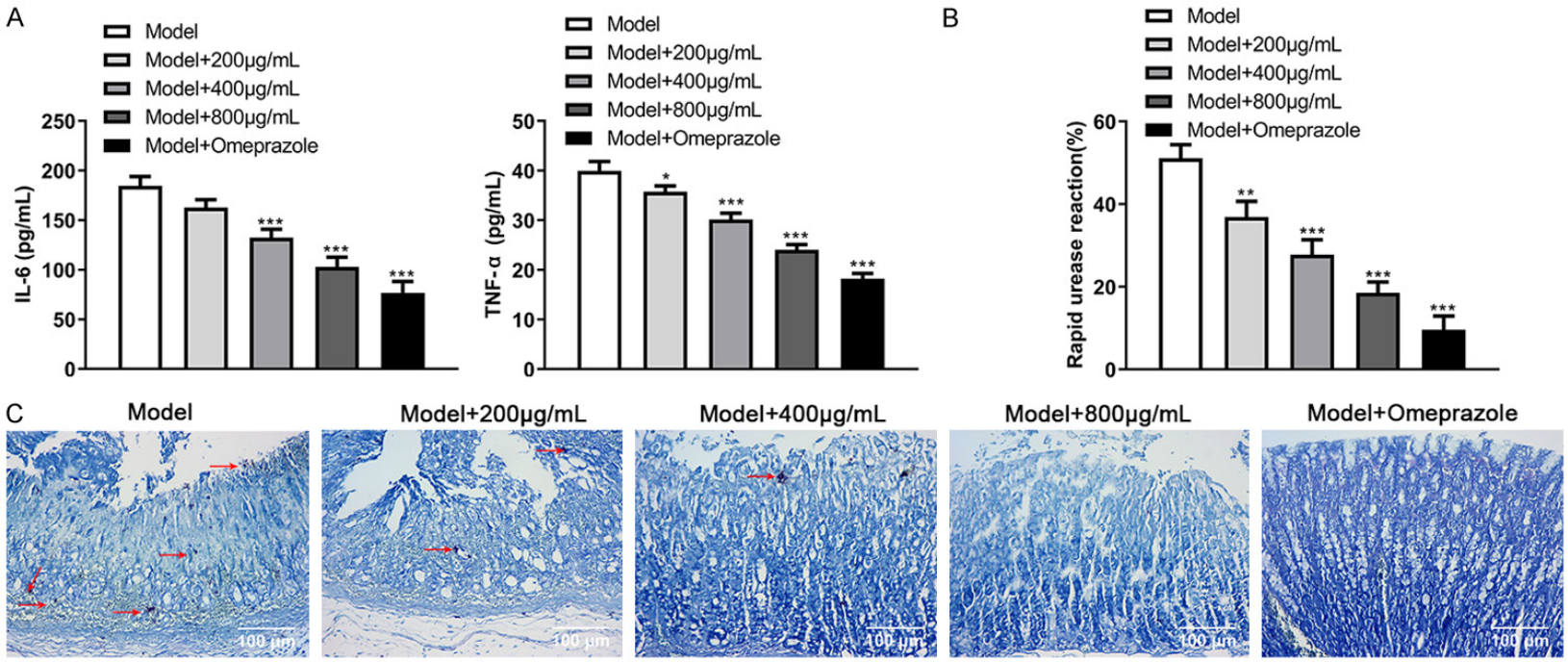

Figure 3. PCA inhibits the infection of *H. pylori* in vivo. A: Levels of IL-6 and TNF-α in the serum of rats in model group, model + 200 μg/mL PCA, model + 400 μg/mL PCA, model + 800 μg/mL PCA treatment groups and model + omeprazole treatment group (n = 10/group); B: The *H. pylori* in gastric tissue was measured by rapid urease test (n = 10/group); C: The presence of *H. pylori* in the gastric mucosa was verified by modified Giemsa staining (n = 3/group). The arrows are pointed to *H. pylori* on the gastric mucosa. Scale bar = 100 μm, magnification × 20. Compared with the model group, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. IL-6: Interleukin-6; TNF-α: Tumor Necrosis Factor-α; PCA: Protocatechuic Acid.

