Guanxinning tablet inhibits the interaction between leukocyte integrin Mac-1 and platelet GPIbα for antithrombosis without increased bleeding risk

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[ABSTRACT] Recent studies have showed that thrombosis is closely related to leucocytes involved in immunity. Interfering with the binding of leukocyte integrin Mac-1 and platelet GPIbα can inhibit thrombosis without affecting physiological coagulation. Mac-1-GPIbα is proposed as a potential safety target for antithrombotic agents. Guanxinning tablet (GXNT) is an oral Chinese patent medicine used for the treatment of angina pectoris, which contains phenolic acid active ingredients, such as salvianolic acids, ferulic acid, chlorogenic acid, caffeic acid, rosmarinic acid, tanshinol, and protocatechualdehyde. Our previous studies demonstrated that GXN exhibited significant antithrombotic effects, and clinical studies suggested that it did not increase bleeding risk. In addition, GXN exerted a significantly regulatory effect on immune inflammation. In the current study, we intended to evaluate the effects of GXN on bleeding events and explore the safety antithrombotic mechanism of GXN based on leukocyte-platelet interaction. First, we established a gastric ulcer model induced by acetic acid in rats and found that GXN not only did not increase the degree of gastrointestinal bleeding when gastric ulcer occurred, but also had a certain promoting effect on the healing of gastric ulcer. Second, in vitro experiments showed that after pretreatment with GXN and activation by phorbol 12-myristate-13-acetate (PMA), the adhesion and aggregation of leucocytes with human platelets were reduced. It was also found that GXN reduced the expression and activation of Mac-1 in leucocytes, and inhibited platelet activation due to leukocyte engagement via Mac-1. Overall, the results suggest that GXN may be a safe antithrombotic agent, and its low bleeding risk mechanism is probably related to inhibited leukocyte-platelet aggregation and its interaction target Mac-1-GPIbα.

[KEY WORDS] Thrombosis; Guanxinning tablet; Phenolic acids; Integrin Mac-1; Glycoprotein Ibα


Introduction

Thrombosis is the pathological basis of cardiovascular diseases, and clinically manifests as coronary heart disease (CHD), myocardial infarction (MI) and ischemic stroke, which has becomes a serious threat to human health and the leading cause of disability and mortality worldwide. Current antithrombotic agents, such as antiplatelet agents and anticoagulants, may inevitably lead to an increased risk of bleeding, resulting in adverse cardiovascular events. Therefore, it is urgent to find new antithrombotic agents with good safety and high efficacy for the prevention and treatment of cardiovascular diseases.

Polyphenols are the most abundant secondary metabolites in natural plants with extensive bioactivity. Phenolic acids, as one of the important polyphenols, are also the key active components in medicinal plants. Phenolic acid-rich Chinese medicinal herbs, such as Danshen (Salvia miltiorrhiza Bge.), Dansguk (Angelica sinensis (Oliv.) Diels), Chishao (Paonia lactiflora Pall.) and Chuanxiong (Ligusticum chuanxiong Hort.), have unique effects in the treatment of cardiovascular diseases. Guanxinning tablet (GXNT) is a new compound preparation composed of traditional Chinese herb-
al medicines of Danshen and Chuanxiong. It is clinically used to treat CHD and angina pectoris through promoting blood circulation, removing blood stasis, dredging the channels and collaterals, and nourishing the heart [6]. In the preliminary study, we found that GXNT inhibited thrombosis induced by phenylhydrazine and arachidonic acid in zebrafish, and protected against myocardial ischemia-reperfusion injury in rats, through decreasing blood viscosity, antplatelet aggregation and anti-myocardial ischemia [8-13]. In addition, GXNT effectively inhibited carotid artery thrombosis induced by FeCl$_3$ in rats, which was related to the MAPK signal pathway [8]. Recently, an ongoing phase 4 clinical trial involving 3000 patients has indicated that, the GXNT intervention group showed only 2% hemorrhagic events, while the control group had 4% hemorrhagic events (defined as grades 3–5 hemorrhagic events based on BARC), suggesting that GXNT might be a potentially safe antithrombotic agent with reduced bleeding risk (unpublished). A previous clinical study demonstrated that the administration of GXNT improved prognosis without increasing bleeding risk in coronary artery disease patients who received aspirin alone in place of dual antplate-let therapy (DAPT) after percutaneous coronary intervention (PCI) [9], which is consistent with the results of the ongoing Phase 4 clinical trial. Thus, we believe that GXNT is an antithrombotic agent without increased bleeding risk, and the mechanism and target need to be further studied.

