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# Effect of patchouli alcohol on *Helicobacter pylori*-induced neutrophil recruitment and activation

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ABSTRACT

Neutrophil infiltration typically occurs in *Helicobacter pylori* (*H. pylori*)-induced acute gastritis; however, this immune response fails to eradicate *H. pylori* in vivo. Moreover, reactive oxygen species (ROS), which are generated by neutrophils, cause severe damage to gastric mucosa. Patchouli alcohol (PA) has been reported to have effective anti-oxidative and anti-*H. pylori* activities, and we investigated its effects on *H. pylori*-induced neutrophil recruitment and activation in this research. In neutrophil recruitment experiment, *H. pylori* was injected into rat air pouch to explore the effects of PA (10, 20 and 40 mg/kg) on acute inflammatory response. The results revealed that PA significantly reduced the weight of exudate and the number of neutrophils in the air pouch. Meanwhile, remarkable decrements in TNF-α and IL-8 levels in exudates were observed. In neutrophil activation experiment, rat neutrophils were isolated and activated by using 50 μg/mL *H. pylori* water-soluble surface protein with or without the treatment of PA (5, 10 or 20 μmol/L). Results indicated that PA not only significantly inhibited the production of ROS, but also reduced the gene and protein expressions of p22/p47-phoxes, and the binding of p22/p47-phoxes. Furthermore, the influence of PA on the neutrophil activation genes of *H. pylori* (*h-nap* and *sabA*) was investigated, and the results showed that expressions of *h-nap* and *sabA* were remarkably decreased after PA treatment. In conclusion, PA reduced the recruitment and activation of neutrophils induced by *H. pylori*, as shown by its inhibition of pro-inflammatory factor generation, p22/p47-phoxes function and *H. pylori* neutrophil activation-related gene expression.

## 1. Introduction

*Helicobacter pylori*, a Gram-negative bacteria which can survive in human stomach, has been listed as type I carcinogenic factor by the International Agency for Research on Cancer since 1994 [1], since *H. pylori* infection was highly associated with digestive system diseases, such as gastritis, gastroduodenal ulcer, gastric-mucosa-associated lymphoid tissue lymphoma and gastric cancer [2]. PPI triple combination therapy (two antibiotics with a proton pump inhibitor) was commonly adopted as the first-line clinical therapy for *H. pylori* infection [3]. Given that antibiotic resistance is becoming an inevitable situation, exploring alternative drugs for treatment of *H. pylori* and its associated gastritis is highly necessary.

In the initial infection of *H. pylori*, the typical *H. pylori* toxin factor cytotoxin-associated gene A (CagA) was injected intracellularly based on its type IV secretion systems, followed by nuclear factor-κB pathway activation and interleukin-8 release, which contributed to neutrophil recruitment [4–6]. Previous research [7,8] found that *H. pylori* water-soluble surface proteins (also known as *H. pylori* water/aqueous extraction), particularly *H. pylori*-neutrophil-activating protein (H-nap) and sialic acid-binding adhesin (SabA), recruited and activated neutrophils [9–11]. In addition, *H. pylori* lipopolysaccharide regulated the expression of NADPH oxidase subunits (gp91-, p22-, p67-, p47- and p40-phoxes) in neutrophils, leading to excessive reactive oxygen

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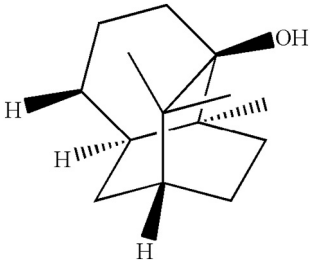


**Fig. 1.** Chemical structure of PA.

species (ROS) production, which caused severe inflammatory injury [12].

Pogostemonis Herba, the dried aerial part of *Pogostemon cablin* (Blanco) Benth. (Labiatae), is a traditional Chinese medicine which has wide therapeutic properties in abdominal pain and gastrointestinal system disease [13]. The main component of Pogostemonis Herba is patchouli alcohol (PA, chemical structure shown in Fig. 1). Previous work from our laboratory has demonstrated that PA possesses gastroprotective effects against ulcer and anti-inflammatory activities against xylene-induced ear oedema in mice and carrageenan-induced paw oedema in rats. It also displayed in vitro and in vivo anti-*H. pylori* activities and *H. pylori* urease inhibition effects [14–16].

In the follow-up study, in vivo and in vitro experiments were conducted to investigate the effects of PA on neutrophil recruitment and activation induced by *H. pylori* and to explore the possible mechanisms. Firstly, a rat air pouch model was established as described elsewhere [17]. The weight of exudate, neutrophil number and inflammatory factor level were determined to manifest the effects of PA on neutrophil recruitment. Secondly, neutrophils were isolated from rat peripheral blood and co-cultured with *H. pylori* water-soluble surface proteins in the presence or absence of PA to explore the effects of PA on neutrophil activation. ROS, p22/p47-phoxes gene and protein expression, and *H. pylori h-nap* or *sabA* gene expression were selected as the indicators.

