



Research Article

Evaluation of the gastroprotective effects of 20 (S)-ginsenoside Rg3 on gastric ulcer models in mice



Kai Zhang^{1,2}, Ying Liu², Cuizhu Wang¹, Jiannan Li², Lingxin Xiong¹, Zhenzhou Wang¹, Jinping Liu^{1,**}, Pingya Li^{1,*}

¹ Research Center of Natural Drug, School of Pharmaceutical Sciences, Jilin University, Changchun, China

² Department of General Surgery, The Second Hospital of Jilin University, Changchun, Jilin, China

ARTICLE INFO

Article history:

Received 7 October 2017

Received in Revised form

15 January 2018

Accepted 3 April 2018

Available online 9 April 2018

Keywords:

Antiinflammation

Antioxidant

20 (S)-ginsenoside Rg3

Gastric ulcer

Ulcer index

ABSTRACT

Background: Gastric ulcer (GU) is a common gastrointestinal disease that can be induced by many factors. Finding an effective treatment method that contains fewer side effects is important. 20 (S)-ginsenoside Rg3 is a kind of protopanaxadiol and has shown superior antiinflammatory and antioxidant effects in many studies, especially cancer studies. In this study, we examined the treatment efficacy of 20 (S)-ginsenoside Rg3 on GU.

Methods: Three kinds of GU models, including an alcohol GU model, a pylorus-ligated GU model, and an acetic acid GU model, were used. Mouse endothelin-1 (ET-1) and nitric oxide (NO) levels in blood and epidermal growth factor (EGF), superoxide dismutase, and NO levels in gastric mucosa were evaluated. Hematoxylin and eosin staining of gastric mucosa and immunohistochemical staining of ET-1, inducible nitric oxide synthase (NOS2), and epidermal growth factor receptors were studied. Ulcer index (UI) scores and UI ratios were also analyzed to demonstrate the GU conditions in different groups. Furthermore, Glide XP from Schrödinger was used for molecular docking to clarify the interactions between 20 (S)-ginsenoside Rg3 and EGF and NOS2.

Results: 20 (S)-ginsenoside Rg3 significantly decreased the UI scores and UI ratios in all the three GU models, and it demonstrated antiulcer effects by decreasing the ET-1 and NOS2 levels and increasing the NO, superoxide dismutase, EGF, and epidermal growth factor receptor levels. In addition, high-dose 20 (S)-ginsenoside Rg3 showed satisfactory gastric mucosa protection effects.

Conclusion: 20 (S)-ginsenoside Rg3 can inhibit the formation of GU and may be a potential therapeutic agent for GU.

© 2018 The Korean Society of Ginseng, Published by Elsevier Korea LLC. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

Gastric ulcer (GU) is one of the major gastrointestinal diseases that occur on the surface of the gastric mucosa. It is a kind of local tissue necrosis because of the formation of ulceration lesions. In recent years, the incidence of gastric ulcer increases because of many external damaging factors, such as smoking, excessive drinking, stress, poor diet, and long-term ingestion of nonsteroidal antiinflammation drugs (NSAIDs) [1]. Generally, the imbalance of the damage to the gastric mucosa caused by exogenous factors and the mucosal self-repair effect lead to the incidence of GU.

Gastric mucosa can be damaged by excessive intake of alcohol. In detail, alcohol can lead to direct injury of mucosa vascular endothelial cells, disrupt the cells continuity, induce the formation of reactive oxygen radicals and inflammatory cytokines, and cause local ischemia of the gastric mucosa [2]. In addition, most of the NSAIDs, such as aspirin and diclofenac sodium, are very common factors in GU formation. The NSAIDs are mainly maintained in a nonionic state in the gastric fluid and can easily pass through the gastric mucosa cell membrane and accumulate within cells. High concentrations of NSAIDs can change the permeability of the cell membrane, which may lead to edema, degeneration, necrosis, and shedding of gastric mucosal epithelial cells. As a result, the barrier

* Corresponding author. School of Pharmaceutical Sciences, Jilin University, Fujin Road 1266, Changchun 130021, China.

** Corresponding author. School of Pharmaceutical Sciences, Jilin University, Fujin Road 1266, Changchun 130021, China.

E-mail addresses: liujp@jlu.edu.cn (J. Liu), lipy@jlu.edu.cn (P. Li).

effect of the gastric mucosa is damaged, and GU forms [3]. Furthermore, poor dieting and many kinds of stress can also lead to gastric hyperacidity and local blood supply disorders of the gastric tissue, which can easily cause GU formation. Therefore, drugs that possess the ability to reduce the amounts of inflammatory and oxidative stress and protect the gastric mucosa from local ischemia injury can be used in the treatment of GU.

Currently, the most common drugs used in GU treatment are proton pump inhibitors and H₂ receptor antagonists. However, the associated undesirable side effects and the recurrence of GU after treatment have attracted increasing attention [4]. Thus, it is still necessary and important to search for an ideal antiulcer drug that possesses fewer side effects and can protect the gastric mucosa well. *Panax notoginseng*, also known as Tianqi or Sanqi in Chinese, is a traditional Chinese medicine that has been used for a long time because of its multiple pharmacological effects [5]. It has been reported that Yunnan Baiyao, in which the main ingredient is *P. notoginseng*, was efficient in treating uterine hemorrhaging, ulcerative colitis, and skin ulcers [6]. The bioactive components of *P. notoginseng*, including many kinds of protopanaxadiol-type saponins, are believed to be ginsenosides [7]. Previous studies have shown that 20 (S)-ginsenoside Rg3, a deglycosylated derivative of the ginsenoside Rb3, exerts obvious antiinflammation, antiischemia, and antioxidative stress effects [8–11]. Based on these findings, we aimed to explore the anti-GU effects of ginsenoside Rg3 in mice. In this study, three GU models, alcohol, pylorus-ligated, and acetic acid, were used to systematically study the GU treatment efficacy of 20 (S)-ginsenoside Rg3.

2. Materials and methods

2.1. Materials

20 (S)-ginsenoside Rg3 (C42H72O13, HPLC > 98%) was purchased from Jilin Yatai Pharmaceutical Co., Ltd. (Jilin, China). Cimetidine was bought from Sigma (St. Louis, MO, USA) and used as the model control in this study. Mouse endothelin-1 (ET-1), nitric oxide (NO), epidermal growth factor (EGF), and superoxide dismutase (SOD) enzyme-linked immunosorbent assay (ELISA) kits were purchased from Longton Co. Ltd. (Shanghai, P. R. China). The antibodies of ET-1, inducible nitric oxide synthase (NOS2), and epidermal growth factor receptor (EGFR) for immunohistochemical staining were bought from Abcam (Cambridge, USA).

2.2. Animals

The animal experiments were conducted based on the guide for the administration of laboratory animals (Directive 86/609/EEC in the Protection of Animals Used for Experimental and Other Scientific Purposes, 1986) and were approved by the Institutional Animal Care and Use Committee of Jilin University (No. SCXK-2013-0001). Male Wistar rats, weighing 180–220 g, were obtained from the Laboratory Animal Center of Jilin University. For each GU model, the animals were divided into six groups: the blank control group, model control group, cimetidine group, low-dose Rg3 group (L-Rg3), moderate-dose Rg3 group (M-Rg3), and high-dose Rg3 group (H-Rg3). Ten animals were included in each group. For rats in the blank control and model control groups, intragastric administration of 10 mL/kg/day 0.9% saline solution was performed. For rats in the cimetidine group, intragastric administration of 2 mg/kg/day cimetidine was performed. For rats in the L-Rg3, M-Rg3, and H-Rg3 groups, intragastric administrations of 5 mg/kg/day, 10 mg/kg/day, and 20 mg/kg/day 20 (S)-ginsenoside Rg3 were performed, respectively.

2.3. Establishment of GU

For the alcohol GU model, intragastric administration was performed for 7 days for each group. Half an hour after the last intragastric administration, the rats were orally treated with 5 mL/kg alcohol (Beijing Chemical Works, Beijing, P.R. China), except for the animals in the blank control group. One hour later, the rats were killed by cervical dislocation, and the gastric tissue was harvested for further evaluation.

For the pylorus-ligated GU model, intragastric administration of different samples was also first performed for 7 days. The rats were fasted for 24 hrs after the last intragastric administration. Then, they were anesthetized with a pentobarbital sodium (50 mg/kg) (J&K Technology Co., Ltd., Beijing, P.R. China) intraperitoneal injection, were fixed, and underwent laparotomy. The stomach pylori was exposed and ligated by surgical sutures, followed by the abdomen suturing layer by layer. Eighteen hours later, the rats were also killed, and their stomachs were collected.

For the acetic acid GU model, rats were first fasted for 24 hrs and then were anesthetized, were fixed, and underwent laparotomy. The stomach was exposed, and 0.3 mL of acetic acid (J&K Technology Co., LTD) was submucosally injected at the junction of the stomach body and pyloric sinus. The stomach was embedded in the large omentum, and the abdomen was sutured. The animals were then treated with intragastric administration of different samples for 7 days. One hour after the last treatment, the rats were killed, and the stomach was harvested, as described previously.

2.4. Body weight measurement

To evaluate the toxicity of Rg3, the body weights of rats in each animal model were measured. For animals in all the three GU models, their body weights were recorded before each intragastric administration for 7 days.

