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ORIGINAL RESEARCH

Clopidogrel-Induced Gastric Injury in Rats is Attenuated by Stable Gastric Pentadecapeptide BPC 157

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Aim: Although Clopidogrel is safe in healthy volunteers, it can induce recurrence of gastric ulcers in high-risk patients. Here, we investigated the protective effect of the natural product, stable gastric pentadecapeptide 157 (BPC 157) on Clopidogrel-induced gastric injury.

Methods: We used acetic acid to induce gastric ulcer in Sprague Dawley rats. Clopidogrel alone or in combination with BPC 157 or L-NAME (nitric oxide system blockade) were administered after healing of acetic acid-induced ulcer. One percent methylcellulose solution was used as control. Ulcer recurrence rate and the ulcer index were compared between these groups. Gastric mucosal apoptosis rate, microscopic inflammation activity and angiogenesis markers vascular endothelial growth factor A (*VEGF-A*) and *CD34* were examined by TUNEL, histological evaluations (HE) and immunohistochemistry (IHC). Pathways involved, expressions of endoplasmic reticulum (ER) stress apoptosis marker *CHOP*, angiogenic markers *VEGF-A* and its receptor *VEGFR1*, and endothelial NO synthase (*eNOS*) were all analyzed by Western blot.

Results: This study indicated that Clopidogrel significantly induced the gastric ulcers recurrence, severe inflammation and ER stress related apoptosis of the gastric mucosa, suppressed the synthesis of angiogenic markers and *eNOS*. Furthermore, Clopidrogel intervention resulted in the activation of protein kinase B (*AKT*) and p38 mitogen-activated protein kinase (*p38/MAPK*). BPC 157 attenuated the gastric mucosal damage caused by Clopidogrel and reversed these molecular effects. However, NO blockade L-NAME weakened the protective effect and thus the molecular effects of BPC 157 on gastric mucosa.

Conclusion: In conclusion, these results suggest that BPC 157 inhibited Clopidogrel-induced gastric mucosa injury partially by inhibition of gastric mucosa cell ER stress-mediated apoptosis and inflammation, and promoting gastric mucosa angiogenesis via *VEGF-A/VEGFR1* mediated-*AKT/p38/MAPK* signaling pathways.

Keywords: clopidogrel, BPC 157, angiogenesis, AKT, p38/MAPK

Introduction

Clopidogrel is an oral irreversible *P2Y12* receptor antagonist which inhibits platelet aggregation and is widely used by patients with acute coronary syndromes or after percutaneous coronary intervention.[1,2] Although Clopidogrel cannot cause damage to the gastric mucosa in healthy volunteers,[3] it can increase the bleeding rate in patients at high risk of ulcers[4,5] and induce recurrence of a healed ulcer during a 6-month follow-up period.[6] The mechanism of Clopidogrel on gastric mucosa injury is still unclear. Previous studies have confirmed that Ticlopidine, another *P2Y12* receptor

antagonist, delays the healing of rats' ulcers by inhibiting ADP-induced platelets aggregation.[7,8] In our previous studies, we found that Clopidogrel inhibits human gastric mucosal epithelial cell line GES-1 proliferation then further disrupt the tight junction structure (TJs) of gastric epithelium,[9] through activation of endoplasmic reticulum (ER) stress-induced apoptosis via *MKP-5*-mediated *p38/MAPK* phosphorylation.[10,11]

It is well known that the reconstruction of epithelial cells and submucosal connective tissues is the pathophysiological basis of gastric ulcer healing, involving cell proliferation and angiogenesis.[12,13] Angiogenesis stimulates wound healing by forming new blood vessels from pre-existing vessels, within a few days, these are organized into a microvascular network throughout the granulation tissue. Numerous papers have reported that stable gastric pentadecapeptide BPC 157 can regulate angiogenesis during the healing process by rescuing the duodenal lesions in rats through interacting with the nitric oxide (NO) system and reducing of free radical formation,[14] and also can resolve pringle maneuver[15] and hippocampal ischemic damage in rats due to ischemia or reperfusion.[16] Its strong angiogenic effect is mainly via activation of the *VEGF-AKT-eNOS* signaling pathway.[17] Furthermore, BPC 157 has also been demonstrated to promote vascular endothelial and tendon cell proliferation and migration.[18,19]

As an anti-ulcer peptidergic agent, BPC 157 is also effective both in the upper and lower gastrointestinal tract and now proven to be safe in inflammatory bowel disease (IBD) in clinical Phase II trials, with no toxicity reported.[20,21] For nonsteroidal anti-inflammatory drugs ($NSAID_S$) cytotoxicity such as gastrointestinal, liver and brain lesions, BPC 157 counteraction includes both *COX-1* and *COX-2* blockers.[22–26] It can even counteract prolongation of bleeding and thrombocytopenia caused by aspirin.[27,28] BPC157 can repair mucosal integrity and endothelial damage better than sucralfate.[29] Besides that, BPC 157 also has a stronger effect on promoting angiogenesis and granulation tissue regeneration than H2-blockers, omeprazole or even sucralfate.[30]

Sprague Dawley rats are the most widely used animal model for human gastrointestinal disorder research, and the physiology of their stomach has been thoroughly studied and is well known. This study aimed to investigate whether BPC 157 has a protective effect on gastric mucosal damage caused by Clopidogrel in Sprague Dawley rats and further elucidate its molecular mechanism.

Materials and Methods

Animals

Adult male Sprague Dawley rats (12-weeks old, 300 ± 10 g) were purchased from Animal Experimental Center, Zhejiang, China. The rats were fed in the standard SPF environment, housed at a temperature of 23°C and in a 12h light-dark cycle. All rats were given standard food and tap water. The Committee for the Use of Live Animals in Southeast University approved the use of animals in this study (No: 20,181,130,007). All animal housing and handling were conducted in compliance with the guidelines of Southeast University and the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

Drugs, Chemicals and Reagent Kits

Clopidogrel was purchased from MedChen Express (New Jersey, MCE, USA) and suspended in 1% methylcellulose. Synthesized BPC 157 and L-NAME were purchased from Modong Gene (Nanjing, Jiangsu, China) and Sigma-Aldrich (Sigma-Aldrich Biotechnology, St. Louis, MO, USA) respectively, both dissolved in saline. Most of the other chemicals and related drugs were obtained from Sigma-Aldrich unless needed to be declared otherwise.