Emerging experimental evidence has indicated the great differences between the molecular mechanisms of physiologic hemostasis and pathologic thrombosis [10]. Thrombosis acts as an intravascular effector of innate immunity, and involves interactions among innate immune cells, platelets, and plasma proteins, especially leukocytes (such as monocytes and neutrophils) and platelets [10, 11]. Inflammation is linked to all stages of the development of thrombosis. After activation, leukocytes bind to platelets to form leukocyte-platelet aggregates (PLAs), which can be used as an indication of thrombosis as a relatively new concept proposed [10]. Leuko-
cyte-platelet interaction is now considered to be an important molecular mechanism that differentiates hemostasis from thrombosis and has gradually attracted increasing attention in antithrombotic research [12]. A recent study by Daniel Simon has revealed that the interaction of the leukocyte integrin receptor macrophage-1 antigen (Mac-1, also known as CR3, integrin αMβ2 or CD11b/CD18) with the platelet surface receptor GPIIbα is a new pathway to form thrombus [13]. Mac-1 binding to GPIIbα can bidirectionally mediate the “outside-in” signal pathway of leukocytes and platelets, thereby promoting thrombosis. Disturbing the Mac-1-GPIIbα interaction can delay thrombosis, but hardly affect physiological hemostasis [13]. Therefore, the Mac-1-GPIIbα is likely to be a novel and safe anti-thrombotic candidate target with reduced risk of bleeding, with important clinical application value. In the current study, we evaluated the effects of GXNT on the healing and bleeding of gastric ulcer using an acetic acid-induced gastric ulcer model of rats, and detected the effects of GXNT on the interaction of Mac-1-GPIIbα using cell experiments in vitro. These findings will provide experimental basis for the prevention and treatment of thrombotic cardiovascular diseases and contribute to the development of GXNT as a safe antithrombotic agent.

Materials and Methods

Drug preparation

GXNT (Guaxinning extract powder, abbreviated GXN, at a raw dose of 12.8 g·g$^{-1}$) was provided by Chiatai Qingchunbao Pharmaceutical Co., Ltd. (Hangzhou, China). GXN was prepared from Danshen and Chuanxiong as described in our previous study [10], where brown powder, salvi-
anolic acid B and ferulic acid were used as quality controls. In animal experiments, GXN was dissolved with 0.9% normal saline (NS) to reach a desired concentration. In cell experiments, GXN was formulated to the required concentration before use and passed through a syringe filter (Sartorius, Gottingen, Germany) with 0.22 μm in diameter for sterilization. Aspirin enteric-coated tablets were purchased from Bayer Healthcare Co., Ltd. (BJ50468, Leverkusen, Germany).

Rats

A total of 48 male Sprague-Dawley (SD) rats (SPF grade), aged 6–8 weeks and weighing 190–210 g, were pur-

chased from Shanghai SLAC Laboratory Animal Co., Ltd. (Certification No. SCXK[Hu]2017-0005; Shanghai, China).

All the animals were maintained in the Laboratory Animal Research Center of Zhejiang Chinese Medical University (Certification No. SYXX [Zhe]2018-0012; Hangzhou, China), housed in individually ventilated cages (IVC) under a 12-h light/dark cycle, with free access to standard chow and water. All animal experiments were conducted according to the requirements of the Ethical Committee of Zhejiang Chinese Medical University (Approval No. IACUC-20201102-08; Hangzhou, China).

Grouping and administration

After acclimation for one week, SD rats were randomly divided into six groups according to their body weights ($n = 8$): a sham-operated group (Sham), a model group (Model), a low-dose GXN group (GXN-L, GXN at 500 mg·kg$^{-1}$), a high-
dose GXN group (GXN-H, GXN at 1000 mg·kg$^{-1}$), a low-
dose aspirin group (Asp-L, aspirin at 50 mg·kg$^{-1}$) and a high-
dose aspirin group (Asp-H, aspirin at 100 mg·kg$^{-1}$). Prior to modeling, rats in each administration group were orally administered with corresponding agents, while those in the sham-operated group and the model group were given equal volumes of NS by gavage. Three days after continuous administration, gastric ulcer operation induced by acetic acid was performed in rats, followed by administration for another five consecutive days. After the final drug administration, rats were deprived of food for 24 h and sacrificed.