## 2. Materials and methods

### 2.1. Materials

Columbia blood agar base and brain heart infusion (BHI) were provided by Oxoid (USA). Sheep blood was procured from Pingrui Biotechnology (China). Poloxamer 407 was obtained from BASF Chemical Ltd. (Germany). Metronidazole (MET) and clarithromycin (CLR) were obtained from Toku-E (Japan). Neutrophil separating solution was obtained from TBD science (China), and TRIzol reagent was procured from Life Technologies (USA). FastQuant reverse transcription (RT) Kit and Super Real premix (SYBR green) were obtained from Qiagen (Germany). RIPA lysis buffer, protease inhibitor cocktail and stripping buffer were purchased from CWBIO (China). All chemical reagents used were of analytical grade. BD trucount™ absolute counting tubes and PE Mouse Anti-Rat CD11b were purchased from BD Biosciences (USA). Bicinchoninic acid assay (BCA) Protein Assay Kit, Cell Counting Kit-8 and intracellular ROS Detection Kit were purchased from Beyotime® Biotechnology (China). OxyBURST Green H2HFF BSA Reagents was obtained from Thermo Scientific™ (USA). P22phox antibody was provided by Santa Cruz Biotechnology (USA). Beta-Actin, P47phox antibody and corresponding secondary antibodies for Western blot analysis were obtained from 4A Biotech (China). Goat anti-Rabbit IgG (H + L), Alexa Fluor 488 and Goat anti-Mouse IgG (H + L), Alexa Fluor 555 used in confocal microscopy were provided by Southern Biotech (USA). Hoechst 33342 Solution antibody was purchased from Thermo Scientific™ (USA). ELISA kits for IL-8, IL-1β, TNF-α and $PEG_2$ detection were purchased from 4A Biotech (China).

### 2.2. Animals

Seventy male Sprague–Dawley rats (6 weeks, 180–200 g) were obtained from Guangdong Medical Laboratory Animal Centre (Guangdong, China). The laboratory animal certification license for the rats was SCXK (YUE) 20130002. Thirty rats were used for neutrophil recruitment assay, and another forty for further assays of the PA effects on neutrophil activation. All animals were kept in a pathogen-free environment with a 12-h light/dark cycle and provided free access to food and water. Experimental protocols were approved by the Animal Experimental Ethics Committee of Guangzhou University of Chinese Medicine (Guangzhou, China).

### 2.3. Patchouli alcohol preparation

Endotoxin-free PA (purity > 99%) was prepared and further confirmed by melting point, infrared (IR), $^{1}H$ and $^{13}C$ nuclear magnetic resonance and mass spectrometry analyses as previously described [14]. In the in vitro study, dimethylsulfoxide (DMSO) was used to dissolve PA and served as a control (DMSO ≤0.1% in all experiments). In the in vivo study, poloxamer 407 was used to prepare a PA solid dispersion by a melting method as previously described [18]. The doses of PA in this study were adopted based on our pilot trial and the in vivo antiulcer experiment [16].

### 2.4. H. pylori culture and H. pylori water-soluble surface protein (HWSP) extraction

*H. pylori* strain NCTC11637 was purchased from American Type Culture Collection. The strain was stored at −80 °C in BHI containing 30% glycerol. In this study, *H. pylori* was cultured in Columbia blood agar base supplemented with 5% sheep blood at 37 °C in a tri-gas incubator (Nuaire Nu-5831E, USA) containing 10% $CO_2$, 5% $O_2$ and 85% $N_2$. *H. pylori* was harvested using aseptic cotton carriers, resuspended in phosphate-buffered saline (PBS) and then transferred to new solid plates for sub-culture. *H. pylori* was sub-cultured twice before each experiment to obtain accurate and stable results. For liquid proliferation, *H. pylori* was inoculated in BHI supplemented with 10% FBS and shaken at 120 rpm under the same air environment.

HWSP was prepared based on the previous research [19]. *H. pylori* was collected by centrifugation at 5000*g* for 10 min, resuspended in PBS and then frozen-thawed once at −80 °C. The suspension was subjected to an ultrasonic standing wave field for five cycles (1-min duration and 1-min interval) and then centrifuged (10,000*g* for 10 min, 4 °C). The supernatant was filtered with a 0.22 μm syringe filter, and stored at −80 °C before the experiment. The protein concentration was determined by the BCA kit.