Induction of Rats' Gastric Ulcers by Acetic Acid

The Sprague Dawley rats' gastric ulcers were induced by acetic acid by following the method of S. Okabe[31] with slight modifications. After the rats were anesthetized with pentobarbital (80 mg/kg i.p.), a laparotomy along the midline was performed and the stomach was fully exposed. Then, one end of the cylindrical plastic tube (10mm diameter) was adhered to the stomach serosal part, and 80% acetic acid was injected at the other end for 1 minute. After surgery, the rats were fasted for 12 h to reduce complications such as abdominal distension and perforation after eating. Then eight rats were sacrificed in every 5 days, and the size of ulcers was measured until the ulcers were completely healed (Figure S1).

Experimental Design

25 days after gastric ulcer induction, all ulcers were determined completely healed then the other rats were randomly divided into four groups each containing 10 rats. Clopidogrel 10 mg/kg intragastric (i.g.) once daily, BPC 157 10 ng/kg intraperitoneally (i.p.) once daily and L-NAME 5 mg/kg intraperitoneally (i.p.) once daily were

given individually or combined at the same time when the rats were under starvation at 8:00am. The administration details are as follows: control group (1% methylcellulose solution i.g. once daily); Clopidogrel group (Clopidogrel 10 mg/kg i.g. once daily);[8] Clopidogrel + BPC 157 group (Clopidogrel 10 mg/kg i.g. combined with BPC 157 10 ng/kg i.p.[25] once daily); Clopidogrel + BPC 157 + L-NAME group (Clopidogrel 10 mg/kg i.g., BPC 157 10 ng/kg i.p. and L-NAME 5 mg/kg i.p.[25] once daily). All rats were sacrificed by cervical dislocation after 30 days of drug administration. The gastric ulcer indexes were calculated according to the method described by Antonisamy et al[32] (Figure S2).

Histological Evaluations (HE) and Immunohistochemistry (IHC)

Gastric tissue samples fixed in paraformaldehyde were embedded in paraffin, then dyed with hematoxylin and eosin (H&E). Histological evaluation was performed under an Olympus BH−2 photomicroscope. Gastric injury was graded using a scoring system as previously described.[33] It was scored using a scale ranging from 0 to 3 (0: none, 1: mild, 2: moderate, and 3: severe) for each criterion. The total score was 12. In addition, IHC for *VEGF-A* (Proteintech, Chicago, IL, USA) and *CD34* (Proteintech, Chicago, IL, USA) were performed using the automated IHC system (Hoffman La-Roche Ltd., Basel, Switzerland). All scores were assessed using the semiquantitative H scoring approach by two experienced pathologists who were blinded for the assessment.

TUNEL Assay

Apoptosis of rats' gastric epithelial cells was evaluated using TUNEL apoptosis assay kit (Vazyme, Nanjing, Jiangsu, China) according to the manufacturers' instructions. The apoptotic cells showed intense dark nuclear staining. The proportion of the dark nuclear staining cells was evaluated by counting the cells stained with TUNEL divided by the total number of cells. One thousand nuclei were evaluated. Three independent experiments were performed blindly by two experienced pathologists.

Western Blotting

The gastric mucosal tissues of rats were lysed in lysis buffer. Total proteins of each sample were separated on a 10% SDS-PAGE gel and electrophoretically transferred onto a PVDF membrane (EMD Millipore, Billerica, MA, USA). Then the membranes were blocked with 5% skim milk for 2 hours. This step was followed by incubation with primary antibody overnight at 4°C. The primary antibodies used were anti-*VEGF-A* (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-*VEGFR1* (Cell Signaling Technology, Berkeley, CA, USA), anti-*pVEGFR1* (Cell Signaling Technology, Berkeley, CA, USA), anti-*AKT* (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-*pAKT* (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-*ERK* (Cell Signaling Technology, Berkeley, CA, USA), anti-*pERK* (Cell SignalingT echnology, Berkeley, CA, USA), anti-*p38* (Cell Signaling Technology, Berkeley, CA, USA), anti-*p-p38* (Cell Signaling Technology, Berkeley, CA, USA), anti-*eNOS* (Proteintech, Chicago, IL, USA), anti-*p-eNOS* (Proteintech, Chicago, IL, USA), anti-*CHOP* (Cell Signaling Technology, Berkeley, CA, USA) and anti-*β-actin* (Cell Signaling Technology, Berkeley, CA, USA). Then the membranes were incubated with secondary antibody at room temperature for 2 hours. The enhanced chemiluminescence (ECL) kit was used for visualization. The results were semi-quantified by the Gel and Graph Digitizing System (Silk Scientific, Orem, UT, USA). All experiments were repeated for three times.

Statistical Analysis

All statistical analyses were performed using SPSS 19.0 (IBM Corporation, Armonk, NY, USA) software. All data were presented as mean ± SD. The independent samples *t*-test was used in analyzing the difference of means between two groups. One-way analysis of variance was used for comparison among three or more groups. Statistical significance was determined at p value < 0.05.