Establishment of a gastric ulcer model induced by acetic acid

A gastric ulcer model was induced by acetic acid in rats according to the modified Okabe method [14]. In brief, all animals were fasted for sixteen hours before operation. Rats in...
each group were intraperitoneally injected with 3% sodium pentobarbital (at 45 mg kg\(^{-1}\) body wt; Merck, Darmstadt, Germany). Then, the abdominal skin was shaved followed by disinfection with iodophor. The abdomen was opened along the midline of the xipohoid process to expose the stomach. Then, 0.1 mL of 20% acetic acid (\(V/V\)) was slowly injected into the subserosal layer approximately 5 mm below the angular incisure on the lesser curvature of the stomach over 60 s. Subsequently, the wound was disinfected, and each layer of the abdominal wall was sutured in sequence to close the opened abdomen. Rats in the sham-operated group underwent open abdominal exposure alone.

**Gross observation and histopathological analysis of gastric tissue**

After the animals were sacrificed, the excised stomachs were cut open along the greater curvature, rinsed with ice-cold NS, and then laid flat. The gastric tissue was placed on the coordinate paper with a scale, 0.5 cm \(\times\) 0.5 cm for each small square, and photographed. The ulcer area was measured by Image J software (National Institutes of Health, Maryland, USA).

Subsequently, the ulcer tissue and normal tissue adjacent to the ulcer were collected and cut into two parts. The tissue near the pylorus was taken in the sham-operated group. One part was placed into a frozen tube and kept at \(-80^\circ\) C for enzyme-linked immunosorbent assay (ELISA). The other part, about 1.0 cm \(\times\) 0.5 cm in size, was fixed in 10% neutral formaldehyde solution for two days, dehydrated, and embedded in paraffin. The paraffin-embedded blocks were cut into 4 μm thick slices by a paraffin microtome (Leica, Wetzlar, Germany), and two sections of each sample were prepared. One was used for HE staining, and the other was for immunofluorescence staining labeled with ICAM-1 fluorescent antibody (Cat No. sc-8439, Santa Cruz, Darmstadt, Germany), according to the manufacturer’s instructions. Then, the sections were scanned by a Nano Zoomer 2.0RS digital slide scanner (Hamamatsu, Japan) to observe the pathological changes in gastric tissues. The expression of ICAM-1 was observed by an Olympus VS120 virtual scanning glass microscope (Olympus, Tokyo, Japan), and quantitative analysis was performed by Image Pro-Plus 6.0 software (Media Cybernetics, Maryland, USA).

**Determination of VEGF and ES in gastric tissue**

The frozen gastric tissues were thawed at 4 °C before use. The gastric tissues, weighing 200 mg, were homogenized in 1800 μL NS and centrifuged at 3000 r min\(^{-1}\) for 20 min. The supernatant was taken and the levels of vascular endothelial growth factor (VEGF) and endostatin (ES) in the stomach were measured by commercially available ELISA kits (Multisciences, Hangzhou, China), according to the manufacturer’s instructions in the absorbance was read at 280 nm using an automated ELISA reader (Thermo, MA, USA).

**Platelet collection**

Healthy male adult subjects were enrolled as the volunteers in this study. The exclusion criteria were no cardiovascular disease, allergy, and metabolic abnormalities, without administration of antiplatelet drugs or other non-steroidal anti-inflammatory drugs two weeks before the experiment. The elbow venous blood samples were collected into a vacuum tube containing 3.8% sodium citrate (9 : 1, \(V/V\)), before centrifugation at 250 \(\times\) g for 20 min to obtain platelet-rich plasma (PRP). Then, PRP was centrifuged at 900 \(\times\) g at room temperature for 10 min to precipitate platelets. The resultant platelets were resuspended with calcium-free Tyrode’s buffer (Yuanye Bio-Technology, Shanghai, China) containing ACD (Yuanye Bio-Technology, Shanghai, China; Tyrode’s : ACD 9 : 1, \(V/V\)) and centrifuged at 800 \(\times\) g for 10 min. Then, the platelets were washed twice with calcium-free Tyrode’s buffer, and the concentration of washed platelet suspension was adjusted to the required density (2 \(\times\) \(10^7\)/mL).

**CCK8 assay**

THP-1 monocyte cells were purchased from the National Collection of Authenticated Cell Cultures (Cat No. SCSP-567, Shanghai, China). THP-1 cells were cultured with RPMI medium 1640 (Gibco, CA, USA) supplemented with 10% (\(V/V\)) fetal bovine serum (Gibco, CA, USA), and incubated in a carbon dioxide incubator (Thermo Forma, MA, USA) with 5% CO\(_2\) at 37 °C.