### 2.5. Neutrophil recruitment assay

A rat air pouch model was established as previously described [17]. In brief, 30 male rats were injected subcutaneously with 20 ml of sterile air in the dorsal skin on the first day and another 10 ml at the same site on the third day. After the last injection, rats were randomly divided into five groups: control (vehicle, 200 mg/kg), model (vehicle, 200 mg/kg) and PA low-, middle- and high-dose (10, 20 and 40 mg/kg) groups. The groups were pre-treated orally with corresponding drugs or vehicle for 3 days. After the final oral administration, 2 ml ($1.2 \times 10^9$ CFU/mL) *H. pylori* suspension was injected into the air pouch, whereas the control group rats received equal amounts of sterile PBS. Six hours later, the rats were anaesthetised with halothane, and the exudate inside the air pouch was collected. The weight of exudate was measured. PE labelled mouse anti-rat CD11b antibody was used to stain neutrophil, and BD Trucount™ absolute counting tube was adopted for neutrophil quantification by flow cytometry. Furthermore, the levels of IL-8, IL-1β, and TNF-α in the exudate and mucous were quantified by using ELISA

Kit.

### 2.6. Neutrophil isolation and identification

Rat whole blood was collected from anaesthetised rats through the abdominal aorta and then placed above TBD neutrophil separating solution at a ratio of 2:3. Neutrophil cells were isolated by centrifugation (500*g* for 30 min at room temperature). The layer containing neutrophils was removed, followed by hypotonic lysis of erythrocytes. Neutrophils were resuspended, cultured in RPMI 1640 and identified by flow cytometry using PE labelled mouse anti-rat CD11b antibody (1:1000). Cell viability was examinated by trypan blue exclusion, and the cell viability in all studies exceeded 90% (Shown in supplementary material).

### 2.7. Effect of PA on neutrophil cell viability

The freshly isolated neutrophils were aliquoted into a 96-well microplate (100 µl, $5 \times 10^3$/well), and treated with DMSO (control) and PA (5, 10 and 20 µmol/L). After incubating at 37 °C, 5% $CO_2$ for 30 or 60 min in a cell incubator (CO-150, UK), CCK-8 reagent (10 µl/well) was added for another 30 min. Then the absorbance was read at 450 nm to determine the effect of PA on neutrophil cells viability using Multiskan Spectrum Microplate Spectrophotometer (Thermo Scientific™, USA).

### 2.8. Neutrophil activation and treatment

The freshly isolated neutrophils were inoculated in 6-well plate ($2 \times 10^6$/well), and divided into following six groups: control (DMSO), model (DMSO +50 µg/mL HWSP), MET (DMSO +50 µg/mL HWSP +0.5 µg/mL MET), PA low-, middle- and high-dose (DMSO +50 µg/mL HWSP +5, 10 or 20 µmol/L PA) groups. According to the result of our pre-experiment, 50 µg/mL HWSP was used in this study (Shown in supplementary material). After treatment at 37 °C in a cell incubator for 30 min, neutrophils were separated by centrifugation at 300*g* for 10 min at room temperature. The cell pellet was washed twice before further detection.

### 2.9. Neutrophil ROS generation determination

Intracellular ROS levels were detected using ROS Detection Kit. In brief, neutrophils were resuspended in RMPI 1640, stained with 2′,7′-dichlorofluorescein diacetate (10 µmol/L) in a cell incubator at 37 °C for 20 min, washed twice with PBS, and then resuspended in RMPI 1640. The fluorescence-labelled neutrophils were treated separately as described in Section 2.8. The intracellular ROS levels were determined as the fluorescence intensity by flow cytometry at 488 nm excitation and 525 nm emission, respectively.

Extracellular ROS levels were detected using OxyBURST Green $H_2$HFF BSA Reagents. In brief, neutrophils were resuspended in pyrogen-free and endotoxin-free Hanks' solution, then were incubated with 10 µg/mL OxyBURST $H_2$HFF Green BSA Reagents in a fluorescence cuvette at 37 °C. After 0, 10, 20, 30, 60 min, ROS releasement was measured using a SpectraMax i3x Multi-Mode reader (Molecular Devices, Austria) at 488 nm excitation and 530 nm emission.

### 2.10. Assay of PA on p22/p47-phoxes gene expression

After treatment as described in Section 2.8, TRIzol reagent was added to the neutrophil to isolate the total RNA. Total RNA was qualitied by reading the value of OD260/280 (range 1.8–2.0) and quantified at 260 nm using a NanoDrop 2000 UV–Vis spectrophotometer (Thermo Scientific™, USA). Following the manufacturer's instructions of FastQuant reverse transcription (RT) kit, 0.5 µg total cellular RNA was added to a 10 µl-volume system containing 2 µl 5× gDNA Buffer and incubated at 42 °C for 3 min to eliminate the genomic deoxyribonuclease. Then, reverse transcription was performed in a final volume of 20 µl containing 2 µl 10× Fast RT Buffer, 1 µl RT Enzyme Mix, 2 µl FQ-RT Primer Mix and RNase-Free dd$H_2O$. After incubation at 42 °C for 15 min, sample was heated for 3 min at 95 °C to end the reaction. Samples were stored at −20 °C. The real-time quantitative PCR was conducted in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, USA) with Super Real premix (SYBR green). The cycling protocol was set at 1 cycle at 95 °C for 15 min, 40 cycles at 95 °C for 5 s and 60 °C for 30 s. The data were analysed using the Pfaffl method, and the 16S gene served as the internal reference [20]. Sequences of the PCR primers are listed in Table 1.