Results

Healing of Acetic Acid-Induced Gastric Ulcers in Rats

Ng's Study has shown that 12% of patients who had a history of gastrointestinal bleeding developed bleeding again after taking Clopidogrel for 1 year, and the lesions during re-bleeding were the same as previous.[34] It suggest that Clopidogrel causes re-bleeding only in patients with underlying mucosal injury or scarring.[6,35] In order to comply with the clinical facts, in this study, we firstly used acetic acid to induce gastric ulcers, then Clopidogrel was introduced after the ulcers were completely healed. We found that after 25 days, the rat gastric mucosa healing rate reached up to 100% (Table 1). The

Table 1 Gastric Mucosal Healing After Gastric Ulcer Induced by Acetic Acid in Rats

Days	Healing Ratio	Ulcer Size (mm^2)
5	0/8	24.48 ± 1.02
10	0/8	18.48 ± 1.06***
15	5/8	7.62 ± 1.00###
20	7/8	3.32 ± 0.63ΔΔΔ
25	8/8	0.00 ± 0.00+++

Notes: ***Indicates $p < 0.001$ vs 5 days; ###Indicates $p < 0.001$ vs 10 days; ΔΔΔIndicates $p < 0.001$ vs 15 days; +++Indicates $p < 0.001$ vs 20 days.

average ulcer size became smaller gradually until healed completely after 25 days ($p < 0.001$).

BPC 157 Protects Gastric Mucosa from Clopidogrel-Induced Ulcer Recurrence

After healing of acetic acid-induced gastric ulcer in 25 days, Clopidogrel was re-used to induce recurrence of the gastric ulcer. Then the effect of BPC 157 on Clopidogrel-induced gastric mucosal damage was assessed. The results showed no visible lesions in control group. Compared to the control group, oral administration of Clopidogrel for 30 days caused gastric ulcer in 60% of rats, serious mucosal erosions and erythemas. However, Clopidogrel co-treatment with BPC 157 significantly reduced the gastric ulcer recurrence rate and ulcer index, and the formations of mucosal erosions and erythemas were also rare. Interestingly, the nitric oxide (NO) system blockade L-NAME can partially reverse this protective effect including reduction of gastric ulcer recurrence rate and ulcer indexes induced by BPC 157 (Table 2, Figure 1). These results clearly demonstrated the protective effects of BPC 157 in Clopidogrel-induced gastric mucosal damage, and the NO system blockade L-NAME could partially attenuate these protective effects of BPC 157 on gastric mucosa. It suggests that the NO system may be involved in the protective effect of BPC 157 on Clopidogrel-induced gastric mucosal injury.

Table 2 The Protective Effect of BPC 157 on Clopidogrel-Induced Gastric Ulcer Recurrence and Mucosal Damage

Group	Ulcer Recurrence Ratio	Ulcer Index
Control	0/10	0.00 ± 0.00
Clopidogrel	6/10	11.83 ± 1.17**
Clopidogrel + BPC 157	2/10	4.50 ± 0.71#
Clopidogrel + BPC 157 + L-NAME	5/10	10.20 ± 0.84Δ

Notes: **Indicates $p < 0.01$ vs Control group; #Indicates $p < 0.05$ vs Clopidogrel group; ΔIndicates $p < 0.05$ vs Clopidogrel + BPC 157 group.

BPC 157 Attenuates Clopidogrel-Induced Gastric Mucosa Cells Inflammation

As presented in Figure 2, the control group had normal architecture and integrity of gastric mucosa and no infiltration of the inflammatory cells in gastric glands (Figure 2A). In contrast, Clopidogrel treatment markedly induced gastric mucosal erosions, featuring severe hyperemia, epithelial cell loss, disturbed glandular structure, damaged crypts, irregular arrangement and inflammatory cells infiltration (Figure 2A). These histological changes were partially reversed by BPC 157 mainly manifested in the reduction in inflammatory cell infiltration, hyperemia and epithelial cell loss (Figure 2A). Not surprisingly, L-NAME could partially weaken the protective effect of BPC 157 on gastric mucosa (Figure 2A). The results of histological microscopic lesion score are shown in Figure 2B.

BPC 157 Attenuates Clopidogrel-Induced Gastric Mucosa Cells Apoptosis

In our previous study, we have confirmed that Clopidogrel can stimulate ER stress response then induce gastric mucosal epithelial cell apoptosis, and further disrupt gastric mucosal epithelial barrier.[10] TUNEL analysis was then used to determine if BPC 157 inhibit gastric mucosa injury via suppression of cell apoptosis. As shown in Figure 3, Clopidogrel induced the apoptosis of gastric mucosal cells compared with the control group (Figure 3A). BPC 157 significantly attenuated the Clopidogrel-induced apoptosis compared with the Clopidogrel-only group (Figure 3A). However, after administration of L-NAME the gastric mucosa apoptotic cells increased again (Figure 3A). The ratio of apoptotic cells in each group is shown in Figure 3B. This data is consistent with other reports showing that the NO system response acts as an anti-inflammatory agent through inhibition of apoptosis on cells.[36]

BPC 157 Promotes Angiogenesis Through NO Response Following Clopidogrel-Induced Gastric Ulcer

Our above results confirmed that BPC 157 has a protective effect on Clopidogrel-induced gastric mucosal damage, and the specific mechanism in particular related with the NO response. We all know that NO