The viability of THP-1 cells was measured using CCK8 assay kits (APExBIO, Texas, USA) according to the standard protocol. The cells in the exponential growth phase were seeded in 96-well plates at a density of 1.0 \(\times\) 10\(^5\) cells/well, and exposed to complete medium as a blank control group or GXN at the concentrations of 50, 100, 200, 400, 800, 1200, and 1600 μg·mL\(^{-1}\). After incubation for 6 h, the cells were treated with or without phorbol 12-myristate-13-acetate (PMA, 1 ng·mL\(^{-1}\)) (Multisciences, Hangzhou, China). Each treatment was performed in sextuplicate. Then, the cells were incubated for 24 h, and then 10 μL CCK8 solution was added at 37 °C for 2 to 4 h. The optical density (OD) at 570 nm was measured by a microplate reader (Thermo, MA, USA).

**Platelet-leukocyte aggregation in vitro**

To visualize monocyte-platelet aggregation, fluorescence microscopy was used. The differentiation of THP-1 cells (10\(^5\)/mL) was induced by treatment with PMA (1 ng·mL\(^{-1}\)) for 24 h prior to use, and human platelets were activated by 1 U·mL\(^{-1}\) thrombin (Sigma) at room temperature for 2 min. THP-1 cells and platelets were labeled with 1 μmol·L\(^{-1}\) BCECF AM (Beyotime, Shanghai, China) and 5 μg·mL\(^{-1}\) Dil (Beyotime, Shanghai, China), respectively, at 37 °C for 1 h. After washing with PBS for three times, monocyte-platelet aggregation was performed with stirring at 1000 r min\(^{-1}\) in an aggregometer (Chrono-log, PA, USA) for 5 min. Cell aggregates were attached to a glass slide which was then mounted with fluorescent media containing DAPI (ZS-G8-BIO, Beijing, China). Fluorescence images were captured by a Laser Scanning Confocal Microscope (ZEISS, Oberkochen, Germany).

Then, aggregation was quantitatively analyzed by flow cytometry (FCM). THP-1 cells (10\(^3\)/mL) were divided into...
five groups: a blank control group \[15\], a model group (Model, PMA at 1 ng·mL\(^{-1}\)), and low-, medium- and high-dose GXN groups (GXN-L, GXN-M and GXN-H at 100, 200 and 400 μg·mL\(^{-1}\), respectively). Each treatment group was exposed to the corresponding dose of drug at 37 °C for 6 h. Then, an aliquot of 10 μL PMA (1 ng·mL\(^{-1}\)) was added before incubation overnight for cell differentiation, except for the blank control group. THP-1 cells in each group were collected and washed with PBS for three times. Subsequently, unactivated platelets were mixed with THP-1 cells in the blank control group, while activated platelets were mixed with THP-1 cells in other groups as described above. THP-1 cells were labeled with PE-conjugated anti-human CD45 antibody (BD Biosciences, New Jersey, USA), and platelets were labeled with FITC-conjugated anti-human CD42b antibody (BD Biosciences, New Jersey, USA). After incubation in the dark at room temperature for 30 min, the aggregation rate was analyzed by a Cytoflex S flow cytometer (Beckman, CA, USA). Leukocytes which bound to platelets were distinguished depending on whether CD42 was positive on the surface of leukocyte. Gates for PE and FITC fluorescents were estimated based on the fluorescence of unstained probes. PLA was represented by CD45 and CD42b double-positive events, and 10,000 events were recorded for each sample. The percentage of PLA was calculated relative to the total number of leukocytes (CD45 positive cells), namely PLA = [(CD45/CD42b double-positive events)/CD45 positive events] × 100 %.

**Adhesion assays for Mac-1 and GPIbα**

The adhesion of THP-1 cells loaded with BCECF AM (1 μmol·L\(^{-1}\)) was analyzed as previously described with certain modifications \[16, 17\]. A 96-well microtiter plate was coated with 100 μL purified soluble GPIbα (R&D Systems, MN, USA; 10 μg·mL\(^{-1}\)) and incubated at 4 °C overnight. THP-1 cells of each group were collected, labeled with BCECF AM (1 μmol·L\(^{-1}\)) at 37 °C for 30 min, washed with PBS, and resuspended. Aliquots (100 μL) of labeled THP-1 cells (5 × 10\(^4\)/mL) were seeded onto each well coated with GPIbα and allowed to adhere at 37 °C for 1 h. After unbound cells were removed by gentle washing, fluorescence intensity was measured at 530 nm by a microplate reader (Thermo, MA, USA) and the number of adherent cells was quantified as the percentage of total loaded cells measured before washing.