2.5. Measurement of ET-1 and NO in blood

In each animal model, blood samples were collected from the heart before sacrifice, and the serum was obtained by centrifugation at 4,000 g for 10 min and then stored at –80°C. Levels of ET-1 and NO were analyzed using ELISA kits.

2.6. Gross evaluation of stomach mucosa

As mentioned previously, after the sacrifice of the animals, stomach tissues were collected and washed clean with a 0.9% saline solution. The stomach was opened along the greater curvature, and the stomach mucosa underwent gross evaluation for any signs of hyperemia, hemorrhage, and ulcers. Ulcer index (UI) scoring was performed according to a previous study [12]. Scoring details are as follows: 0 = normal stomach; 0.5–1 = mucosa congestion; 1–2 = hemorrhage; 2–3 = one to five small ulcers; 3–4 = many small ulcers; 4–5 = one to five small and one to three large ulcers; 5–6 = many small and large ulcers; 6–7 = full of ulcers. UI ratios were also performed to analyze the antiulcer efficiency of Rg3. The UI of the control group was defined as “1”, and the UI ratio was defined as the ratios of the ulcer indexes of treated samples and the model control group. In addition, the percentage of inhibition was also calculated as follows: $[(\text{UI model control group} - \text{UI treated group}) / \text{UI model control group}] \times 100\%$.

2.7. Measurement of EGF, SOD, and NO in the gastric mucosa

After gross evaluation of the GUs, the gastric mucosa was collected and homogenized. Then, the supernatant was obtained by

centrifugation at 2,500 g for 10 min, and ELISA kits were used to analyze the levels of EGF, SOD, and NO in the gastric mucosa.

2.8. Histological and immunohistochemical analyses

The stomach ulcer tissues were rinsed with phosphate buffered saline, fixed in 4% (W/V) phosphate buffered saline–buffered paraformaldehyde, and finally embedded in paraffin. The tissues were serially sectioned at 5.0- μ m intervals and stained with hematoxylin and eosin (H&E). ET-1, NOS2, and EGFR immunohistochemical staining were also performed to analyze the GU treatment efficacy of different samples. The positive stained cells were recorded, and the percentage occupying the total counted cells was calculated.

2.9. Molecular docking of 20 (S)-ginsenoside Rg3

To clarify the mode of action of 20 (S)-ginsenoside Rg3 on EGF and NOS2, a molecular docking study was carried out to measure the relative binding energies and localized binding sites in the active pocket. The study was performed using GLIDE (Grid-based Ligand Docking with Energetics) (GLIDE, version 6.7, Schrödinger, LLC, New York, USA, 2015) software developed by Schrödinger. Maestro Elements (2015-2) was used for all the steps involving protein and ligand preparation, receptor grid generation, and docking. The X-ray crystal structure of EGF [Protein Data Bank (PDB) code: 3RCD] and NOS2 (PDB code: 3EAI) were retrieved from the PDB database (<http://www.rcsb.org/pdb>) according to previous studies [13–15].

The Protein Preparation Wizard in the GLIDE software was used to prepare the receptors. The structures of EGF and NOS2 were optimized after a series of processes, including assigning bond orders and water orientations, removing water, adding hydrogen, and creating zero-order bonds to metals and disulphide bonds [16].

Crystal coordinates of 20 (S)-ginsenoside Rg3 (ligand) were pre-drawn in Maestro Elements (Maestro Elements, 2.2) before this molecular docking study. Three-dimensional structure of the compound was generated using LigPrep module (2015-2) of Schrödinger Suite by assigning the bond orders and angles. In addition, the ligand was subjected to minimization using the OPLS3 force field. For GLIDE docking, the prepared structure of EGF, NOS2, and ligand (Rg3) were imported to the workspace using GLIDE v6.7 from Schrödinger Suite [17–19]. Extra precision (XP) docking was carried out, and the parameters of scaling factor and partial charge cutoff were set at the default values 0.80 and 0.15, respectively [20]. Figures of the docking results were subsequently prepared using PyMOL (Schrödinger).

2.10. Statistical analyses

The results were presented as the mean \pm standard deviation. Data were analyzed using GraphPad Prism 7.0 software (GraphPad Inc., San Diego, CA) using Student *t* test. Statistical significance was set as **p* < 0.05, and high statistical significance was set as ***p* < 0.01 and ****p* < 0.001.

3. Results

3.1. Body weight change

For animals in alcohol and pylorus-ligated GU models, the body weights gradually increased, and there was no statistical difference among all the groups (Figs. 1A, 1B). For animals in the acetic acid GU model, the body weights of rats in the model control group severely decreased. But the body weights of the animals in the other groups slightly decreased during the first 3 or 4 days and then gradually increased (Fig. 1C). At the 7th day after treatment, there was

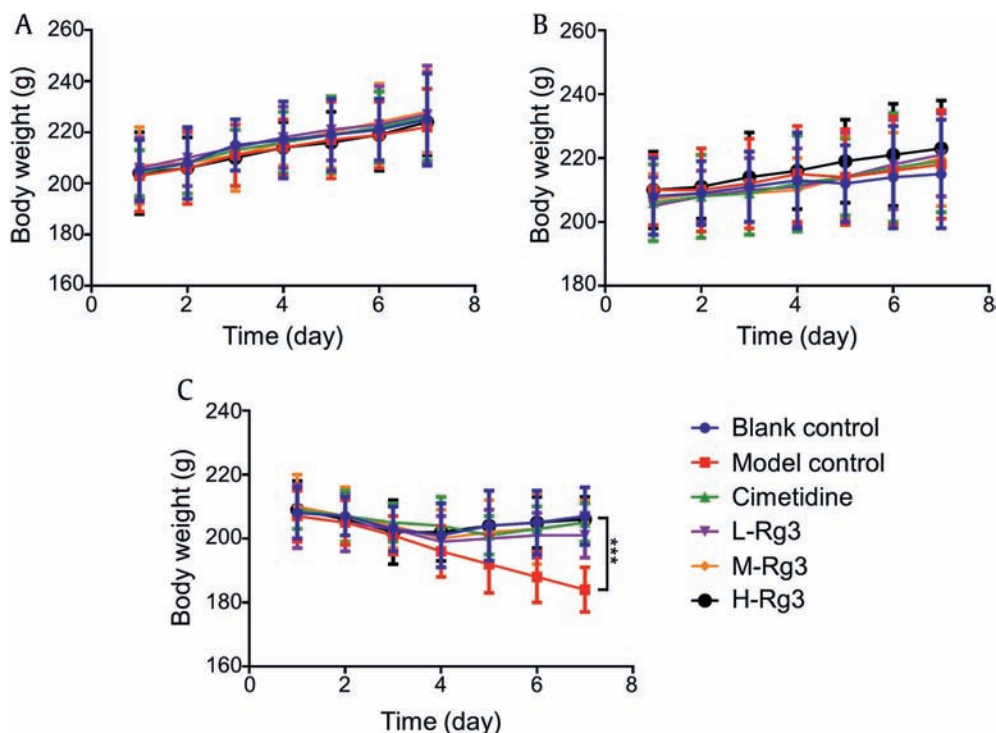


Fig. 1. Body weights of animals in (A) alcohol, (B) pylorus-ligated, (C) and acetic acid GU models. Data are presented as the mean \pm SD (*n* = 10; compared with the model control group, **p* < 0.05, ***p* < 0.01, ****p* < 0.001).

GU, gastric ulcer; H-Rg3, high-dose Rg3 group; L-Rg3, low-dose Rg3 group; M-Rg3, moderate-dose Rg3 group; SD, standard deviation.

significant difference between the model control group and H-Rg3 group in animal body weights ($p < 0.001$).

3.2. ET-1, NO, EGF, and SOD levels

To assess the GU inhibition efficacy of 20 (S)-ginsenoside Rg3, ET-1 and NO levels in the blood and EGF, SOD, and NO levels in the gastric mucosa were evaluated in the three GU models.

Fig. 2 shows ET-1, NO, EGF, and SOD levels in the alcohol GU model. The results indicated that the levels of ET-1 decreased as the amounts of 20 (S)-ginsenoside Rg3 increased, and the blood ET-1 levels decreased greatly in the H-Rg3 group compared with those of the model control group ($p < 0.001$). 20 (S)-ginsenoside Rg3 also changed the blood NO levels significantly. However, when treated

with low-dose 20 (S)-ginsenoside Rg3, the amounts of NO did not increase compared with those of the model control group. However, as for the moderate- and high-dose 20 (S)-ginsenoside Rg3-treated groups, the blood NO levels increased in comparison with those of the model control group ($p < 0.05$). Similarly, only H-Rg3 group increased the mucosa NO levels compared with the model control group ($p < 0.05$). Interestingly, cimetidine did not change the EGF levels in the mucosa tissues in this animal model, and EGF levels only increased in the H-Rg3 groups compared with the model control group ($p < 0.05$). It was obvious that 20 (S)-ginsenoside Rg3 was efficient in changing SOD levels in the gastric mucosa tissues. SOD levels increased significantly in all 20 (S)-ginsenoside Rg3-treated groups in comparison with those of the model control group.