**Table 1**
Primer sequences.

| Gene | Forward primer (5′-3′) | Reverse primer (5′-3′) |
|---|---|---|
| *p22phox* | AATTACTACGTCCGGGCTGTC | AGGTAGATCACACTGGCAATGG |
| *p47phox* | CACAACATAACCTTGGCATGGG | AGTCAGCACGGTTTCTTTGC |
| *Gapdh* | TGGCCTCCAAGGAGTAAGAAAC | TGGAATTGTGAGGGAGATGCTC |
| *sabA* | ATCAAATCGGCGAAGCGGT | TTGCTTCAACGAAGCCACTTG |
| *h-nap* | GCCTTCTTTTTCAGCGGTGT | AAGCGCTCAAACTCACTCGT |
| *16s* | CGATGGATGCTAGTTGTTGGAG | GTCCCCGTCTATTCCTTTGAGTT |

### 2.11. p22/p47-phoxes protein expression

#### 2.11.1. Western blot analysis

Neutrophil cell pellet was resuspended by using RIPA lysis buffer and protease inhibitor cocktail (9:1). Lysates were prepared by pipetting, vortexing and sonically disruption. Insoluble material was removed by centrifugation (10,000*g*, 10 min, 4 °C). The protein concentration was determined by using BCA Kit. 10 µg protein samples were loaded to 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and then transferred onto polyvinyl difluoride membranes. Bovine serum albumin (5%, BSA) was used to block the membrane in tris-buffered saline, and then incubated with respective primary antibodies (p22 or p47-phoxes, 1:1000) at 4 °C overnight. The membrane was washed with tris-buffered saline with tween 20 and then incubated with secondary antibody (1:2000) for 2 h at room temperature. Finally, the antibody reactive bands were visualized by chemiluminescence (Immobilon Western Chemilum HRP Substrate, Germany). The band intensity of each signal was quantified by densitometric analyses using Quantity One software (Bio-Rad).

#### 2.11.2. Immunofluorescence confocal microscopy

Neutrophils were resuspended by PBS and dropped to an adhesion microscope slide (drew a limited area on the microscope slide in advance using a pap pen) at room temperature for 20 min. Then the microscope slide with neutrophil adhered was softly immersed and fixed with 4% paraformaldehyde at room temperature. After 20 min, the fixed cells were washed three times with PBS (washed three times with PBS between each step mentioned blow), and treated with 1% Triton X-100 for cell permeabilization. Finally, cells were blocked, double-labelled with P22-phox/P47-phox primary antibodies and incubated with corresponding secondly antibodies. BSA (5%) dissolved in PBS was used as blocking buffer and dilution for primary/secondary antibodies. The primary antibodies used were mouse anti-p22phox and goat-anti-p47phox antibody (1:200). Secondary antibodies were Alexa Fluor 488-conjugated goat anti-rabbit, and Alexa Fluor 555-conjugated goat anti-mouse antibody (1:200). Finally, the samples were stained with Hoechst 33342 (1:1000) and placed on LSM-800 confocal microscope (Carl Zeiss) for image acquisition.