**Mac-1 mRNA expression and activation assay**

THP-1 cells of each group were collected, and total RNA was extracted and synthesized into cDNA using the primer-Script RT reagent kit (TaKaRa, Osaka, Japan) according to the manufacturer’s instruction. The primer sequences of Mac-1 were 5′-ACTTATGACCTGGCTCTGAACCTC-3′ and 5′-GTCGCTGCTGCGTGCTTGTC-3′. The primer sequences of GAPDH were 5′-CATGAAGATGATGACACAGCTC-3′ and 5′-AGTCCCTCCACGATAACAAAGT-3′. Real-time polymerase chain reaction (RT-PCR) was performed in triplicate and all information was collected by a PTC-200 PCR instrument (Bio-Rad, CA, USA). The cycle threshold method was used for relative quantification, with GAPDH as a housekeeping gene for normalization.

Furthermore, the activation of Mac-1 was analyzed by Western blot and FCM \[18\]. Briefly, THP-1 cells of each group were collected and total protein was extracted by a commercially available kit (KeyGEN, Nanjing, China). Cell lysates were prepared and quantified by immunoblotting using specific primary antibodies, such as rabbit anti-CD11b (Cell Signaling Technology, MA, USA) and β-actin (ProteinTech, Wuhan, China). For FCM analysis, THP-1 cells were incubated with PE-conjugated anti-human CD11b (CBRM1/5) antibody (eBioscience, CA, USA) at room temperature for 30 min. The fluorescence intensity was detected by FCM. CytExpt software (Beckman, CA, USA) was used to obtain data for analysis.

**Platelet activation assay**

THP-1 cells in each group were collected, washed, and resuspended. Then, unactivated human platelets were added to THP-1 cells mentioned above at a ratio of 1 : 300 and co-incubated at 37 °C on a horizontal shaker for 30 min. They were stained in the dark at room temperature for another 30 min, with a fluorescent antibody mix of 2 μL anti-human CD62P-FITC (Biolegend, CA, USA) and 2 μL anti-CD41a-PE (eBioscience, CA, USA) for labeling platelets. Finally, the samples were subjected to FCM.

**Statistical Analysis**

All experimental results were analyzed using SPSS 24.0 software (SPSS, Chicago, IL, USA). Data are expressed as the mean ± standard error (SEM). Group comparisons were performed by the one-way analysis of variance (ANOVA) followed by LSD analysis. Values of P < 0.05 were considered statistically significant.

**Results**

**Effects of GXN on gastric ulcer by gross observation**

Gross observation results are shown in Fig. 1. In the sham-operated group, the gastric tissue was intact and smooth, without ulcers, erosion, and bleeding points. In the model group, the gastric ulcer and erosion were obvious, without bleeding point. The gastric ulcer and erosion in the GXN groups were partially attenuated to various degrees, and no obvious bleeding point was found. In the aspirin groups, the gastric ulcer, erosion, and congestion were observed, with various degrees of bleeding symptoms.

Furthermore, the ulcer area was quantitatively analyzed. Compared with the model group, the ulcer area in the GXN groups at different doses decreased to various degrees, without statistical difference (P > 0.05). The ulcer area in the aspirin groups showed an elevated trend and significantly increased in the high-dose aspirin group (P < 0.05).

**Effects of GXN on gastric ulcer by histopathological examination**

The pathological changes of gastric tissues are shown in Fig. 2. In the sham-operated group, the structure of the gastric mucosa, lamina propria, and muscularis was complete and...
continuous, while the glands were closely arranged without inflammatory cell infiltration. In the model group, the gastric tissue exhibited edema and erosion in the mucosa, loosening and shedding of surface epithelial cells, inflammatory cell infiltration, disruption in the lamina propria gland, capillary dilatation, congestion, and focal hemorrhage. Treatment with GXN at different doses showed mild erosion in the mucosa, slight inflammatory cell infiltration, and occasional loss of mucosal integrity, without focal hemorrhage. In contrast, after aspirin administration at different doses, the gastric ulcer showed severe disruption in the mucosa, with edema and erosion, visible congestion, and serious hemorrhage.

**Effects of GXN on immunofluorescence of microvessels in gastric ulcer tissues**

The blood vessels were labeled with the endothelial cell marker ICAM-1. The mean of integrated optical density (IOD) of each group was analyzed. The results showed that the vessels were intact, and a few red blood cells were observed in the blood vessels in the sham-operated group (Fig. 3). The gastric ulcer appeared in the model group, with the presence of congestion and hemorrhage, and IOD obviously increased, compared with those in the sham-operated group ($P < 0.01$). After treatment with GXN, hemorrhage and IOD were significantly reduced compared with those in the model group ($P < 0.05$). However, the aspirin group presented more serious symptoms and higher IOD than the model group ($P < 0.05$), indicating that aspirin can exacerbate congestion and bleeding.