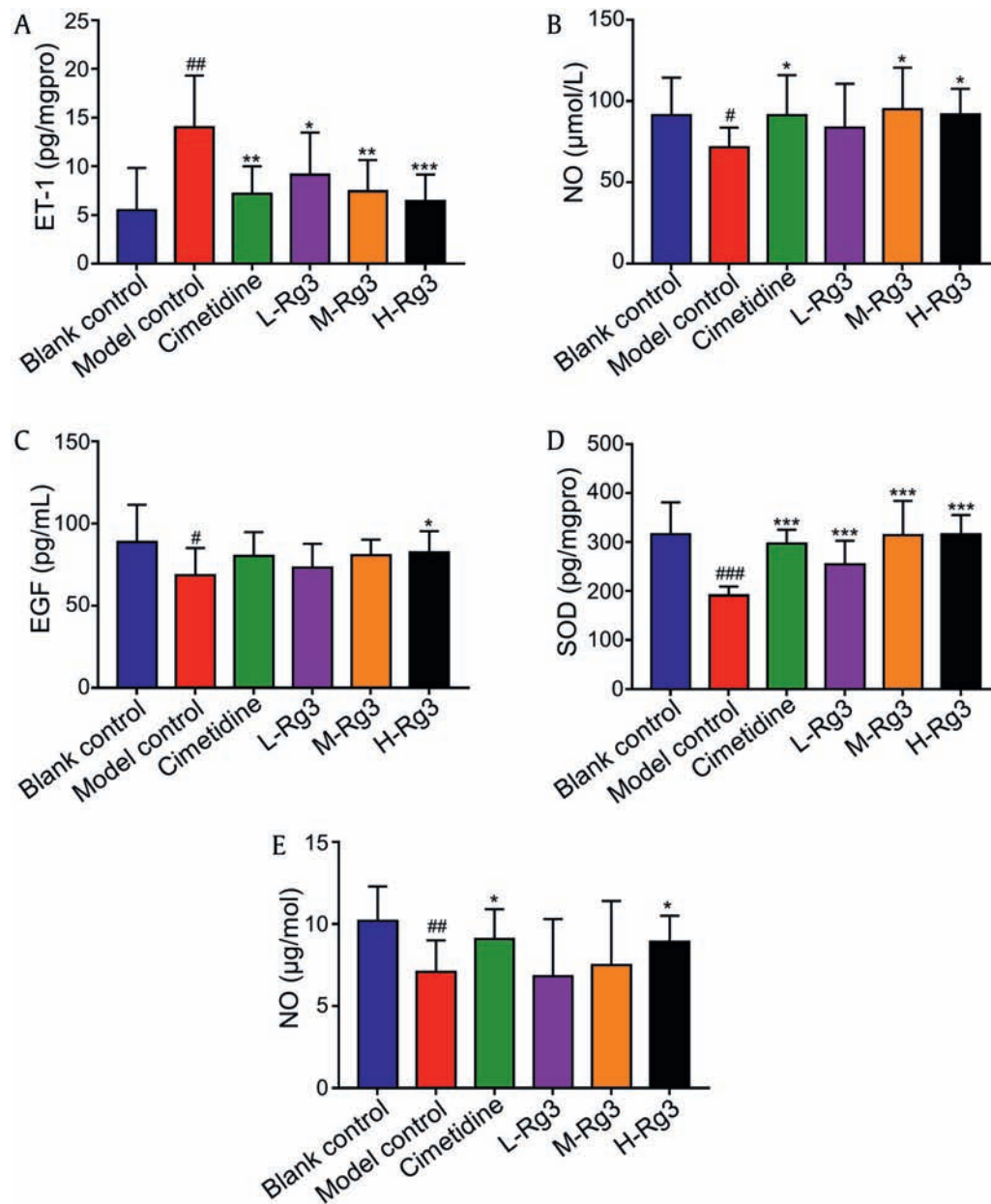


Fig. 2. (A) ET-1 and (B) NO levels in blood and (C) EGF, (D) SOD, and (E) NO levels in the gastric mucosa in the alcohol GU model. Data are presented as the mean \pm SD ($n = 10$; compared with the blank control group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$; compared with the model control group, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

EGF, epidermal growth factor; ET-1, endothelin-1; GU, gastric ulcer; H-Rg3, high-dose Rg3 group; L-Rg3, low-dose Rg3 group; M-Rg3, moderate-dose Rg3 group; NO, nitric oxide; SD, standard deviation; SOD, superoxide dismutase.

Fig. 3 shows ET-1, NO, EGF, and SOD levels in the pylorus-ligated GU model. Low and moderate doses of 20 (S)-ginsenoside Rg3 had no effect on ET-1 and NO values in the blood. However, blood ET-1 levels decreased and NO levels increased significantly in the H-Rg3 group compared with those of the model control group ($p < 0.05$). Cimetidine also had no effect on EGF levels in the gastric mucosa tissues, whereas high-dose 20 (S)-ginsenoside Rg3-treated rats showed increased EGF levels compared with the 0.9% saline solution-treated animals ($p < 0.05$). The SOD levels increased in the M-Rg3 group ($p < 0.05$) and H-Rg3 group ($p < 0.01$) compared with those of the model control group. The mucosa NO levels increased significantly compared with those of the model control group ($p < 0.05$).

Fig. 4 shows ET-1, NO, EGF, and SOD levels in the acetic acid GU model. Blood ET-1 levels decreased in all 20 (S)-ginsenoside Rg3-

treated groups, whereas both blood and gastric mucosa NO levels only increased in the H-Rg3 group compared with the model control group. Moderate and high doses of 20 (S)-ginsenoside Rg3 increased the EGF levels greatly in gastric mucosa tissues compared with the 0.9% saline solutions. SOD levels increased in all 20 (S)-ginsenoside Rg3-treated groups compared with those in the model control group.

3.3. Antiulcer efficacy of 20 (S)-ginsenoside Rg3

Two observers who were blind to the identity of the samples evaluated the UI scores of the gastric mucosa. The blank control group, which only received 0.9% saline solution, appeared to have no gastric lesions. As shown in Fig. 5, all the 20 (S)-ginsenoside Rg3-treated groups showed less UI scores than the model control

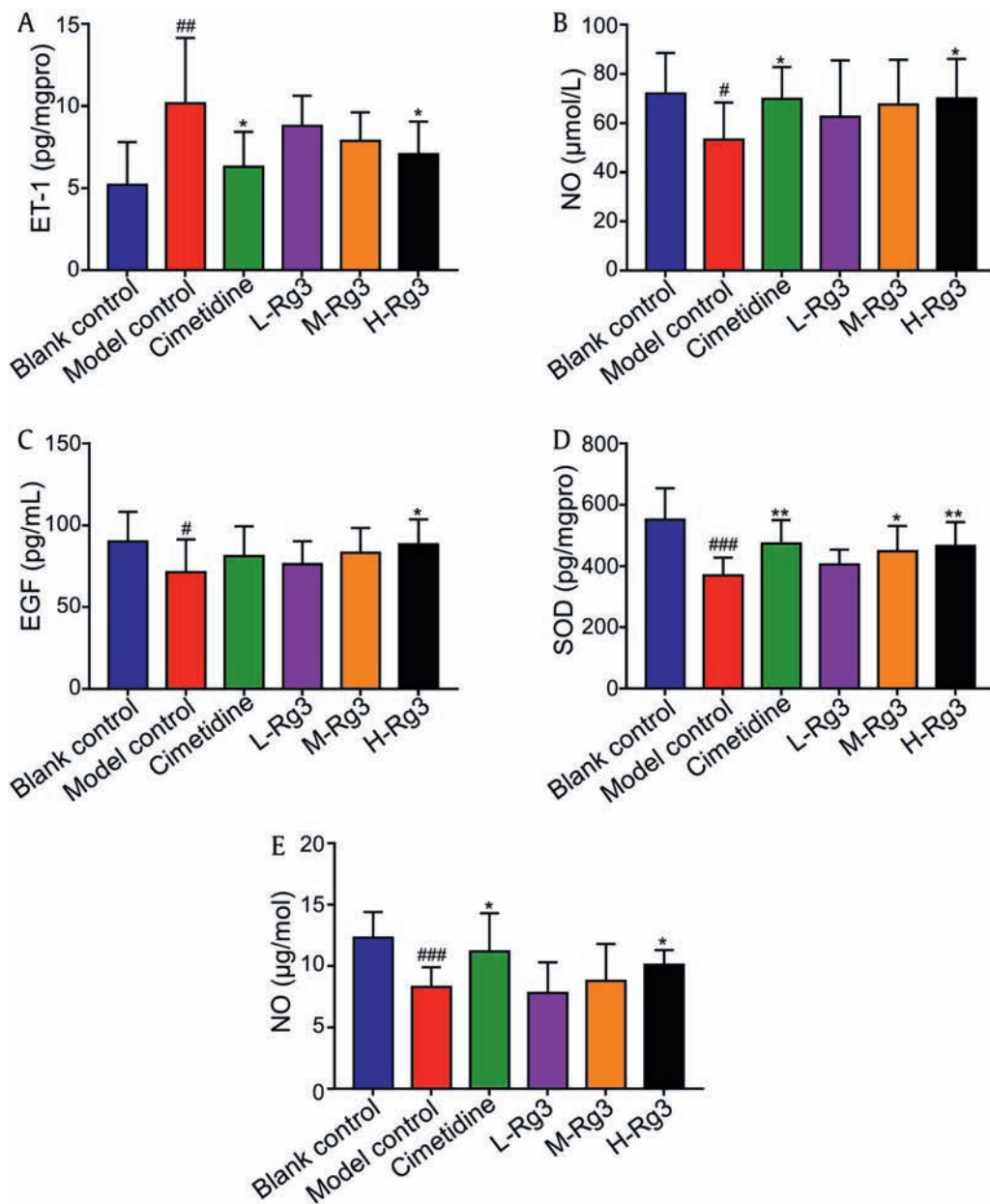


Fig. 3. (A) ET-1 and (B) NO levels in blood and (C) EGF, (D) SOD, and (E) NO levels in the gastric mucosa in the pylorus-ligated GU model. Data are presented as the mean \pm SD ($n = 10$; compared with the blank control group, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$; compared with the model control group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). EGF, epidermal growth factor; ET-1, endothelin-1; GU, gastric ulcer; H-Rg3, high-dose Rg3 group; L-Rg3, low-dose Rg3 group; M-Rg3, moderate-dose Rg3 group; NO, nitric oxide; SD, standard deviation; SOD, superoxide dismutase.