### 2.12. H. pylori h-nap or sabA gene expression

The effect of PA on *h-nap* and *sabA* gene expression was measured as

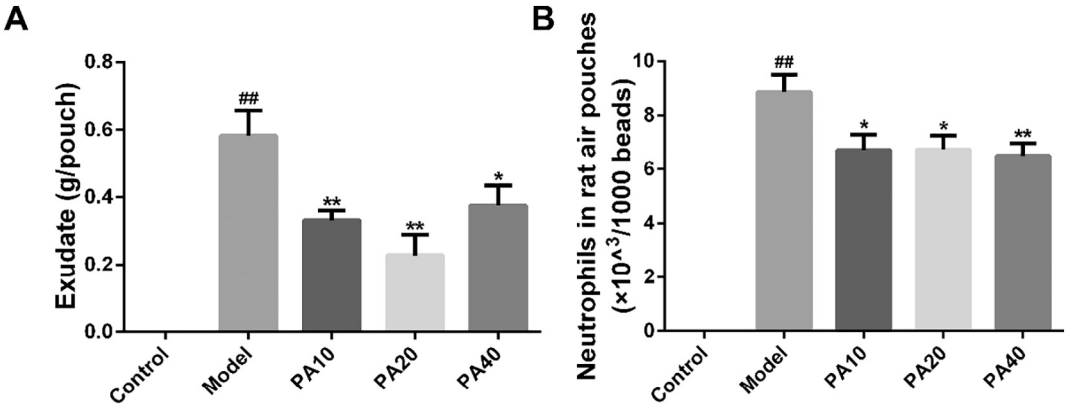


**Fig. 2.** Effect of PA on exudate and neutrophil recruitment caused by *H. pylori*. Exudate was collected and weight. 200 μl exudate from each group was removed for neutrophils counting by using BD absolute counting tubes through flow cytometry. Analysis was administered by comparing the number of neutrophil when 1000 beads was collected during flow. A: exudate weight; B: neutrophil number. Data were presented as the mean ± S.E.M. (n = 6). $^{\#\#}P < 0.01$ vs control, $^{*}P < 0.05$, $^{**}P < 0.01$ vs model.

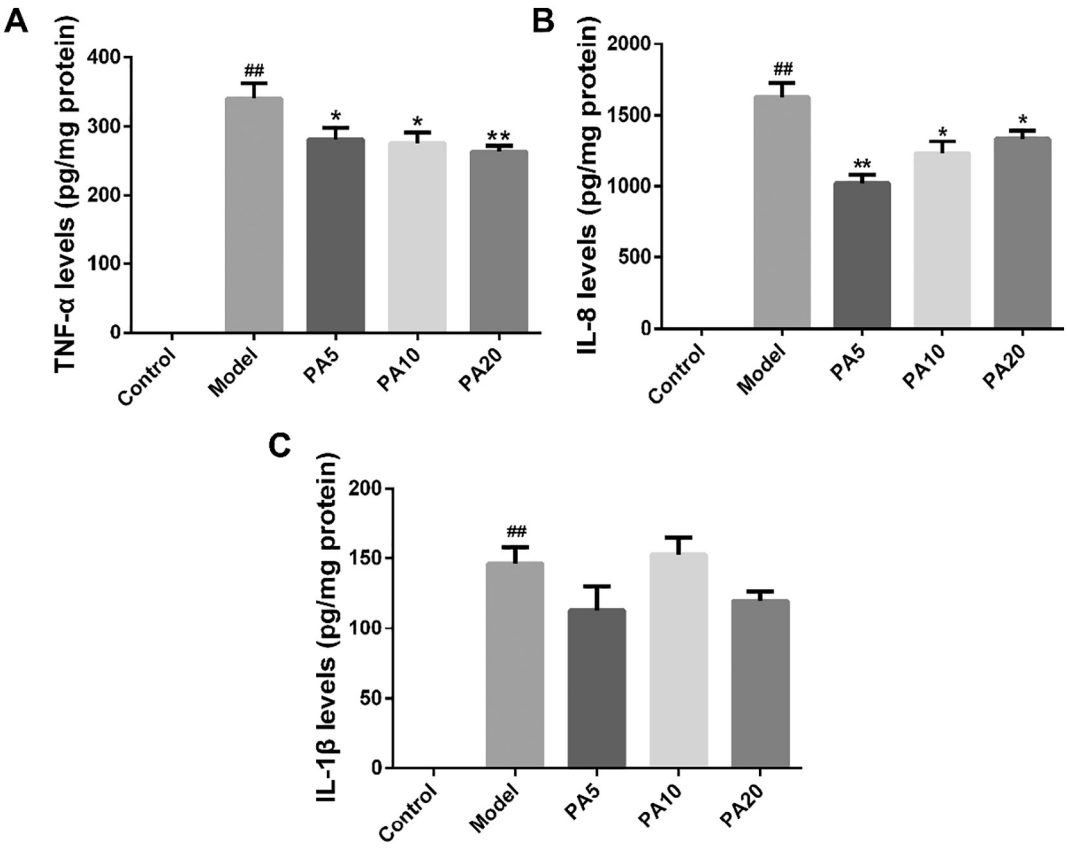


**Fig. 3.** Effect of PA on pre-inflammatory factors releasement in exudate. 200 μl exudate from all groups were used for total protein extraction using RIPA lysis buffer. Protein concentration was determined by BCA quantitative method. Pre-inflammatory factor levels were detected using ELISA kits and equalized by total protein concentration. A: TNF-α; B: IL-8; C: IL-1β. Data were presented as the mean ± S.E.M. (n = 6). $^{\#\#}P < 0.01$ vs control, $^{*}P < 0.05$, $^{**}P < 0.01$ vs model.

previously described [15]. *H. pylori* was resuspended in BHI supplemented with 10% FBS, and then treated with MET (0.5 μg/mL),5, 10 and 20 μmol/L PA. DMSO served as the control. After incubated at 37 °C for 1 h in the tri-gas incubator, bacteria were collected by centrifugated at 2500 rpm for 5 min, and TRIzol reagent was adopted to isolate RNA. RNA isolation, reverse transcription and qRT-PCR were performed following the manufacturer's instructions. The cycling protocol was set at 1 cycle at 95 °C for 15 min and 40 cycles at 95 °C for 5 s and 60 °C for 30 s. The data were analysed using the Pfaffl method, and the 16 s gene served as the internal reference. Sequences of the PCR primers are listed in Table 1.