**Effects of GXN on the expression of VEGF and ES in gastric ulcer tissues**

Angiogenesis is usually regulated by positive and negative regulators. Positive regulators promote angiogenesis and growth, while negative regulators inhibit angiogenesis. VEGF is one of the most important pro-angiogenesis factors, while ES is the most effective endogenous angiogenesis inhibitor. The effects of GXN on the expression of VEGF and
ES in gastric tissue are shown in Fig. 4. Compared with the sham-operated group, the model group presented a significantly increased level of VEGF ($P < 0.05$, Fig. 4A), without significant changes in ES levels ($P > 0.05$, Fig. 4B). After treatment with GXN, the levels of VEGF and ES did not significantly change, but administration of high-dose aspirin resulted in a significantly decreased level of VEGF ($P < 0.01$) and an increased level of ES ($P < 0.01$), compared with those in the model group.

**Effects of GXN on the viability of THP-1 cells**

The CCK8 assay was used to evaluate the viability of THP-1 cells after GXN treatment, as shown in Fig. 5. THP-1 cells were incubated with increasing concentrations of GXN (50, 100, 200, 400, 600, 800, 1200, and 1600 μg·mL$^{-1}$) in the absence (Fig. 5A) or presence (Fig. 5B) of 1 ng·mL$^{-1}$ PMA for 24 h, and the cell viability was ≥ 90%, indicating robust cell viability. The results demonstrated that GXN showed no cytotoxicity towards THP-1 cells at the above concentration range.

**Effects of GXN on platelet-leukocyte aggregation**

According to previous methods [19], THP-1 cells and platelets were aggregated with stirring at 1000 r·min$^{-1}$ to simulate venous shear stress in vivo, and then platelet-leukocyte aggregation was analyzed by electron microscopy and FCM.

The aggregation of THP-1 cells and platelets was observed under a laser confocal microscope. As shown in Fig. 6A, platelets (red fluorescent) were clearly attached to THP-1 cells (green fluorescent).
Fig. 5 Effects of GXN on the viability of THP-1 cells. The viability of THP-1 cells treated with increasing concentrations of GXN in the absence (A) or presence (B) of 1 ng·mL\(^{-1}\) PMA for 24 h was evaluated by CCK8 assay. Data are presented as the mean ± SEM (n = 6). GXN: Guanxinning; and PMA: phorbol 12-myristate-13-acetate

Fig. 6 Effects of GXN on platelet-leukocyte aggregation. (A) Observation of platelet-leukocyte aggregation by laser confocal microscopy (640 ×). The figure shows the adhesion of THP-1 cells and platelets. (A1) Green represented THP-1 cells labeled with BCECF AM (1 μmol·L\(^{-1}\)); (A2) Red represented platelets labeled with Dil (5 μg·mL\(^{-1}\)); (A3) Blue represented nuclei labeled with DAPI; (A4) Merge images by (A1), (A2) and (A3), scale bar: 10 μm. (B) Representative scatter plots of THP-1 cell and platelet aggregation by flow cytometry. Taking the model as an example, THP-1 cells and platelets were labeled with CD45 PE and CD42b FITC, respectively. Fluorescence intensity of each sample was detected and 10000 events were recorded. (B1) The cells were divided into two groups by FSC/SSC scatter plot, and gate P1 represented THP-1 cells; (B2) CD45 PE/FSC scatter plot, and gate P2 represented CD45 positive THP-1 cells; (B3) CD42b FITC/CD45 PE scatter plot showed the percentage of CD45 and CD42b double-positive particles in positive monocyte particles. (C) Platelet-leukocyte aggregation treated with GXN at different doses. Data are expressed as the average of three independent experiments performed in triplicate (*P < 0.05, **P < 0.01 vs the blank group). FSC: forward scatter; SSC: side scatter; and GXN: Guanxinning

Then, the effects of GXN on platelet-leukocyte aggregation were measured by FCM. The cells were divided into two groups by FSC/SSC scatter plot (Fig. 6B1), and gate P1 represented THP-1 cells. Gate P2 in CD45 PE/FSC scatter plot represented CD45-positive THP-1 cells (Fig. 6B). Gate P3 in CD42b FITC/CD45 PE scatter plot showed the percentage of...
CD45 and CD42b double-positive particles in the total number of positive monocytes (Fig. 6B3). The aggregation rate in the model group significantly increased, compared with that in the control group ($P < 0.01$, Fig. 6C). The aggregation rate in the medium- and high-dose GXN groups were lower than that in the model group ($P < 0.05$, $P < 0.01$).