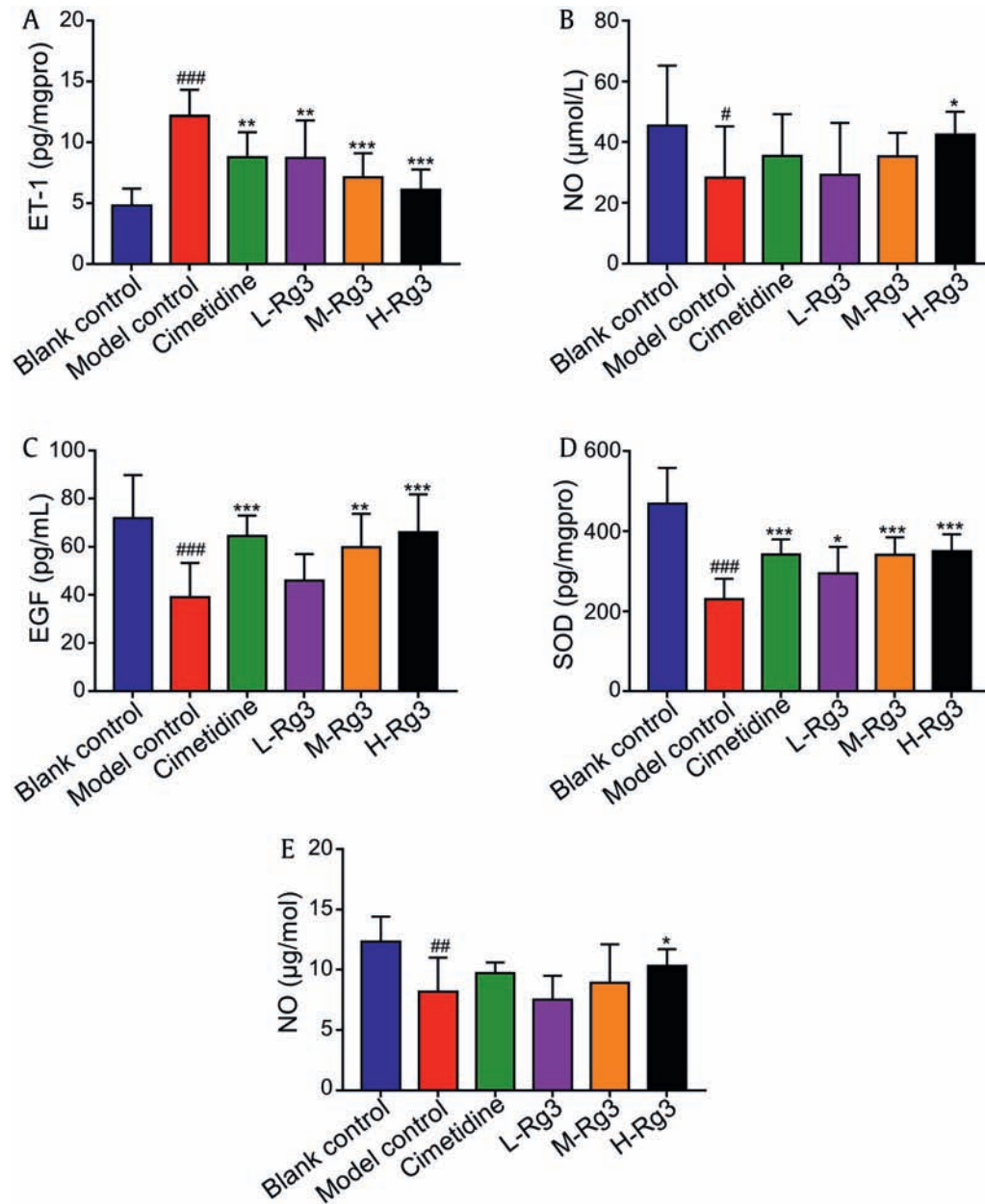


Fig. 4. (A) ET-1 and (B) NO levels in blood and (C) EGF, (D) SOD, and (E) NO levels in the gastric mucosa in the acetic acid GU model. Data are presented as the mean \pm SD ($n = 10$; compared with the blank control group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; compared with the model control group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). EGF, epidermal growth factor; ET-1, endothelin-1; GU, gastric ulcer; H-Rg3, high-dose Rg3 group; L-Rg3, low-dose Rg3 group; M-Rg3, moderate-dose Rg3 group; NO, nitric oxide; SD, standard deviation; SOD, superoxide dismutase.

group in these three GU models. The M-Rg3 and H-Rg3 groups greatly decreased the UI scores when compared with the model control group ($p < 0.001$). The result of the UI ratios was similar to that of UI scores. However, there were no differences in UI ratios between L-Rg3 and model control groups in the alcohol GU model and acetic acid GU model. In addition, the inhibition rates (%) of GU in the cimetidine, L-Rg3, M-Rg3, and H-Rg3 groups were 35.1%, 16.2%, 44.1%, and 63.1% in the alcohol GU model; 52.8%, 26.4%, 48.8%, and 64.8% in the pylorus-ligated GU model; and 38.2%, 8.8%, 42.2%, and 62.7% in the acetic acid GU model, respectively. The GU was inhibited better with increases in the administration of 20 (S)-ginsenoside Rg3. High-dose 20 (S)-ginsenoside Rg3 showed better GU prevention efficacy.

3.4. Effects of 20 (S)-ginsenoside Rg3 on histopathological changes of the gastric mucosa

Histopathological alterations of the gastric mucosa of different groups in the alcohol GU model, pylorus-ligated GU model, and acetic acid GU model are shown in Fig. 6. Normal stomach tissues, including the mucosa, submucosa, muscular layer, and serosa, were obvious in the blank control group in all the three GU models. The animals in the model control group were only treated with 0.9% saline solution, and severe histopathological changes were observed in the gastric specimens. H&E staining of the stomach tissues of the model control group showed large ulcer-induced mucosa lesions, large amounts of inflammation responses, gastric

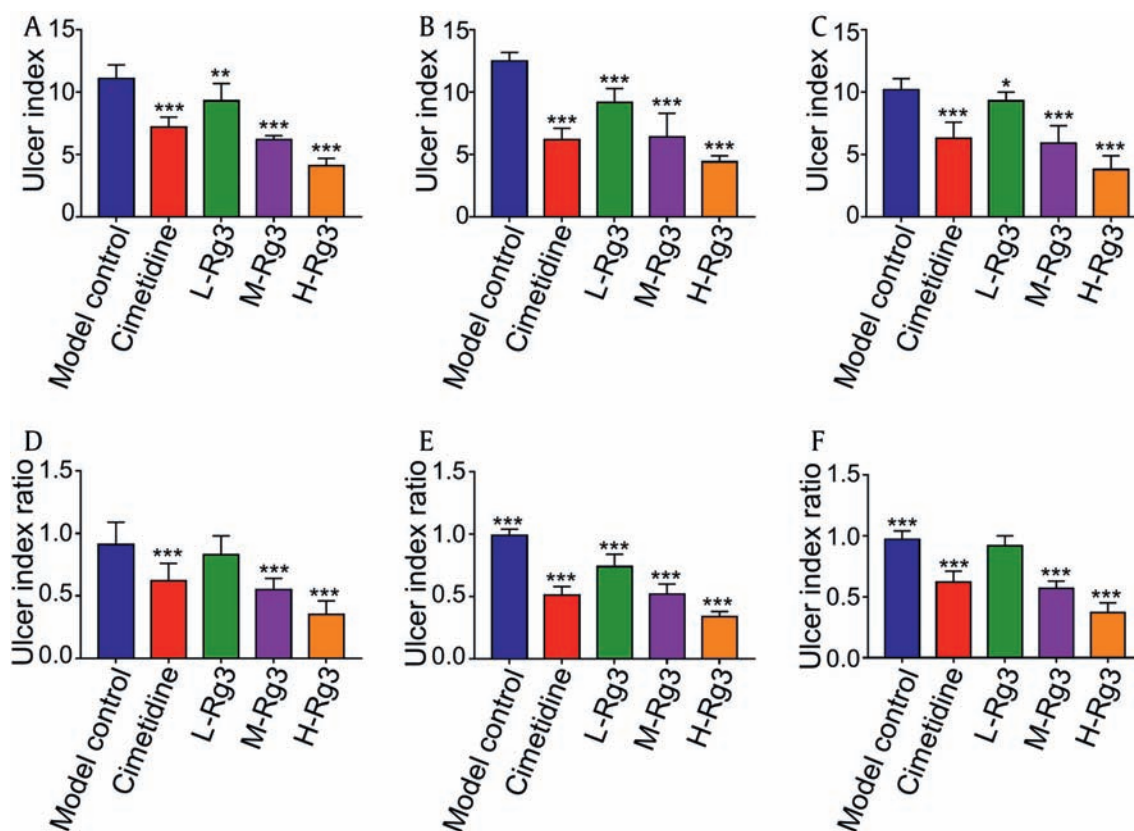


Fig. 5. Ulcer index scores and ulcer index ratios of (A and D) the alcohol GU model, (B and E) pylorus-ligated GU model, and (C and F) acetic acid GU model. Data are presented as the mean \pm SD ($n = 10$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