### 2.13. Statistical analysis

All results were presented as means ± standard errors of the means and analysed using ANOVA in SPSS 21.0. The least significant difference test or Dunnett's test was used for multiple groups based on homogeneity of variance. Statistical significance was considered at $P < 0.05$.

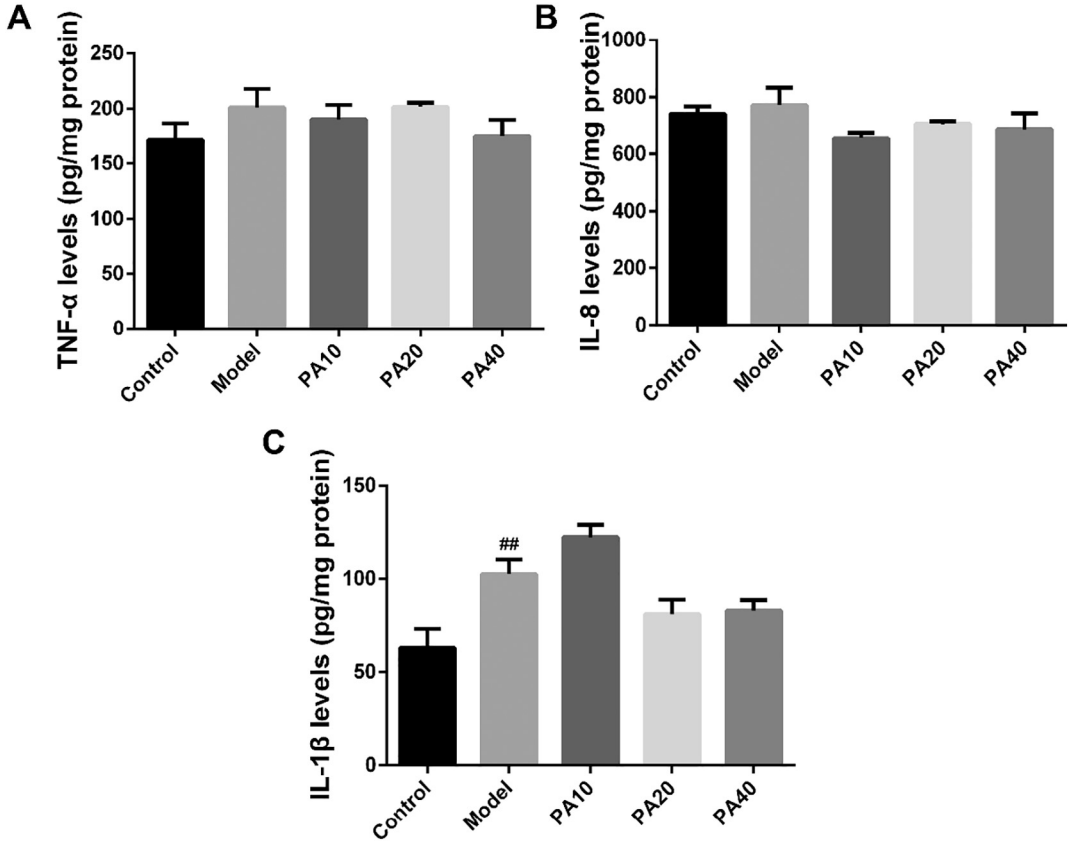


**Fig. 4.** Effect of PA on pre-inflammatory factors in mucous. Total protein was extracted with RIPA lysis buffer and concentration was determined by BCA quantitative method. Pre-inflammatory factor levels were detected using ELISA kits and equalized by total protein concentration. A: TNF-α; B: IL-8; C: IL-1β. Data were presented as the mean ± S.E.M. (n = 6). $^{\#\#}P < 0.01$ vs control.

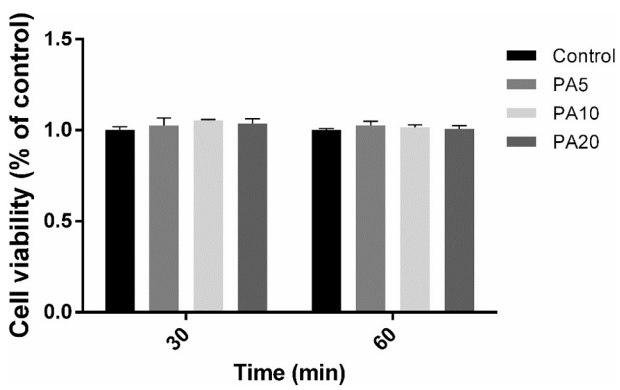


**Fig. 5.** Effect of PA on neutrophil cell viability. Cells were treated with PA (5, 10 and 20 μmol/L) for 30 or 60 min, and then the cell viability was assessed by CCK-8 assay. The results were presented as compared with control viability. Data were expressed as mean ± S.E.M. (n = 4).