### Effects of GXN on the adhesion of leukocyte Mac-1 to platelet GPIIbα

It has been found that the eM1-domain of leukocyte integrin Mac-1 can directly interact with GPIIbα, indicating that platelet GPIIbα can bind to Mac-1 on the surface of leukocytes. To evaluate the effect of GXN on the binding site, THP-1 cells expressing Mac-1 were used for adhesion assay. As shown in Fig. 7, compared with the control group, the model group showed significantly enhanced adhesion of Mac-1 to GPIIbα ($P < 0.01$). Compared with the model group, the medium- and high-dose GXN groups produced significantly weakened adhesion of Mac-1 to GPIIbα ($P < 0.05$ and $P < 0.01$).

### Discussion

GXNT is composed of extracts from Danshen and Chuanxiong. It is clinically used for the treatment of stable or unstable coronary heart disease and angina pectoris due to thrombosis $^{[4, 20]}$. In the current study, we found that GXN exerted no significant adverse effect on ulcer healing, while no obvious ulcer bleeding was observed in the gastric ulcer model induced by acetic acid in rats. In cell experiments in vitro, we found that GXN inhibited the expression and activation of Mac-1 in THP-1 cells, and interfered with the interaction between Mac-1 and GPIIbα. Furthermore, GXN inhibited platelet activation caused by the interaction of Mac-1-GPIIbα, preventing the formation of thrombus. These findings indicated that GXN is a potential antithrombotic agent, and does not increase bleeding risk, which is of great significance for the prevention and treatment of thrombotic cardiovascular diseases.

Thrombosis is a complex pathological process, which involves a series of critical events such as blood coagulation, platelet adhesion, activation and aggregation $^{[3]}$. Inhibiting platelet function or preventing blood coagulation can prevent thrombosis. The currently available antiplatelet and anticoagulant agents, including cyclooxygenase-1 inhibitors, P2Y purinoreceptor 12 (P2Y12) antagonists, glycoprotein IIb/IIIa (GPIIb/IIIa) antagonists, and protease-activated receptor 1 antagonists, are associated with increased risks of major bleeding, major gastrointestinal bleeding and intracranial bleeding, with comparable absolute risk estimates. Aspirin is a nonsteroidal anti-inflammatory drug. A total daily dose of aspirin from 75–100 mg can increase the absolute risk of gastrointestinal ulcer bleeding by 0.15–0.40% $^{[22]}$, and even a dose of 10 mg daily can significantly reduce prostaglandin levels leading to gastric erosion $^{[23]}$. Rats with gastric ulcer induced by acetic acid is the most similar to human ulcers in
Inflammation and thrombosis often appear together in many diseases. Leukocyte counts and activation can predict the occurrence of myocardial infarction [1]. PLAs can be a new indication of thrombosis. Clinical studies have found that platelet-monocyte aggregates (PMAs) can also predict myocardial infarction and is a more sensitive marker of platelet activation in vivo than platelet surface P-selectin [13]. Circulating PMA levels significantly increased in patients with acute myocardial infarction, and PMAs were significantly correlated to vascular thromboembolism [14]. THP-1 monocytes are a human leukemia monocyte cell line, which has been extensively used to explore monocyte/macrophage functions, mechanisms, and signaling pathways [27]. Co-culture of platelet-monocyte can be a common model which reflects the interaction between platelets and monocytes. THP-1 cells can be effectively differentiated after phorbol ester induction for aggregating with platelets, with elevated Mac-1 expression [28]. In the current study, THP-1 cells and platelets from healthy volunteers were used to observe the effects of GXN on the aggregation of leukocytes and platelets. The results showed that GXN decreased the aggregation.

Mac-1 is expressed on the surface of leukocytes and mediates numerous key innate immune responses. Mac-1 receptor contributes to leukocyte recruitment, tight adhesion, and transendothelial migration at vascular injury sites and promotes tissue inflammation [29]. Studies have shown that integrin Mac-1 bound to platelet surface receptor GP Ibα which is the counter receptor for Mac-1 [30]. Mac-1 exists in an inactive conformation on the surface of leukocytes, and after stimulation, its conformational changes result in an activated state capable of binding to ligands with high affinity. αM I-do-
main is the main ligand-binding domain of Mac-1, which can specifically bind to GPIbα. Plow [10] found that intervention with the Mac-1-GPIbα pathway can regulate thrombosis and mediate blood coagulation, without affecting hemostasis. In addition, previous work showed that Mac-1 bound to GPIbα, regulating inflammation in various models [13]. Inhibiting the interaction between Mac-1 and GPIbα or an antibody targeting Mac-1 was shown to block inflammation [11]. Taking these works together, researchers then proposed that Mac-1-GPIbα interaction is critical for the regulation of thrombosis. Therefore, Mac-1-GPIbα becomes a new therapeutic target for thrombosis without increased bleeding risk, as well as an inflammatory regulatory target. In the current study, we also observed that GXN inhibited Mac-1 expression and αM I-domain activation in leukocytes, and inhibited the adhesion of Mac-1 to GPIbα, which suggested that the low bleeding risk for GXN may be related to inhibiting Mac-1 expression and activation, and then inhibiting Mac-1-GPIbα adhesion. GXN may also play an anti-inflammatory role through inhibiting Mac-1-GPIbα, so as to alleviate thrombotic complications.