GU, gastric ulcer; H-Rg3, high-dose Rg3 group; L-Rg3, low-dose Rg3 group; M-Rg3, moderate-dose Rg3 group; SD, standard deviation.

pit cell damages, mucosa congestion and edema, and even muscular layer injuries. When the rats were treated with low-dose 20 (S)-ginsenoside Rg3, even though the mucosa injuries were not as obvious as those of the model control group, we could still observe small or large mucosal lesions, mucosal congestion and edema, and inflammation responses. Similar results were observed in the cimetidine and M-Rg3 groups in which only some small mucosa lesions with slight inflammation responses were found. High-dose 20 (S)-ginsenoside Rg3 improved these gastric alterations and only resulted in some very small mucosa lesions.

3.5. Effects of 20 (S)-ginsenoside Rg3 on immunohistochemical changes of ET-1, NOS2, and EGFR

ET-1 is known to be one of the most effective materials for the contraction of blood vessels, and it is essential for GU formation. Fig. 7A shows the immunohistochemical staining of ET-1 of different groups in the three GU models. The results indicated that compared with the blank control group, the model control group expressed greater amounts of ET-1 ($p < 0.001$) (Figs. 7B–7D). The cimetidine group showed less ET-1 levels than the model

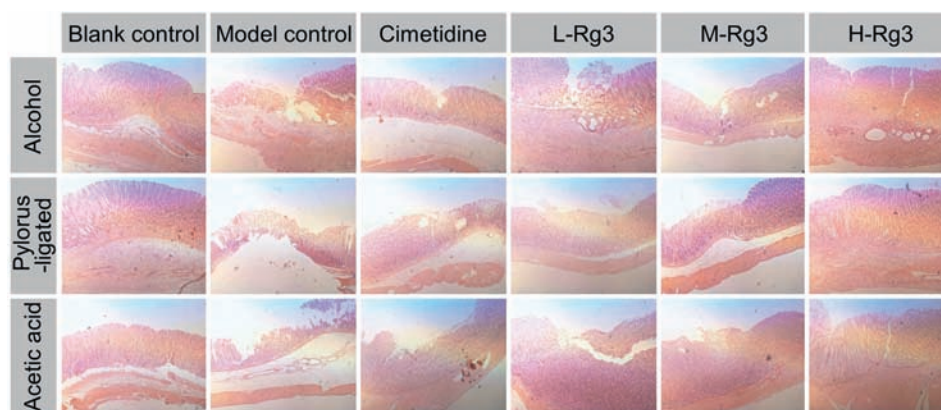


Fig. 6. H&E staining of the gastric mucosa of different groups in the alcohol GU model, pylorus-ligated GU model, and acetic acid GU model ($\times 40$). GU, gastric ulcer; H&E, hematoxylin and eosin; H-Rg3, high-dose Rg3 group; L-Rg3, low-dose Rg3 group; M-Rg3, moderate-dose Rg3 group.

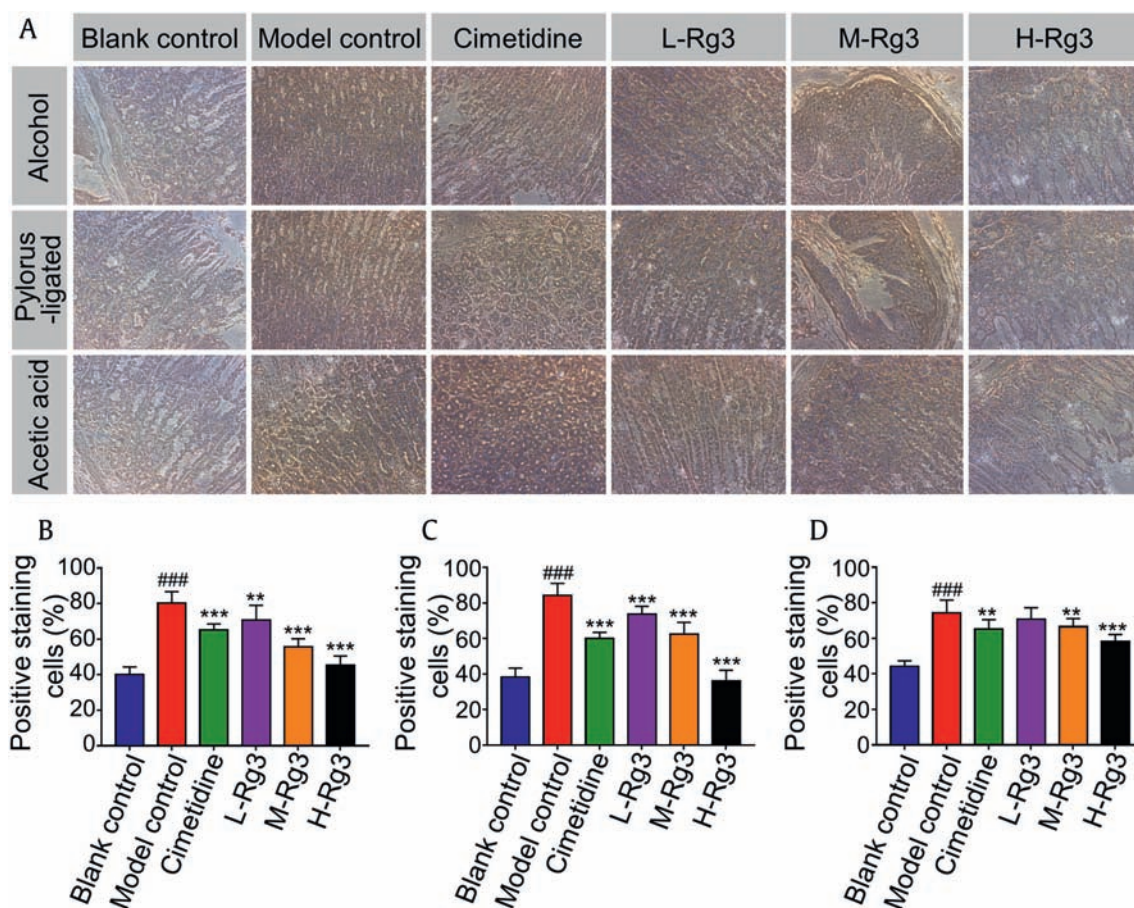


Fig. 7. Effects of 20 (S)-ginsenoside Rg3 on ET-1 expressions. (A) Immunohistochemical analyses of ET-1 in different groups in the alcohol GU model, pylorus-ligated GU model, and acetic acid GU model ($\times 200$). Positive ET-1 staining cells of different groups in the (B) alcohol GU model, (C) pylorus-ligated GU model, and (D) acetic acid GU model. Data are presented as the mean \pm SD ($n = 10$; compared with the blank control group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; compared with the model control group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

ET-1, endothelin-1; GU, gastric ulcer; H-Rg3, high-dose Rg3 group; L-Rg3, low-dose Rg3 group; M-Rg3, moderate-dose Rg3 group; SD, standard deviation.

control group. Moderate and high doses of 20 (S)-ginsenoside Rg3 greatly decreased the ET-1 levels in the gastric mucosa. ET-1 also expressed less in the L-Rg3 group of the alcohol and pylorus-ligated GU models, but not in the acetic acid GU model.

The expression of NOS2 in the gastric mucosa in all the three GU models is shown in Fig. 8. The positive immunohistochemical staining of NOS2 cells were stained brown, and large amounts of brown cells were found in the model control and L-Rg3 groups, whereas there were fewer brown cells in the cimetidine, M-Rg3, and H-Rg3 groups (Fig. 8A). By calculating the positive NOS2 staining cells, we could find that animals treated with moderate and high doses of 20 (S)-ginsenoside Rg3 showed lower amounts of NOS2 expression than those of the model control group ($p < 0.001$). L-Rg3 did not have any effect on NOS2 in the pylorus-ligated GU model, whereas it had decreased expression in the other two GU models.

The EGFR expression was also analyzed in Fig. 9. Cells with positive EGFR expression were also stained brown (Fig. 9A). All the 20 (S)-ginsenoside Rg3-treated groups showed increased EGFR expression compared with that of the model control group (Figs. 9B–9D). With increasing amounts of 20 (S)-ginsenoside Rg3, the number of the positive EGFR staining cells increased.