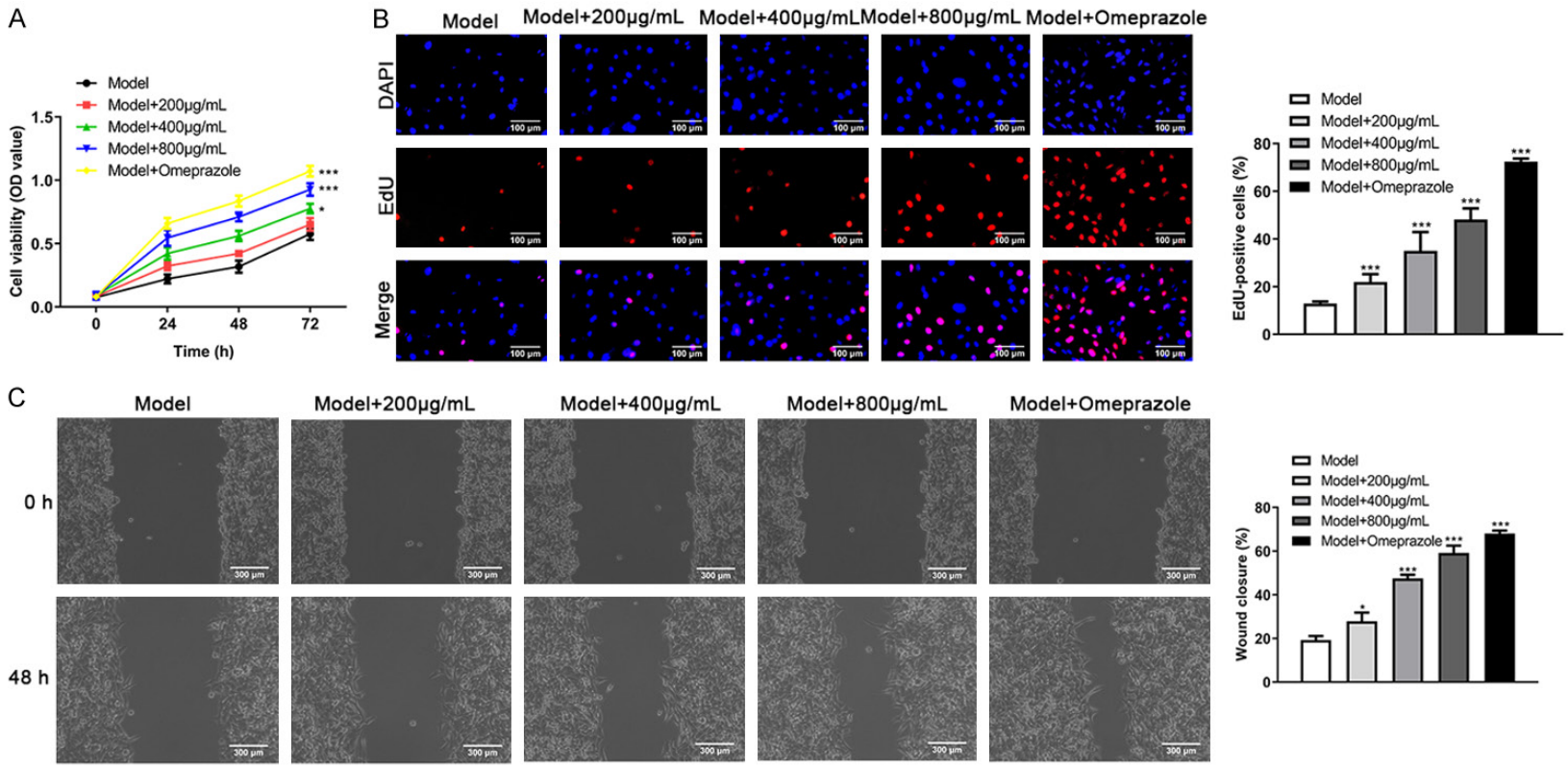

Figure 4. PCA promotes the proliferation and migration of GES-1 cells in vitro. A: CCK-8 assay was performed to detect the proliferation of GES-1 cells after PCA treatment (6 duplications/group); B: EdU assay was performed to detect the proliferation of GES-1 cells after PCA treatment (6 duplications/group). Scale bar = 100 μm, magnification × 100; C: A wound-healing assay was conducted to detect the migration of GES-1 cells (6 duplications/group). Scale bar = 300 μm, magnification × 40. Compared with the model group, $^{*}P<0.05$, $^{***}P<0.001$. PCA: Protocatechuic Acid.