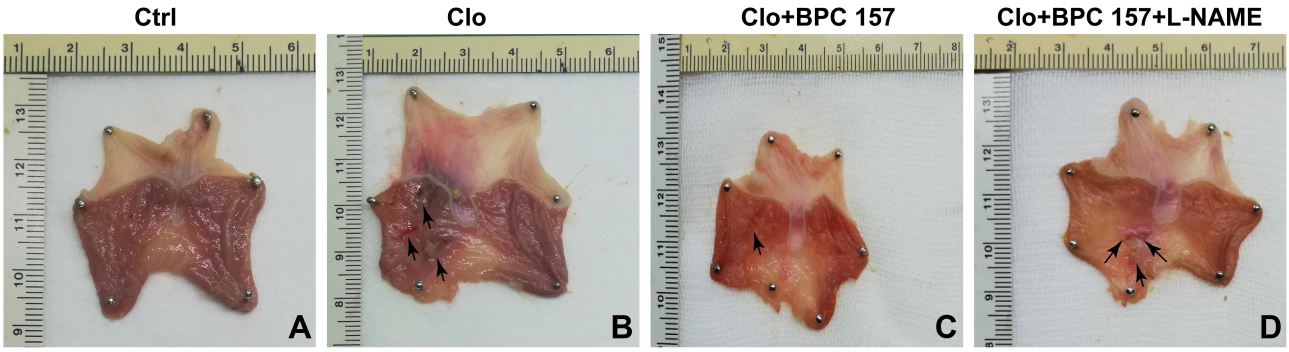

Figure 1 Representative gross morphologies of stomach at the time of necropsy. (**A**) Control group (1% methylcellulose solution) without visible lesions. (**B**) The Clopidogrel (10 mg/kg) group with severe lesions and deep ulcers. (**C**) Clopidogrel (10 mg/kg) plus BPC 157 (10 ng/kg) treatment group with very few lesions and superficial ulcers. (**D**) L-NAME (5 mg/kg) group with more lesions and ulcers compared with the Clopidogrel (10 mg/kg) plus BPC 157 (10 ng/kg) treatment group. Arrows: lesions and ulcers.

plays a crucial role in *VEGF-A*-dependent angiogenesis[37] and the angiogenesis is necessary and most important for ulcer repair. So, next, we performed the immunohistochemical examination of *rats*' gastric mucosa for the angiogenesis markers *VEGF-A* and *CD34* (Figure 4). In Clopidogrel group, both the levels of *VEGF-A* and *CD34* were decreased compared with control group. In the BPC 157 co-treatment group, the levels of these two angiogenesis markers were all up-regulated compared with Clopidogrel-only group. The NO synthase blocker L-NAME inhibited up-regulation of angiogenesis markers *VEGF-A* and *CD34* by BPC 157, suggesting that BPC 157 promotes angiogenesis and then further repair the gastric mucosa through NO response.

The Angiogenesis is Promoted by BPC 157 on Gastric Mucosa via VEGF-A Mediated AKT and P38/MAPK Signaling Pathways

Considering that *AKT*, *p38/MAPK* and extracellular regulated protein kinases (*ERK/MAPK*) are downstream targets of *VEGF-A*[38–40] and also the classic pathways that regulate apoptosis and inflammation, we sought to investigate the molecular mechanism by using western-blot analysis. Our results showed that Clopidogrel down-regulated the *VEGF-A* and*VEGFR1*, subsequently inactivated *AKT* signaling pathway. However, it was also associated with phosphorylation of *p38/MAPK* and *ERK/MAPK*; furthermore, BPC 157 could significantly

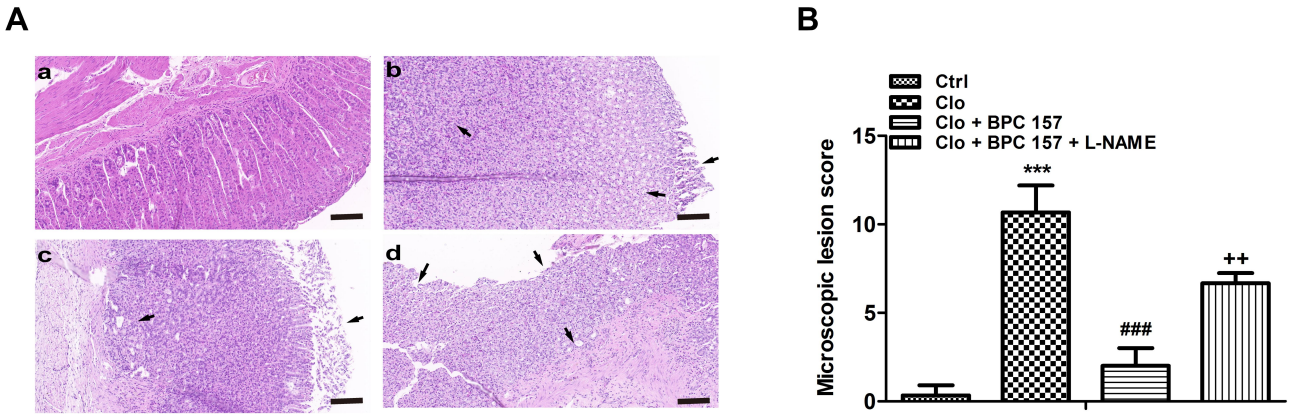

Figure 2 (**A**) Histopathological features and lesion score of stomach. a, Control group showed intact mucosal lining of flattened epithelial cells. Mucosal crypts appear arranged normally; b, The Clopidogrel group showed denudation of lining epithelium, areas of hyperemia, disturbed glandular structure, damaged crypts, irregular arrangement and inflammatory cells infiltration; c, Clopidogrel plus BPC 157 group with minimal changes compared to Clopidogrel group; d, L-NAME exposed mucosal tissue showed similar pathological changes as in Clopidogrel group. Scale bar = 200 μm. The lesion score is shown in (**B**). $^{***}P < 0.001$ vs Control; $^{\#\#\#}P < 0.001$ vs Control + Clopidogrel; $^{++}P < 0.01$ vs Control + Clopidogrel + BPC 157.