3.6. Results of molecular docking

The relative binding energies and localized binding sites in the active pocket were measured in the molecular docking. As shown in

Fig. 10A, the first model provides the most probable binding sites and structural configurations of 20 (S)-ginsenoside Rg3 in EGF. Three hydrogen bonds were observed with 20 (S)-ginsenoside Rg3 at GLU-812, LEU-726, and SER-1007 residues in EGF. The most probable binding sites and structural configurations of the compound in NOS2 were observed in the second model (Fig. 10B), and the three hydrogen bonds at GLN-257, ARG-260 and GLU-371 were marked clearly.

4. Discussion

GU is a kind of inflammatory necrotizing lesion that occurs between the cardia and pylorus of the stomach. It is also a common clinical disease that can occur at any age. Patients with GU always experience chronic and rhythmic upper abdominal pain, stomach bleeding, stomach perforation, pyloric constriction, and other concurrent diseases [21,22]. However, researchers have not reached a conclusion on the exact pathogenesis and mechanism of GU. The levels of a variety of plasma inflammatory factors change during the development of GU, and this can reflect the disease conditions in some cases [23,24]. Oxygen-free radicals are important pathogenic factors associated with GU formation. When the gastric mucosa is damaged, large amounts of oxygen-free radicals will form, which will trigger the oxidation reaction, promote the inflammatory response, and increase the formation of GU [25,26].

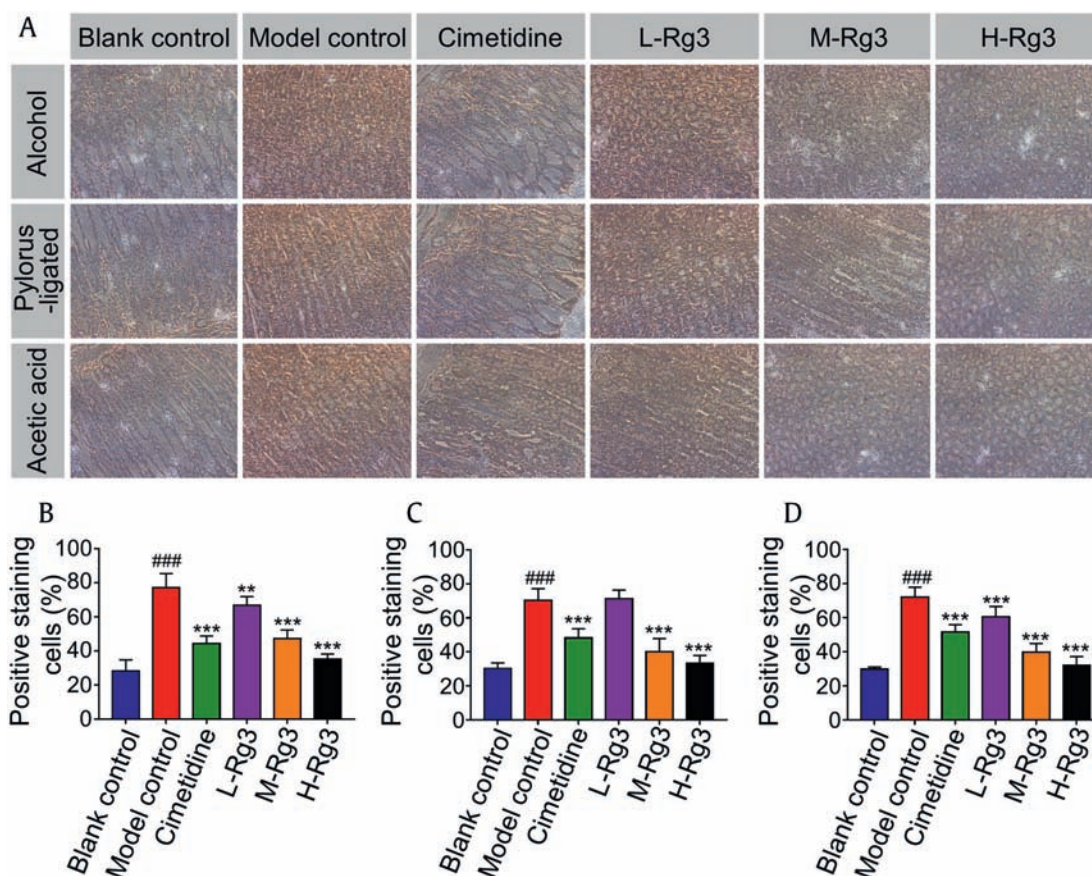


Fig. 8. Effects of 20 (S)-ginsenoside Rg3 on NOS2 expressions. (A) Immunohistochemical analyses of NOS2 in different groups in the alcohol GU model, pylorus-ligated GU model, and acetic acid GU model ($\times 200$). Positive NOS2 staining cells of different groups in the (B) alcohol GU model, (C) pylorus-ligated GU model, (D) and acetic acid GU model. Data are presented as the mean \pm SD ($n = 10$; compared with the blank control group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; compared with the model control group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

GU, gastric ulcer; H-Rg3, high-dose Rg3 group; L-Rg3, low-dose Rg3 group; M-Rg3, moderate-dose Rg3 group; NOS2, nitric oxide synthase; SD, standard deviation.

Ginsenoside Rg3, classified as protopanaxadiol 1, is a dammarane-type tetracyclic terpene sapogenin, which possesses effective antiinflammatory and antioxidant effects in many *in vitro* and *in vivo* studies. Kim et al investigated the treatment efficacy of 20 (S)-ginsenoside Rg3 on spinal cord injury [8]. They found that 20 (S)-ginsenoside Rg3 could suppress the proinflammatory cytokines of tumor necrosis factor- α , interleukin (IL)-6, and IL-1 β . In addition, 20 (S)-ginsenoside Rg3 also decreased the over-expression of cyclooxygenase-2 and NOS2. Cheng et al investigated 20 (S)-ginsenoside Rg3 for the treatment of hypertrophic scars and found that the therapeutic effects of 20 (S)-ginsenoside Rg3 on the inflammatory phase of wound healing and hypertrophic scar formation were pretty good [27]. In another study, the authors found that 20 (S)-ginsenoside Rg3 possessed significant inhibitory effects on nuclear factor kappa B and IL-1 β and showed an antiinflammatory effect on human asthmatic lung tissue [28]. Many other studies also reported the antiinflammatory and antioxidant effects of 20 (S)-ginsenoside Rg3 [9,29,30]. As a result, we would like to investigate the therapeutic effects of 20 (S)-ginsenoside Rg3 on GU formation in this study. Three GU models were used to better investigate the treatment efficacy of 20 (S)-ginsenoside Rg3 on GU.

There was no obvious toxicity of 20 (S)-ginsenoside Rg3 in this study, and it was safe to be used *in vivo*. Rg3 did not induce toxicity in rats because there was no significant difference in the body weights among all the groups in alcohol and pylorus-ligated GU models. As for the animals in the acetic acid GU model, the body

weights of rats seriously decreased in the model control group because of the laparotomy injury and GU formation. No GU formed in the blank control group, and the body weights of animals in this group decreased first and then increased. The body weights of animals in cimetidine, L-Rg3, M-Rg3, and H-Rg3 groups all decreased during the first 3 or 4 days and then increased. We thought that the laparotomy injury and GU formation resulted in the decrease of the body weights at the early stage. But cimetidine and Rg3 gradually played positive effects on GU healing which slightly increased the body weights at the late stage.

ET-1 is known to be the most effective blood vessel contractive material. In a stress state, the secretion of ET-1 and its active receptors in the gastric mucosa can be significantly increased, which will lead to severe contraction of the gastric mucosa vessels, thereby decreasing the blood supply of gastric tissues and causing local hypoxia and acidosis. In addition, local hypoxia and acidosis can also increase the expression of ET-1, which can further increase injury to the gastric mucosa and finally induce GU [31,32]. NO is a type of endogenous vasodilator that can inhibit the secretion of ET-1, expand the gastric mucosa vessels, inhibit the aggregation of platelets, change the vascular permeability, and regulate the secretion of gastric acid [33]. In our study, the expression of NO in the blood and gastric mucosa was increased in the H-Rg3 group in all the three GU models, but low and moderate doses of 20 (S)-ginsenoside Rg3 did not show an obvious effect on NO expression. However, even the low-dose 20 (S)-ginsenoside Rg3 can decrease the ET-1 levels in serum, except