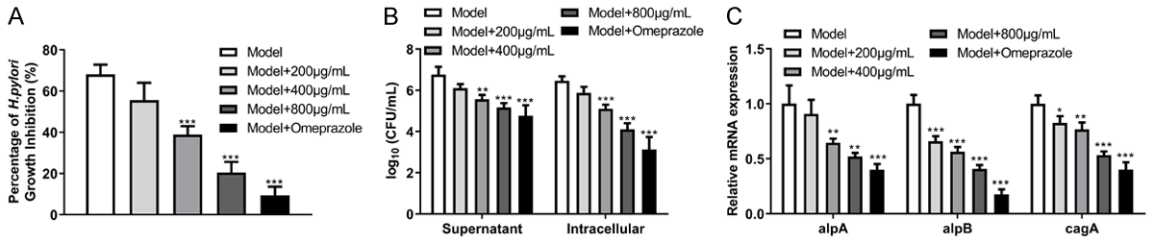

Figure 5. PCA inhibits the adhesion and infection of *H. pylori* to GES-1 cells *in vitro*. A: PCA inhibits the growth of *H. pylori in vitro* (6 duplications/group); B: Counting of the bacteria in cell culture supernatant and the in GES-1 cells after infected by *H. pylori* (6 duplications/group); C: The effect of PCA treatment on the expression levels of the adhesion gene alpA, alpB and the virulence gene cagA of *H. pylori* was evaluated by RT-qPCR (6 duplications/group). Compared with the model group, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. PCA: Protocatechuic Acid.

PCA inhibited H. pylori adhesion and infection to GECs

PCA was proved to inhibit *H. pylori* infection *in vivo*, but the mechanism is still unclear. To this end, we first evaluated the inhibition ability of PCA on the growth of *H. pylori* cultured *in vitro*. After adding different concentrations of PCA into the *H. pylori* solution, the growth of *H. pylori* was inhibited to varying degrees. The inhibition rate of 800 μg/mL PCA on *H. pylori* was around 20.5%, and the inhibition rate of omeprazole on *H. pylori* was around 9.4% (**Figure 5A**). After *H. pylori* infected GES-1 cells in an MOI of 100, the number of bacteria in cell culture supernatant and the intracellular bacteria decreased gradually with the increase of PCA concentration (**Figure 5B**). *H. pylori* can be colonized in the gastric mucosa by secreting a variety of adhesion factors, causing gastric mucosa injury and abnormal gastric acid secretion through secreting virulence factors. Therefore, we studied the influence of PCA on the expression of the adhesion and virulence genes of *H. pylori*. As shown in **Figure 5C**, 400 and 800 μg/mL PCA significantly inhibited the expression levels of adhesion genes alpA, alpB and virulence gene cagA of *H. pylori*.

PCA regulated GECs through the Smad, VEGFA and TNF signaling pathways

We further investigated the possible mechanism by which PCA promoted the proliferation and migration of GECs. Firstly, the CTD database was used to screen out the target sites of PCA in GU (**Figure 6A**), and the protein-protein interaction network analysis on the screened target pathway proteins was conducted with the aid of String database (**Figure 6B**). Next, we detected target pathway protein genes in GES-1 cells after PCA treatment. The expression level of the VEGFA gene in GES-1 cells increased significantly, while the expressions of TNF, smad7, smad3, smad2 and smad4 genes decreased significantly (**Figure 6C**). Compared with sh-NC group, the relative expression of VEGFA and TNF were decreased significantly when VEGFA and TNF were knocked down (**Figure 6D**). In addition, we analyzed the interaction relationship between target pathways by Western blot. Compared with the control group, expressions of Smad family proteins were all reduced when VEGFA was knocked down, while the expressions of Smad family proteins were up-regulated when TNF was knocked down (**Figure 6E**).

Discussion

GU is a kind of chronic ulcer from the cardia to the pylorus, mainly occuring in the pyloric region and is a common digestive system disease [24]. *H. pylori* is a common cause of GUs. At present, the drug resistance of *H. pylori* has gradually increased with the wide application of antibiotics, and traditional Chinese medicine has played an essential role in the treatment of *H. pylori* infection combined with GUs [25]. Chen et al. investigated the effects of BanxiaXiexin Decoction on *H. pylori*-related peptic ulcers and found that the decoction may exert therapeutic effects through the TGF-β/Smad signaling pathway [26]. Yang et al. used ChuyouYuyang granule to treat patients suffering from GUs and found that the damaged gastric mucosa was significantly repaired, and the secretion of pro-inflammatory factors IL-18 and TNF-α was decreased [27]. Although increasing studies have confirmed the significance of tra-