Leukocyte-platelet interaction can induce bidirectional signals that enhance proinflammatory and prothrombotic cellular responses, suggesting that the combination of Mac-1 and GPIbα is essential for thrombosis. Mac-1-GPIbα binding can induce the “outside-in” Mac-1 signals that leads to the phosphorylation of protein kinase C-δ and down-regulates the Foxp1 signaling pathway in THP-1 monocytes, thereby promoting the expression of tissue factors and contributing to thrombosis [13]. Mac-1-GPIbα interaction can also induce platelet “outside-in” signaling and platelet activation through Akt phosphorylation [10]. Therefore, blocking the initial aggregation mediated by Mac-1-GPIbα may prevent the amplification of bidirectional signals for thrombosis, which is also the reason why Mac-1-GPIbα inhibitors can effectively reduce occlusive thrombosis in vivo. GPIIb/IIIa (CD41a) and P-selectin (CD62p) are activated on the surface of platelets and their expression is the most reliable indicators of platelet activation. In the current study, we found that GXN inhibited the expression of platelet activation marker CD41a after leukocytes bound to platelet GPIbα, indicating that GXN can inhibit the activation, aggregation, and adhesion of platelets, and exert anti-thrombotic effects.

In our preliminary work, we identified 13 active ingredients in GXN using liquid chromatography–mass spectrometry. This novel study showed that GXN can specifically inhibit the expression and activation of Mac-1, as well as inhibit platelet activation marker CD41a and CD62p, which may explain the low bleeding risk of GXN and the improved blood coagulation. Therefore, GXN may provide an alternative treatment option for thrombosis without increased bleeding risk.

Fig. 9 Effects of GXN on platelet activation. (A) Effect of GXN on the expression of platelet activation marker CD41a; (B) Effects of GXN on the expression of platelet activation marker CD62p. Data are expressed as the mean ± SEM from three separate experiments using platelets from blood donors (##P < 0.01 vs the blank group; *P < 0.05 vs the model group)
metric technology (LC-MS), including tanshinol, senkyunolide B, phenylalanine, chlorogenic acid, caffeic acid, ferulic acid, protocatechualedehyde, rosinamic acid, senkyunolide I, salvianolic acid B, salvianolic acid D, isoisoamino acid A, and isoisoamino acid C [8]. Among them, the phenolic acid active ingredients of salvianolic acids [32], ferulic acid [33], chlorogenic acid [34], caffeic acid [35], rosinamic acid [36], tanshinol [37], and protocatechualedehyde [38] exerted antiplatelet and antithrombotic effects, where caffeic acid possessed both antihaemostatic and antithrombotic effects [39]. Therefore, the antithrombotic effects of GXN may be related to the effective substances mentioned above. The underlying mechanism of GXN for anti-thrombosis may be multiple due to the presence of complicated components. Currently, bleeding remains a major focus and limitation of antithrombotic therapies. In recent studies, new strategies which may inhibit thrombosis while maintaining haemostasis have been identified. These agents target phosphatidylinositol 3-kinase-β (PI3Kβ), protein disulfide-isomerase, activated GPIIb/IIIa, protease-activated receptors, platelet GPVI-mediated adhesion pathways, and factor XI (FXI) [39, 40]. Besides Mac-1:GPIbα, GXN is likely to act as a safe antithrombotic agent via multiple targets mentioned above. In a word, the study provides experimental evidence of attenuating thrombosis without disrupting hemostasis for GXN.

Conclusions

According to our previous studies, GXN plays a significant role in preventing thrombosis. In the current study, GXN does not affect the healing process of gastric ulcer in rats, without obvious inhibitory effects on the formation of gastric microvessels, indicating a reduced bleeding risk, which can be attributed to phenolic acids. Furthermore, in vitro cell experiments reveal that the reduced bleeding risk for GXN may be associated with inhibited leukocyte-platelet aggregation and its interaction target Mac-1:GPIbα. GXN can also inhibit the activation of platelets, but the related material basis and mechanism need to be further studied.

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