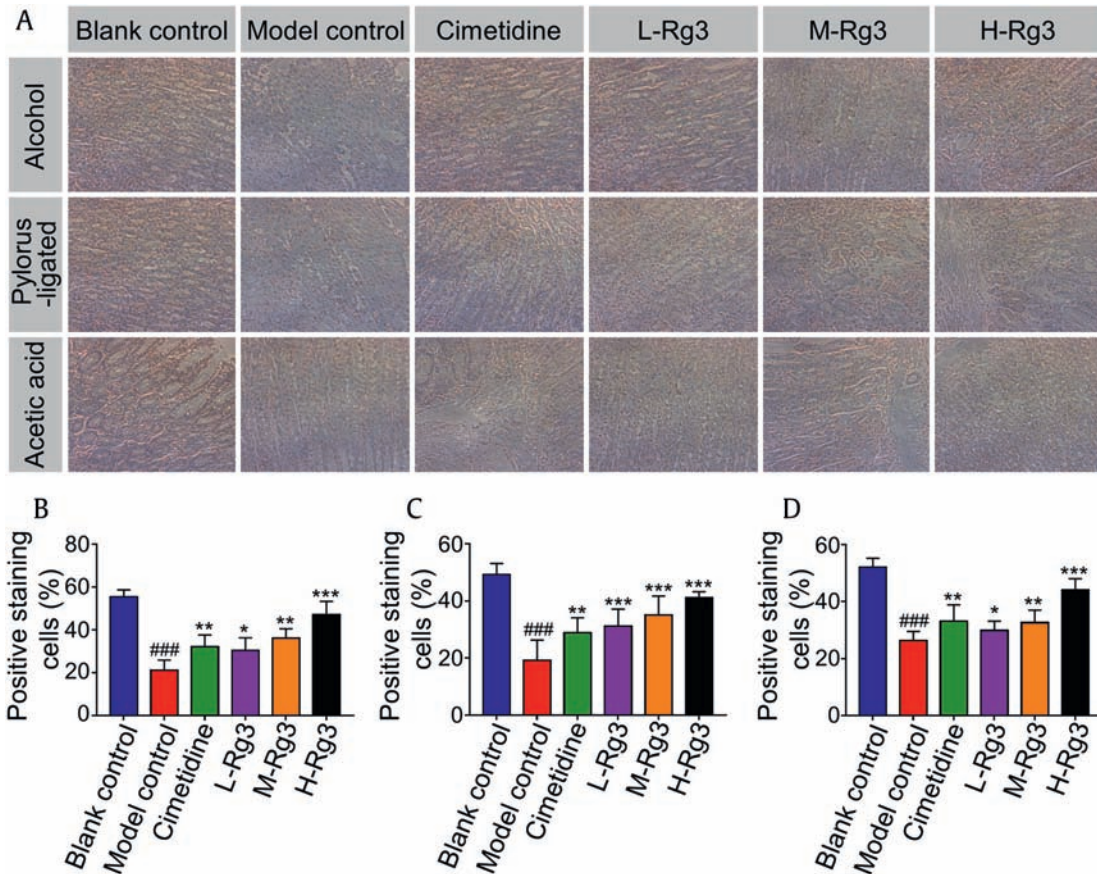


Fig. 9. Effects of 20 (S)-ginsenoside Rg3 on EGFR expressions. (A) Immunohistochemical analyses of EGFR in different groups in the alcohol GU model, pylorus-ligated GU model, and acetic acid GU model ($\times 200$). Positive NOS2 staining cells of different groups in the (B) alcohol GU model, (C) pylorus-ligated GU model, and (D) acetic acid GU model. Data are presented as the mean \pm SD ($n = 10$; compared with the blank control group, * $p < 0.05$, ### $p < 0.01$, *** $p < 0.001$; compared with the model control group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

EGFR, epidermal growth factor receptor; GU, gastric ulcer; H-Rg3, high-dose Rg3 group; L-Rg3, low-dose Rg3 group; M-Rg3, moderate-dose Rg3 group; SD, standard deviation.

in the pylorus-ligated model in which only a high dose of 20 (S)-ginsenoside Rg3 suppressed the expression of ET-1. The immunohistochemical analyses also indicated that there were ET-1 inhibition effects of 20 (S)-ginsenoside Rg3 on the gastric mucosa. These results demonstrated that 20 (S)-ginsenoside Rg3, especially used in high doses, can increase the expression of NO and decrease the ET-1 levels in GU models.

Even though NO can inhibit the effects of ET-1 and regulate the blood supply of the gastric mucosa, too much expression of NO can interact with oxygen-free radicals, leading to peroxidation damage of the cells, thus causing injuries to the gastric mucosa. Infections, endotoxins, and cytokines can stimulate the expression of NOS2, which is a kind of precursor of NO. The increased NOS2 can lead to large amounts of NO secretion and induce severe

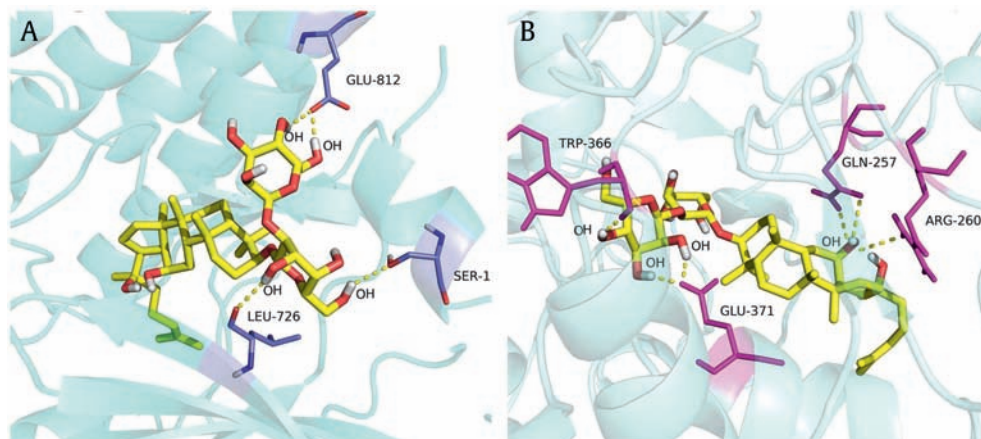


Fig. 10. Docking of (A) 3RCD and (B) 3EAI in 20 (S)-ginsenoside Rg3.

damage to many kinds of tissues [8]. In our study, NOS2 levels in the gastric mucosa were decreased nearly with all doses of 20 (S)-ginsenoside Rg3 in all the three GU models, and even the low-dose 20 (S)-ginsenoside Rg3-treated group showed less NOS2 expression in the alcohol and acetic acid GU models. The molecular docking study also indicated that 20 (S)-ginsenoside Rg3 might regulate the expression of NOS2 to treat GU, and this was consistent with the results of the animal study. In our opinion, the decreased NOS2 levels ensure inhibition of the overexpression of NO. When the animals were in GU conditions, the blood and mucosa NO levels were slightly increased in the H-Rg3 group compared with those of the model control group. However, there was no significant difference in NO levels between the blank control group and H-Rg3 group ($p < 0.05$). This finding indicated that 20 (S)-ginsenoside Rg3 can slightly upregulate the NO levels and protect the gastric mucosa while decreasing the NOS2 levels and ensuring the prevention of the overexpression of NO, which will lead to tissue damage.

As mentioned previously, infections and tissue injuries can increase the expression of oxygen-free radicals, which will lead to even more severe tissue damage. SOD is the main antioxidant material to inhibit oxygen-free radical damage *in vivo*. Our study indicated that 20 (S)-ginsenoside Rg3 increased the SOD levels significantly in all the three GU models. This can further demonstrate that 20 (S)-ginsenoside Rg3 can decrease the contents of oxygen-free radicals, reduce the lipid peroxidation reaction, protect the gastric mucosa, and promote ulcer healing.

EGF is a kind of gastrointestinal nutrient peptide, which is also a kind of antiulcer factor. EGF can inhibit the secretion of gastric acid, increase the blood supply to gastric mucosa, and promote epithelial proliferation and tissue repair. In this study, we investigated the EGF levels in blood and EGFR levels in the gastric mucosa. We found that 20 (S)-ginsenoside Rg3, especially high-dose 20 (S)-ginsenoside Rg3, can significantly increase the EGF levels in blood. This was also consistent with the results of molecular study which demonstrated that 20 (S)-ginsenoside Rg3 might inhibit GU by modulating the expression of EGF. However, the EGFR levels in the gastric mucosa were upregulated in all 20 (S)-ginsenoside Rg3-treated groups.

Based on these mechanisms, the treatment efficacy of 20 (S)-ginsenoside Rg3 on GU was evaluated by UI scores and H&E staining of the gastric mucosa. UI scores and UI ratios showed that moderate and high doses of 20 (S)-ginsenoside Rg3 possessed a satisfactory GU inhibition effect. However, H&E staining further proved the gastric mucosa protection effect of 20 (S)-ginsenoside Rg3.

5. Conclusions

In conclusion, this study demonstrated that 20 (S)-ginsenoside Rg3 effectively inhibited GU formation and protected the gastric mucosa by decreasing NOS2 levels, slightly increasing the NO expression, inhibiting ET-1 levels, promoting SOD expression, and stimulating EGF and EGFR expressions. As a result, this study suggests that 20 (S)-ginsenoside Rg3 can be a candidate for the treatment of GU.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Acknowledgment

This work was supported by Talents Team Major Program of Jilin Province of China (JRCBTZ. [2016] No. 3).