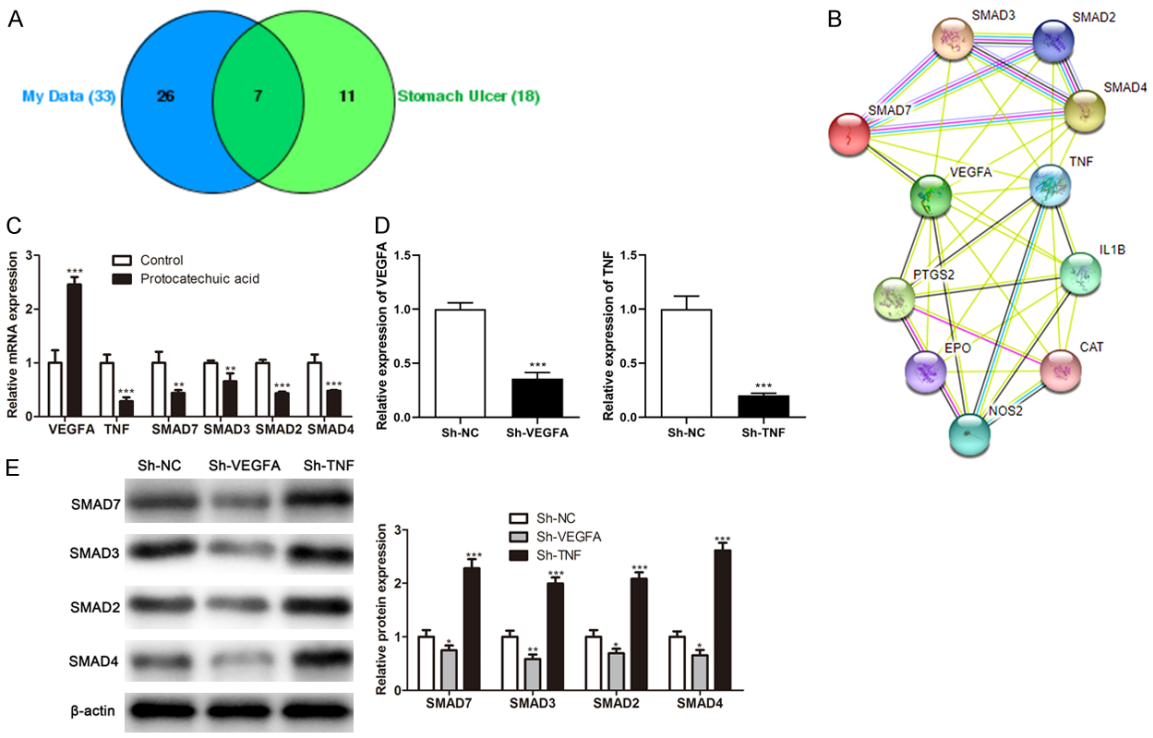

Figure 6. Signaling pathways in which PCA may act in GES-1 cells. A: The CTD database predicted the targets of PCA in GUs; B: The String software was used to predict the protein-protein interaction network of target pathway proteins; C: RT-qPCR detected the expression of target genes after PCA treatment. Compared with the control group (6 duplications/group), **P<0.01, ***P<0.001; D: The relative expressions of VEGFA and TNF were analyzed by ELISA. Compared with the sh-NC group (6 duplications/group), *P<0.05, **P<0.01, ***P<0.001; E: The interaction between target pathways was detected via Western blot. Compared with the sh-NC group (6 duplications/group), *P<0.05, **P<0.01, ***P<0.001. CTD: Comparative Toxicogenomics database; PCA: Protocatechuic Acid; GU: Gastric Ulcer.

ditional Chinese medicine in the treatment of GUs, the critical issue is which components play the role and through which mechanisms of GUs are treated. Therefore, we used the TCMSP database to predict and screen out PCA as the effective monomers of Wuqi Powder.

PCA is a water-soluble phenolic acid and an effective active ingredient in many traditional Chinese medicines. Studies have shown that PCA has various biological activities, such as antioxidant, anti-cancer, anti-inflammatory and antibacterial [28]. Salami et al. demonstrated that PCA had an excellent cytoprotective effect on GUs induced by potassium bromate [29]. Liu et al. verified the anti-*H. pylori* activity of PCA *in vitro* [30]. Our study found that PCA alleviated GUs induced by AA and *H. pylori*.