## 3. Results

### 3.1. Effect of PA on neutrophil recruitment

As shown in Fig. 2A, compared with control group (scarcely any exudate was collected in control group), a large amount of inflammatory exudate was obtained ($P < 0.01$) when animal injected with *H. pylori* ($1.2 \times 10^9$ CFU/mL for 6 h). Pre-treatment with PA at 10, 20, 40 mg/kg distinctly reduced exudate generation ($P < 0.01$ or $P < 0.05$). Moreover, an equal amount of exudate from each group was removed for neutrophil counting. In model group, a vast number of neutrophils were detected ($P < 0.01$). By contrast, PA showed reliable effect on reducing neutrophil number in exudate ($P < 0.01$ or $P < 0.05$) (Fig. 2B). Taken together, PA inhibited the recruitment of neutrophil induced by *H. pylori*.

### 3.2. Inhibition of PA on the level of neutrophil recruitment-related pre-inflammatory factors

As shown in Fig. 3A–C, the significant increment of TNF-α, IL-8, IL-1β levels in exudate were observed in model group ($P < 0.01$). Pre-treatment with PA significantly decreased the levels of TNF-α and IL-8 in exudate ($P < 0.01$ or $P < 0.05$). In mucous, the level of IL-1β was remarkably increased in model group, whereas PA exerted no significant effect on IL-1β releasement (Fig. 4C).

### 3.3. Effect of PA on neutrophil cell viability

As shown in Fig. 5, no significant difference was observed on the viability of neutrophil treated with PA in 5–20 μmol/L. Therefore, 5, 10, or 20 μmol/L PA were adopted for further experiments.

### 3.4. Effect of PA on ROS generation

As shown in Fig. 6, in the graphs of flow cytometry, the peak values of model or MET group shifted to the right side as compared with control group, whereas the peak values of PA (5, 10, or 20 μmol/L) group slightly shifted to the left as compared with model group. For quantitative analysis, the DCF fluorescence intensity in the model group was significantly increased when compared with control group ($P < 0.01$), proving that 50 μg/mL HWSP effectively caused intracellular ROS production. No significant difference between drug groups and model group.