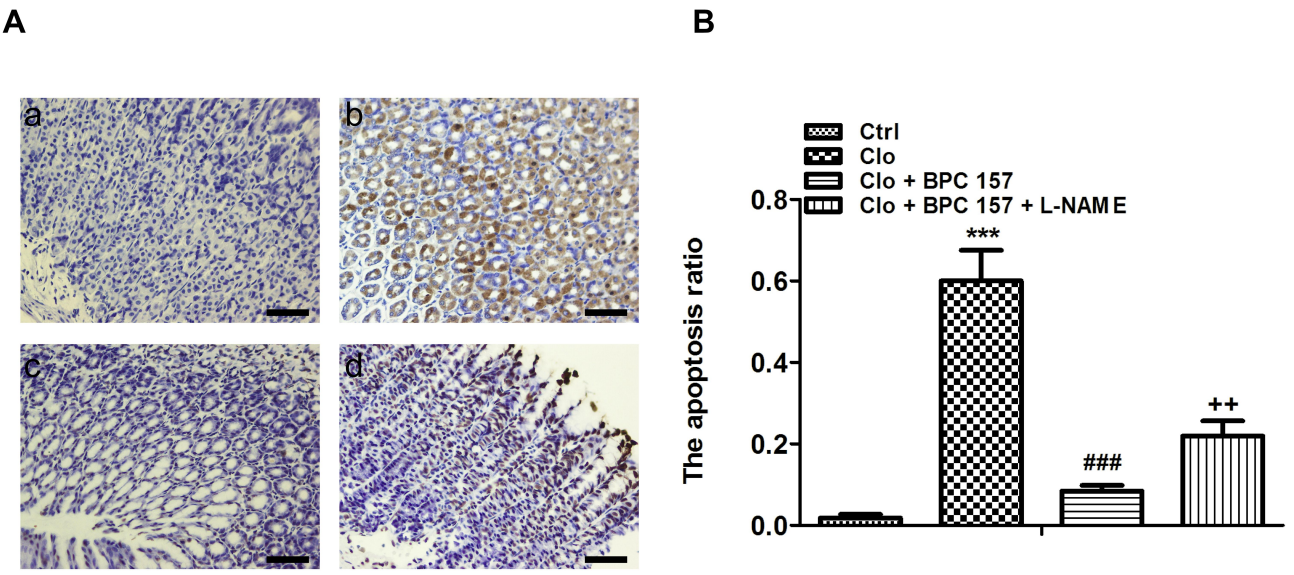

Figure 3 TUNEL staining. Epithelial apoptosis in gastric mucosa was evaluated by TUNEL staining. (**A**) Representative images of TUNEL staining of stomach from rats in four groups. a, Control group; b, Clopidogrel group; c, Clopidogrel plus BPC 157 group; d, Clopidogrel plus BPC 157 and L-NAME group. Scale bar = 200 μm. (**B**) Ratio of TUNEL-positive (brown) cells in each group was shown. $^{***}P < 0.001$ vs Control; $^{\#\#\#}P < 0.001$ vs Clopidogrel; $^{++}P < 0.01$ vs Clopidogrel + BPC 157.

up-regulate the *VEGF-A* and *VEGFR1*, paralleled by the phosphorylation of *AKT*, and inactivation of *p38/MAPK* and *ERK/MAPK* signaling pathways. More importantly, the NO synthase blocker L-NAME can reverse these effects of *AKT* and *p38/MAPK* signaling pathways induced by BPC 157, but cause no changes on *ERK/MAPK*. Therefore, we concluded that BPC 157 affects on angiogenesis by activating the NO system through *VEGF-A* mediated *AKT* and *p38/MAPK* signaling pathways (Figure 5A and B).

BPC 157 Attenuates Clopidogrel-Induced ER Stress and Enhances the eNOS Activation

Previous researches have demonstrated that endothelial NOS (*eNOS*) is the downstream target gene of AKT and p38/MAPK signaling pathway. Upon activation of these two pathways, eNOS leads to the production of NO that translates to an enhanced vasodilation, vascular remodeling and angiogenesis.[41,42] In addition, Wang et al[43] confirmed that ER stress decreases the *eNOS* phosphorylation and the generation of NO in rats' aortic endothelial cells, which can be reversed by ER stress inhibitors. As our previous study confirmed that Clopidogrel activates the *p38/MAPK* signaling pathway followed by up-regulation of C/EBP homologous transcription factor (*CHOP*), and culminating with ER stress apoptosis in human gastric mucosal epithelial cell line GES-1.[10] Therefore, in the current work, we focused on detecting the expression of *CHOP* and *eNOS* proteins. As shown in Figure 5C, Clopidogrel increased the ER stress marker *CHOP* expression and inhibited the *eNOS* phosphorylation compared with the control group, and these changes were both reversed by BPC 157 treatment. The addition of L-NAME partly abolished the effect of BPC 157. These indicated that Clopidogrel induced ER stress not only promotes the gastric mucosal cells apoptosis but also reduce the release of *eNOS*-mediated NO products. And BPC 157 can inhibit ER stress and increase *eNOS*-mediated NO product release. The effects of ER stress inhibition and *eNOS* increased induced by BPC 157 can be partially abolished when using the NO blocker L-NAME.

Discussion

Clopidogrel is one of the most widely used medicines world-wide due to its antiplatelet property. However, recent studies have revealed that Clopidogrel can cause various side effects in the gastrointestinal tract, including gastrointestinal ulcers, bleeding and perforation, and these complications may be fatal.[4] Thus, chronic Clopidogrel users are often provided with prophylactic therapy aimed at neutralizing gastric acidity such as PPI to prevent gastric mucosal damage. But, prolonged PPI use can cause set of long-term side effects like osteoporosis-related fractures and reduce the antiplatelet effect of Clopidogrel for