PCA could inhibit the growth of *H. pylori*, inhibit the secretion of adhesion factors AlpA, AlpB and virulence factor CagA, thus inhibiting the infection of *H. pylori* to the gastric mucosa and reducing the levels of inflammatory cytokines IL-6 and TNF-α in rats. In addition, AlpA and AlpB are encoded by highly homologous genes alpA and alpB. Therefore, AlpA and AlpB can bind to the laminin of the host to mediate the adhesion of *H. pylori* to gastric mucosa [31]. We observed the decreased adhesion and infection of *H. pylori* to GES-1 cells after PCA treatment, suggesting that PCA could inhibit the adhesion and infection of *H. pylori* to GES-1 cells. Furthermore, the RT-qPCR results showed that the mRNA levels of adhesion molecules AlpA, AlpB and virulence factor CagA were down-regulated after PCA treatment, suggesting that PCA could suppress the colonization of *H. pylori* in gastric mucosa by inhibiting the expression of AlpA and AlpB and inhibiting the injection of toxic protein CagA into GECs.

Besides, this research showed that PCA promoted the proliferation and migration of GECs by inhibiting the Smad signaling pathway, thus accelerating the repair of the gastric mucosa.

Based on the CTD database, we predicted that VEGFA, TNF, Smad7, Smad3, Smad2 and Smad4 were the targets of PCA in the treatment of GUs. PCA increased the expression of the VEGFA gene in GES-1 cell, while expressions of TNF, Smad7, Smad3, Smad2 and Smad4 genes were down-regulated. VEGF is the most potent known angiogenesis promoter, which can specifically act on endothelial cells through tyrosine kinase receptors, promote the proliferation of endothelial cells, and promote angiogenesis [32]. The high expression of VEGFA is beneficial to the repair of epithelial tissue and the generation of new blood vessels in ulcer site, thereby accelerating the healing of GUs [33]. The TNF pathway is related to the inflammatory response, which challenges the healing of GUs and induces relapse [34]. The down-regulation of TNF indicates that PCA can inhibit the inflammatory response. Smads are crucial intracellular downstream molecules in TGF-β1 signal transduction, which transmits TGF-β1 signals from membrane receptors to nucleus and then regulates the transcription of target genes. Smad2, 3, 4 and 7 are involved in the TGF-β1 signaling pathway [35]. TGF-β1, as a vital tissue fibrosis factor, can activate human fibroblasts to transform into myofibroblasts, leading to smooth muscle hyperplasia. Studies have shown that TGF-β1 has a certain promoting effect on the occurrence of GUs.

Moreover, Smads family proteins promote the above effects of TGF-β1. It was known that TGF-β1 binds to TβRII and activates TβRI, leading to the phosphorylation of Smad2 and 3. Then the activated Smad2 and 3 form complexes with Smad4 translocated into the nucleus and regulate the expression of target genes. Smad7 is a suppressor protein in the Smads family, which TGF-β1 induces. Under normal circumstances, Smad7 can prevent the excessive activation of the TGF-β1 signaling pathway by competing binding TβRI, preventing Smad2/3 phosphorylation, and recruiting E3 ligase Smurf1/2, thus playing a negative feedback role [36, 37]. The activation of the Smad pathway promotes the epithelial-mesenchymal transition of GECs, while the inhibition of the pathway promotes cell proliferation [38].

Although our research confirmed that the active ingredient PCA in Wuqi Powder could inhibit *H. pylori* infection and promote the repair of gastric mucosa, there are still some limitations in this study that need to be improved. Firstly, in the screening of the effective ingredients in Wuqi Powder, only the common effective ingredients of Schisandra Chinensis and Bletilla striata were included, which makes the study of Wuqi Powder incomplete. In addition, this study confirmed the inhibition of PCA on the growth of *H. pylori in vitro* while it lacked the determination of the minimum inhibitory concentration of PCA on the standard and clinical strains of *H. pylori*. Moreover, advanced studies are needed to explore the effect of PCA on the motor capacity of *H. pylori*, such as the formation of flagella and the expression of motion-related genes flaA and flaB.

Conclusion

This article screened out the active ingredient PCA in Wuqi Powder and confirmed its excellent alleviating effect on GUs induced by AA and *H. pylori* in rats. PCA inhibited the growth of *H. pylori in vivo* and *in vitro* and reduced inflammatory factors IL-6 and TNF-α in rats. Furthermore, PCA inhibited the infection and adhesion of *H. pylori* to GECs by inhibiting the level of adhesion genes alpA, alpB and toxicity gene cagA. In addition, PCA may accelerate the proliferation and migration of GECs through inhibiting the Smad and TNF signaling pathways and activating the VEGFA signaling pathway, thus accelerating the repair of the gastric mucosa.

Disclosure of conflict of interest

None.

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