References

- [1] Shen Y, Sun J, Niu C, Yu D, Chen Z, Cong W, Geng F. Mechanistic evaluation of gastroprotective effects of Kangfuxin on ethanol-induced gastric ulcer in mice. *Chemico-biolog Interact* 2017;273:115–24.
- [2] Marotta F, Tajiri H, Safran P, Fesce E, Ideo G. Ethanol-related gastric mucosal damage: evidence of a free radical-mediated mechanism and beneficial effect of oral supplementation with bionormalizer, a novel natural antioxidant. *Digestion* 1999;60(6):538–43.
- [3] Goswami SK, Rand AA, Wan D, Yang J, Inceoglu B, Thomas M, Morisseau C, Yang G-Y, Hammock BD. Ethanol-related gastric mucosal damage: evidence of a free radical-mediated mechanism and beneficial effect of oral supplementation with bionormalizer, a novel natural antioxidant. *Digestion* 1999;60(6):538–43.
- [4] Kangwan N, Park J-M, Kim E-H, Hahm KB. Quality of healing of gastric ulcers: natural products beyond acid suppression. *World J Gastrointest Pathophysiol* 2014;5(1):40–7.
- [5] Lee CH, Kim J-H. A review on the medicinal potentials of ginseng and ginsenosides on cardiovascular diseases. *J Ginseng Res* 2014;38(3):161–6.
- [6] Chen QS. Pharmacological studies on notoginseng saponins isolated from the fibrous root of *Panax notoginseng*. *Zhong Yao Tong Bao (Beijing, China)* 1981;12(3):45–7.
- [7] Lee MR, Yun BS, Sung CK. Comparative study of white and steamed black *Panax ginseng*, *P. quinquefolium*, and *P. notoginseng* on cholinesterase inhibitory and antioxidative activity. *J Ginseng Res* 2012;36(1):93–101.
- [8] Kim D-K, Kweon K-J, Kim P, Kim H-J, Kim S-S, Sohn N-W, Maeng S, Shin J-W. Ginsenoside Rg3 improves recovery from spinal cord injury in rats via suppression of neuronal apoptosis. *Pro-inflamm Mediat Microglial Activ Mol* 2017;22(1).
- [9] Zhang L-P, Jiang Y-C, Yu X-F, Xu H-I, Li M, Zhao X-Z, Sui D-Y. Ginsenoside Rg3 improves cardiac function after myocardial ischemia/reperfusion via attenuating apoptosis and inflammation. *Evid Based Complement Altern Med* 2016.
- [10] He B, Chen P, Yang J, Yun Y, Zhang X, Yang R, Shen Z. Neuroprotective effect of 20(R)-ginsenoside Rg(3) against transient focal cerebral ischemia in rats. *Neurosci Lett* 2012;526(2):106–11.
- [11] Li G, Zhang X-X, Lin L, Liu X-N, Ma C-J, Li J, Wang C-B. Preparation of ginsenoside Rg3 and protection against H₂O₂-induced oxidative stress in human neuroblastoma SK-N-SH cells. *J Chem* 2014.
- [12] Singh S, Khajuria A, Taneja SC, Khajuria RK, Singh J, Johri RK, Qazi GN. The gastric ulcer protective effect of boswellic acids, a leukotriene inhibitor from *Boswellia serrata*, in rats. *Phytomedicine* 2008;15(6–7):408–15.
- [13] Xia G-Y, Yao T, Zhang B-Y, Li Y, Kang N, Cao S-J, Ding L-Q, Chen L-X, Qiu F. Withapubesides A-D: natural inducible nitric oxide synthase (iNOS) inhibitors from *Physalis pubescens*. *Organic Biomol Chem* 2017;15(47):10016–23.
- [14] Garcin ED, Arvai AS, Rosenfeld RJ, Kroeger MD, Crane BR, Andersson G, Andrews G, Hamley PJ, Mallinder PR, Nicholls DJ, et al. Anchored plasticity opens doors for selective inhibitor design in nitric oxide synthase. *Nat Chem Biol* 2008;4(11):700–7.
- [15] Ishikawa T, Seto M, Banno H, Kawakita Y, Oorui M, Taniguchi T, Ohta Y, Tamura T, Nakayama A, Miki H, et al. Design and synthesis of novel human epidermal growth factor receptor 2 (HER2)/epidermal growth factor receptor (EGFR) dual inhibitors bearing a Pyrrolo 3,2-d pyrimidine Scaffold. *J Med Chem* 2011;54(23):8030–50.
- [16] Zhang N-Q, Wang C-Z, Wang Z-Z, Li Z, Sai J-Y, Meng Y, Wang F, Li P-Y, Liu J-P. Anti-myocardial ischaemic effect of pseudoginsenoside F11 by inhibiting expression of beta1-adrenoceptor in rats with coronary artery ligation. *J Funct Foods* 2017;36:224–32.
- [17] Friesner RA, Banks JL, Murphy RB, Halgren TA, Klicic JJ, Mainz DT, Repasky MP, Knoll EH, Shelley M, Perry JK, et al. Glide: a new approach for rapid, accurate docking and scoring. 1. Method and assessment of docking accuracy. *J Med Chem* 2004;47(7):1739–49.
- [18] Friesner RA, Murphy RB, Repasky MP, Frye LL, Greenwood JR, Halgren TA, Sanschagrin PC, Mainz DT. Extra precision glide: docking and scoring incorporating a model of hydrophobic enclosure for protein-ligand complexes. *J Med Chem* 2006;49(21):6177–96.
- [19] Halgren TA, Murphy RB, Friesner RA, Beard HS, Frye LL, Pollard WT, Banks JL. Glide: a new approach for rapid, accurate docking and scoring. 2. Enrichment factors in database screening. *J Med Chem* 2004;47(7):1750–9.
- [20] Xiong L, Qi Z, Zheng B, Li Z, Wang F, Liu J, Li P. Inhibitory effect of triterpenoids from *panax ginseng* on coagulation factor X. *Molecules (Basel, Switzerland)* 2017;22(4).
- [21] Chang X, Luo F, Jiang W, Zhu L, Gao J, He H, Wei T, Gong S, Yan T. Protective activity of salidroside against ethanol-induced gastric ulcer via the MAPK/NF-kappa B pathway in vivo and in vitro. *Int Immunopharmacol* 2015;28(1):604–15.
- [22] Sakatoku Y, Fukaya M, Fujieda H, Kamei Y, Hirata A, Itatsu K, Nagino M. Tracheoesophageal fistula after total resection of gastric conduit for gastro-aortic fistula due to gastric ulcer. *Surg Case Rep* 2017;3.
- [23] Li S-L, Zhao J-R, Ren X-Y, Xie J-P, Ma Q-Z, Rong Q-H. Increased expression of matrix metalloproteinase-9 associated with gastric ulcer recurrence. *World J Gastroenterol* 2013;19(28):4590–5.
- [24] Li X, Wang L, Li G, Zheng X, Duan C. Expression of miR-204 and MMP-9 in *Helicobacter pylori*-associated gastric ulcer. *Int J Clin Exp Med* 2016;9(5):7928–36.

- [25] Biswas K, Bandyopadhyay U, Chattopadhyay I, Varadaraj A, Ali E, Banerjee RK. A novel antioxidant and antiapoptotic role of omeprazole to block gastric ulcer through scavenging of hydroxyl radical. *J Biolog Chem* 2003;278(13): 10993–1001.
- [26] Al Batran R, Al-Bayat F, Al-Obaidi MMJ, Abdulkader AM, Hadi HA, Ali HM, Abdulla MA. In vivo antioxidant and antiulcer activity of *Parkia speciosa* ethanolic leaf extract against ethanol-induced gastric ulcer in rats. *Plos One* 2013;8(5).
- [27] Cheng L, Sun X, Hu C, Jin R, Sun B, Shi Y, Cui W, Zhang Y. In vivo early intervention and the therapeutic effects of 20(S)-ginsenoside Rg3 on hypertrophic scar formation. *Plos One* 2014;9(12).
- [28] Lee I-S, Uh I, Kim K-S, Kim K-H, Park J, Kim Y, Jung J-H, Jung H-J, Jang H-J. Anti-inflammatory effects of ginsenoside Rg3 via NF-kappaB pathway in A549 cells and human asthmatic lung tissue. *J Immunol Res* 2016;2016. 7521601–7521601.
- [29] Kang H, Hwang Y-G, Lee T-G, Jin C-R, Cho CH, Jeong H-Y, Kim D-O. Use of gold nanoparticle fertilizer enhances the ginsenoside contents and anti-inflammatory effects of red ginseng. *J Microbiol Biotechnol* 2016;26(10):1668–74.
- [30] Park JY, Choi P, Kim T, Ko H, Kim H-K, Kang KS, Ham J. Protective effects of processed ginseng and its active ginsenosides on cisplatin-induced nephrotoxicity: in vitro and in vivo studies. *JAgric Food Chem* 2015;63(25):5964–9.
- [31] Du Y, Zhao W, Lu L, Zheng J, Hu X, Yu Z, Zhu L. Study on the antiulcer effects of *Veronicastrum axillare* on gastric ulcer in rats induced by ethanol based on tumor necrosis factor-alpha (TNF-alpha) and endothelin-1 (ET-1). *Asian Pacific J Trop Biomed* 2013;3(12):925–30.
- [32] Nishida T, Tsuji S, Kimura A, Tsujii M, Ishii S, Yoshio T, Shinzaki S, Egawa S, Irie T, Yasumaru M, et al. Endothelin-1, an ulcer inducer, promotes gastric ulcer healing via mobilizing gastric myofibroblasts and stimulates production of stroma-derived factors. *Am J Physiol Gastrointest Liver Physiol* 2006;290(5):G1041–50.
- [33] Iaquinto G, Giardullo N, Taccone W, Leandro G, Pasquale L, De Luca L, Szabo S. Role of endogenous endothelin-1 in ethanol-induced gastric mucosal damage in humans. *Dig Dis Sci* 2003;48(4):663–9.