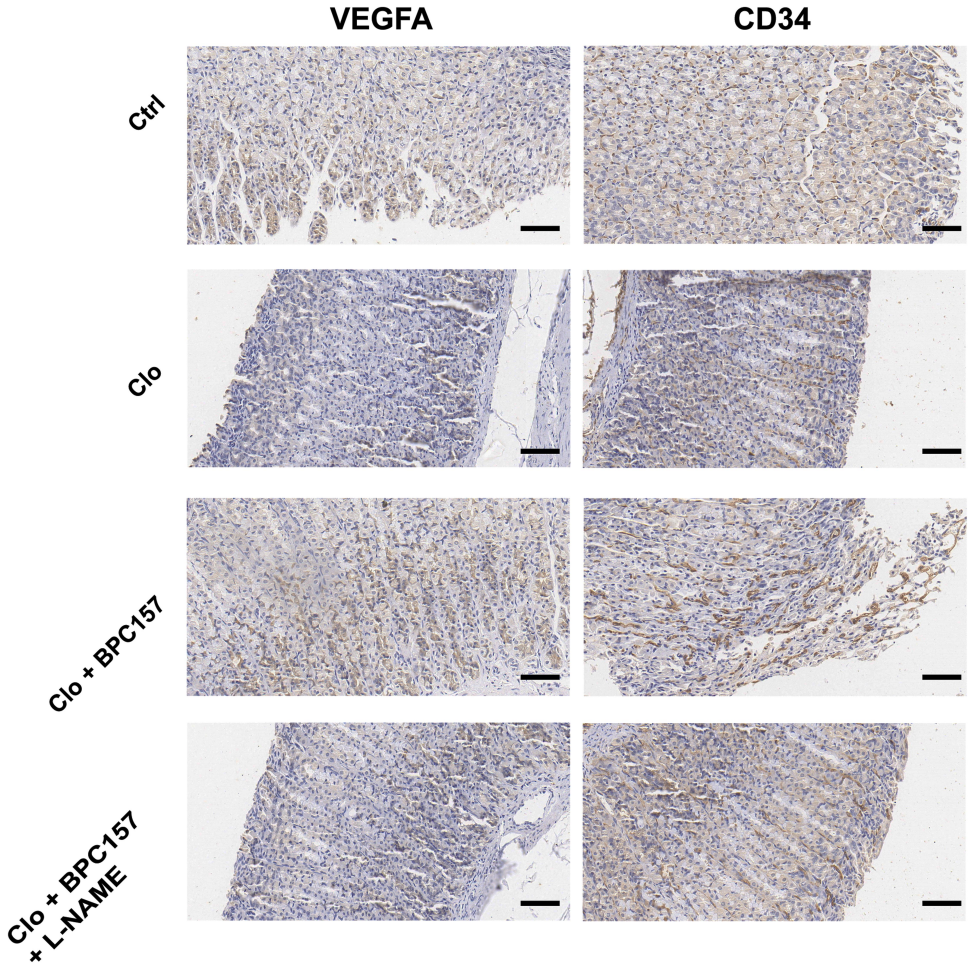

Figure 4 Immunohistochemical detection for the angiogenesis markers *VEGF-A* and *CD34*. IHC staining for *VEGF-A* and *CD34* in *rats*' gastric mucosal tissue. The levels of *VEGF-A* and *CD34* were highly expressed in Control group. In Clopidogrel group, the levels of *VEGF-A* and *CD34* were both decreased compared with control group. And in the BPC 157 co-treatment group, the levels of these two angiogenesis markers were all up-regulated compared with Clopidogrel group. But the NO synthase blocker L-NAME inhibited up-regulation of angiogenesis markers *VEGF-A* and *CD34* induced by BPC 157. Dark brown represents positive result. Scale bar = 50 μm.

preventing cardiovascular events recurrence.[44] Here, we explored the utility of a natural product, BPC 157, in Clopidogrel-induced gastric injury.

Pentadecapeptide BPC 157 is a pentadecapeptide containing partial sequence of the body protection compound, which is derived from the human gastric juice. It appears to be beneficial to most of the human organs without any side effect or toxicity. Research showed that BPC 157 had anti-ulcer and anti-inflammatory effects on various gastrointestinal lesions, and also been reported to have protective effect on pancreas, endothelium, liver injuries, heart damage and pseudo arthrosis.[45] The pathogenesis underlying gastric ulcer is a very complicated process. It is well known that epithelial cell proliferation/apoptosis balance plays an essential role in the multiple progressions of gastric diseases.[46] In our previous study, we found Clopidogrel can cause gastric mucosal epithelial cells apoptosis then further destroy the gastric mucosal epithelial barrier.[9] In accordance with these, in this study, we first observed that BPC 157 can significantly reduce the Clopidogrel-induced gastric ulcer recurrence rate in rats, and inhibit gastric mucosal epithelial cells apoptosis and inflammation. In addition, the NO blockade by L-NAME could partially reverse the protective effect of BPC 157 on gastric mucosa, so we believe that the NO system is related to the mechanism of how BPC 157 protect Clopidogrel-induced gastric mucosal injury. Since Sikiric et al[47] firstly demonstrated that NO generation in gastric mucosa contributes to the anti-ulcer effect of BPC 157 in gastric lesion, subsequently, several reports analyzed the role of NO modulation in the healing effect of BPC 157 in different tissue injuries by using NO-synthase-blockade L-NAME or NO-system overstimulation L-arginine. For the treatment of colitis, ischemia and reperfusion in rats,

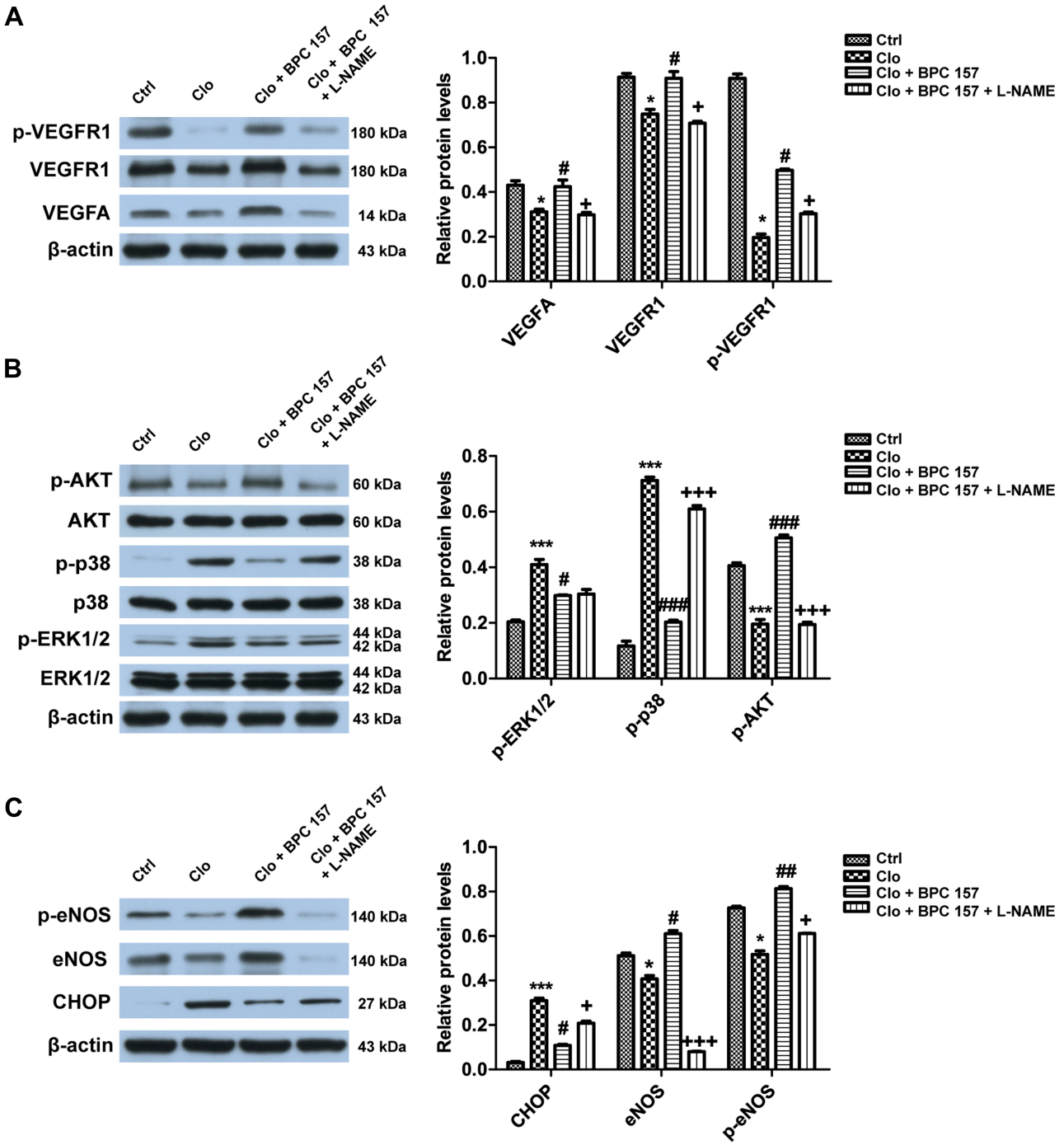

Figure 5 Role of *VEGF-A*-mediated-*AKT* or *p38/MAPK* signaling pathway in the inhibition of BPC 157 on Clopidogrel-induced gastric mucosal injury. (**A**) Clopidogrel group down-regulated the *VEGF-A* and *VEGFR1* compared with Clopidogrel group. But after administration of BPC 157, the *VEGF-A* and *VEGFR1* were up-regulated. However, the L-NAME reversed this effect of BPC 157. $^{*}P < 0.05$ vs Control; $^{\#}P < 0.05$ vs Clopidogrel; $^{+}P < 0.05$ vs Clopidogrel + BPC 157. (**B**) As the *VEGF-A* was downregulated, Clopidogrel dephosphorylated the *AKT* signaling pathway, phosphorylated *p38/MAPK* and *ERK/MAPK* signaling pathways; but the effect of BPC 157 on these three signal pathways was completely opposite to that of Clopidogrel. Notably, L-NAME reversed the effect of *AKT* and *p38/MAPK* induced by BPC 157, but not *ERK/MAPK*. $^{***}P < 0.001$ vs Control; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ vs Clopidogrel; $^{+++}P < 0.001$ vs Clopidogrel + BPC 157. (**C**) Clopidogrel increased the expression of *CHOP* and decreased the *p-eNOS*. These were reversed by BPC 157 treatment. But L-NAME partly abolished the effect of BPC 157. $^{*}P < 0.05$, $^{***}P < 0.001$ vs Control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs Clopidogrel; $^{+}P < 0.05$, $^{+++}P < 0.001$ vs Clopidogrel + BPC 157.

BPC 157 quickly restores blood supply to the chemically injured area and counteracts worsening effects induced by L-NAME or L-arginine.[48] Drmic et al[25] found that high-dose celecoxib administration, as a result of NO system dysfunction, induced gastrointestinal, liver and brain lesions in rats can be attenuated by BPC 157 or

L-arginine. Similarly, BPC 157 also works against cyclophosphamide-induced hemorrhagic cystitis with severe disturbance of the NO system aggravated by using L-NAME.[49]

Angiogenesis plays a key role in the healing of gastric ulcers, because the neovasculature provides blood and nutrients to the healing tissues. Konturek et al[50] showed that endogenous NO can not only increase blood flow at the edge of the ulcer but also stimulate capillary angiogenesis, so it plays an important role in the healing of gastric ulcers. Therefore, in this study, we next detected angiogenesis markers, *VEGF-A* and *CD34*. Our immunohistochemical results showed that BPC 157 did promote the *VEGF-A* and *CD34* protein synthesis, and this effect can be suppressed by L-NAME. We concluded that BPC 157 initiated angiogenesis and then further repaired the gastric mucosa through NO response. This is consistent with Domagoj Drmic's[25] study which showed that Celecoxib-induced gastrointestinal mucosa injury in rats could be counteracted by BPC 157, but aggravated by L-NAME; and Zeljko Djakovic's[51] study which demonstrated that the fistula formed after esophagogastric anastomosis in rats could be cured by BPC 157 and aggravated by L-NAME.

Angiogenesis is regulated by angiogenic growth factors, such as vascular endothelial growth factor (VEGF)-A.[52] *VEGF-A* is reported to play a crucial role in the healing of gastric ulcer. Injection of a plasmid overexpressing VEGF-A in rats can promote the healing of ulcers, while the anti-*VEGF-A* antibody antagonizes this effect.[53] The biological function of *VEGF-A* depends on its specific receptors, such as *VEGFR1*, *VEGFR2* and *VEGFR3*. *VEGFR1* and *VEGFR2* are endothelial cell specific, whereas *VEGFR3* has non-endothelial-cell functions. *VEGF-A* binds to *VEGFR1* with a 10-fold higher affinity than *VEGFR2*, and in Takehito Sato's[54] study it was shown that *VEGFR1* signaling pathway is involved in the healing of NSAID induced gastric ulcer. We hypothesized that *VEGF-A-VEGFR1* may influence the healing of the ulcer induced by Clopidogrel. So in this study, we examined the relative contributions of *VEGF-A* and *VEGFR1* to gastric ulcer healing in rats. The results elucidated that Clopidogrel induced recurrence of gastric ulcers and inhibited the expression of *VEGF-A* and *VEGFR1* while BPC 157 promoted the healing of ulcers and upregulated both of these proteins, and the L-NAME delayed the gastric ulcer healing through downregulating the *VEGF-A* and *VEGFR1*.

VEGFR1 was confirmed to autophosphorylate at Tyr-1169, 1213, 1242, 1327 and 1333, and *PI-3K* is a potential candidate mediating biological effects after *Tyr-1213* autophosphorylation.[55] Several distinct signaling pathways such as *AKT*, *p38/MAPK* and *ERK/MAPK* are activated in a *PI-3K*-dependent manner. This study found that BPC 157 could reverse Clopidogrel-induced *AKT* dephosphorylation, *p38/MAPK* and *ERK/MAPK* phosphorylation. Moreover, inhibition of NO activity by L-NAME attenuated the effects of BPC 157 on *AKT* and *p38/MAPK* but not the *ERK/MAPK* signaling pathway, indicating that BPC 157 promotes angiogenesis and repairs gastric mucosa by activating NO activity and are related to *AKT* and *p38/MAPK* signaling pathways. The *AKT* and *p38/MAPK* pathways are both critical for cellular growth and survival.[56,57] Besides these, it is well known that the *AKT* and *p38/MAPK* signaling pathways are involved in angiogenesis, and these pathways participate in multiple stages of this process. Our data are consistent with recent data generated in human umbilical vein endothelial cells such as inhibition of *AKT* and *p38/MAPK* pathways has antiangiogenic effects.[58] Up-regulation of *AKT* and *MAPK* signaling pathways were reported to promote angiogenesis and enhance wound healing in the male diabetic mice skin wound models.[59]

Considering that phosphorylation of *AKT* activates endothelial NOS (*eNOS*) subsequently, then leads to NO production, which can simulate vasodilation, vascular remodeling and angiogenesis;[60] also increased phosphorylation of *p38/MAPK* and *ERK/MAPK* contributes to the decreased *eNOS* activity,[42] we hypothesized that *eNOS*-derived NO production may influence the healing of the gastric ulcer. Therefore, in the present study, we examined the relative contributions of *eNOS* to gastric ulcer healing after BPC 157 treatment in rats and, as expected, the *AKT* phosphorylation, *p38/MAPK* dephosphorylation and the phosphorylated *eNOS* expression was increased significantly compared to Clopidogrel group. The L-NAME obviously inhibited the *eNOS* expression.

In our previous study, *p38/MAPK* was phosphorylated by Clopidogrel in human gastric mucosal epithelial cell line GES-1, and the cell proliferation was inhibited by ER stress related transcription factor *CHOP* activation.[10] ER stress is proven to induce endothelial dysfunction by disrupting vasoactive homeostasis of the endothelium. Loinard et al[61] have confirmed that phosphorylated *eNOS* is upregulated in *CHOP*−/− mice and *CHOP* binds to *eNOS* promotor, subsequently inhibiting NO production in endothelial cells. Recent study has shown that the

novel *P2Y12* receptor antagonist, Ticagrelor upregulated the expression of phosphorylated *eNOS* and alleviated ER stress in the rat aortic endothelial cells, however this effect can be reversed by ER stress inhibitors.[37] In our study, we found that Clopidogrel downregulated the expression of phosphorylated *eNOS* and induced ER stress in the rat gastric mucosa cells and the BPC 157 protected against Clopidogrel-induced gastric mucosa dysfunction by alleviating ER stress and promoting *eNOS*-mediated angiogenesis whereas the effect of L-NAME is completely the opposite. It is interesting that, same as *P2Y12* blockers, Ticagrelor and Clopidogrel have the opposite role in ER stress and *eNOS* phosphorylation. We speculated whether this difference may be due to the different types of cells. The network of ER stress and the NO system will be further improved in our future research.

Conclusion

In summary, our study demonstrated that BPC 157 was able to inhibit ER stress-induced apoptosis and inflammation of gastric mucosal cells, stimulate gastric angiogenesis and then alleviate the Clopidogrel-induced gastric mucosal damage. The specific mechanism may be by activating *VEGF-A*/*VEGFR1*-mediated *AKT* and *p38/MAPK* signaling pathways to upregulate downstream *eNOS* expression, which may increase the NO product synthesis thus promote angiogenesis of gastric mucosa, and also interact with ER stress. We hope that our results in this present study could provide further evidence for the anti-ulcer effects of BPC 157 and its underlying molecular mechanisms, and also a theoretical basis for the prevention and treatment of gastric damage caused by Clopidogrel. In future, we plan to use the RNAi (RNA interference) method both at the cellular and animal levels to further investigate in-depth about the mechanism, especially the network between ER stress and the NO system.

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Disclosure

The authors report no conflicts of interest in this work.

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