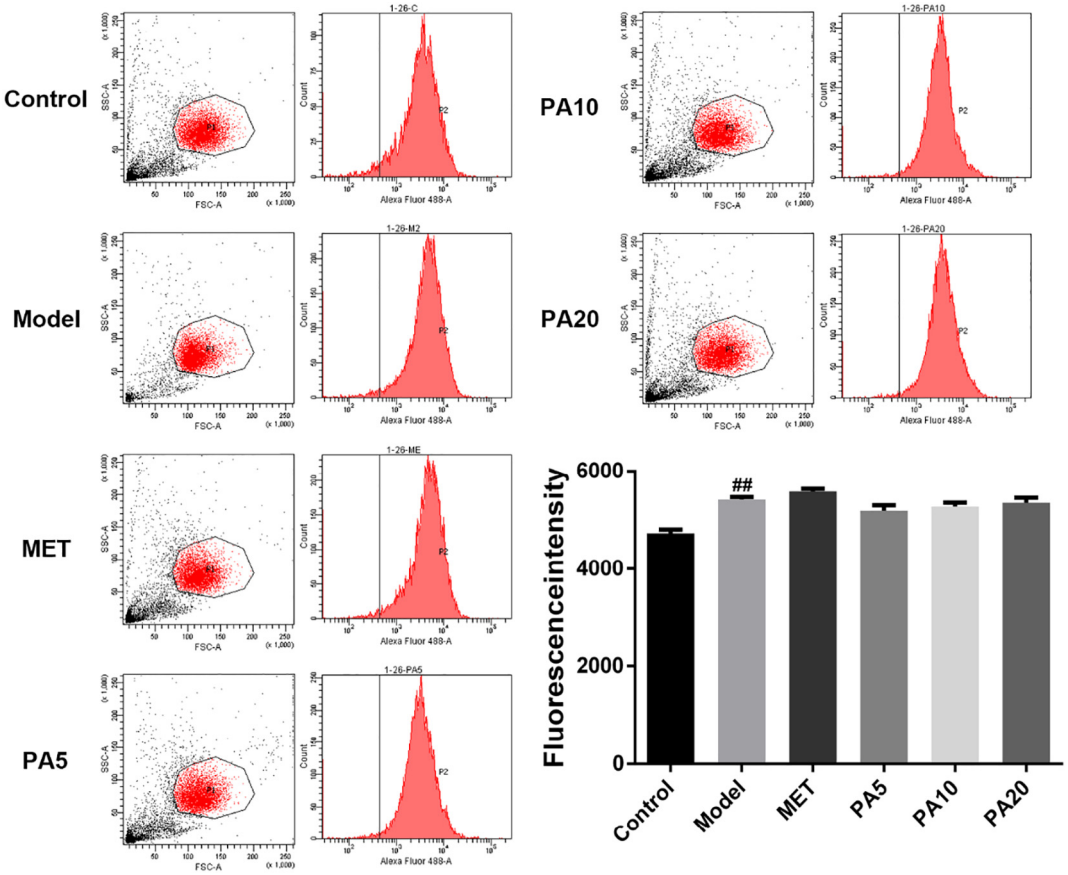


**Fig. 6.** Effect of PA on HWSP-induced intracellular ROS generation in neutrophil measured by flow cytometry. Neutrophil intracellular ROS was stained with DCF-DA for 20 min at 37 °C. And then, neutrophil was activated by 50 μg/mL HWSP (equal amount PBS for control group), and treated with MET (0.5 μg/mL) or PA (5, 10, or 20 μmol/L). 30 min latter, samples were subjected to FACS to analyse the intracellular ROS accumulation. Data were presented as the mean ± S.E.M. (n = 8). $^{\#\#}P < 0.01$ vs control.

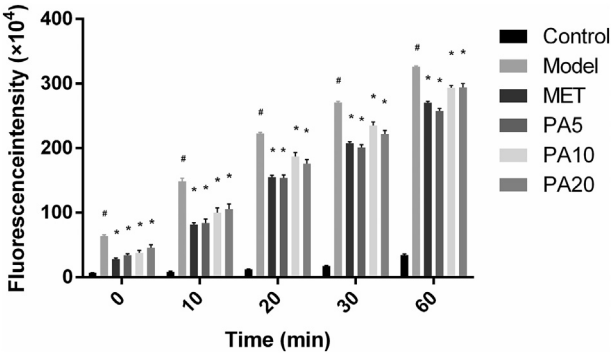


**Fig. 7.** Effect of PA on HWSP-induced extracellular ROS generation in neutrophil measured by immunofluorescence. Neutrophil was activated by 50 μg/mL HWSP (equal amount of PBS for the control group), and treated with MET (0.5 μg/mL) or PA (5, 10, or 20 μmol/L) at 37 °C for 30 min. And then samples were incubated with OxyBURST Green $H_2$HFF BSA reagents. Extracellular ROS accumulation was detected using SpectraMax i3x Multi-Mode reader and measured at 0, 10, 20, 30, 60 min. Data were presented as the mean ± S.E.M. (n = 8). $^{\#}P < 0.01$ vs control, $^{*}P < 0.01$ vs model. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

As shown in Fig. 7, from 0 to 60 min, a significant increase of fluorescence intensity was observed in the model group as compared with control group ($P < 0.01$), indicating that the extracellular ROS production was obviously increased in the model group. Treatment with PA or MET inhibited extracellular ROS generation clearly ($P < 0.01$).

From the results of ROS generation experiment, we can apparently conclude that HWSP could induce intracellular and extracellular ROS generation in neutrophil. Treatment with PA or MET reduced extracellular ROS production as compared with the model group, while no similar tendency was observed in intracellular ROS experiment.

### 3.5. Effect of PA on p22phox/p47phox protein and gene expression

As shown in Fig. 8A–C, 50 μg/mL HWSP significantly increased the expression of p22phox/p47phox protein ($P < 0.01$), while the remarkable inhibition on the p22phox/p47phox levels were observed after treatment of PA at 5, 10 and 20 μmol/L ($P < 0.01$). Significant decrease was also observed as compared MET group with model group. Similar results were shown in qRT-PCR assay, indicating that the alteration of those two protein levels was attributed to the down-regulation on *p22phox* and *p47phox* gene expression ($P < 0.01$).

As shown in Fig. 9, p22phox (red) and p47phox (green) were obviously decreased with the treatment of PA at 5, 10 and 20 μmol/L. Colocalization (orange) of p22phox and p47phox was analysed using image pro plus software by calculating the Pearson's correlation coefficient and Manders' overlap coefficient [21]. As presented in Fig. 9, both of the Pearson's correlation coefficient and Manders' overlap coefficient were increased in model group, whereas the remarkable decrements were observed in PA low-, middle- and high dose groups.

All these data strongly confirmed that PA can reduce HWSP-induced ROS generation through its inhibition on p22phox/p47phox gene and protein expression, as well as protein assembling in both protein and gene levels.

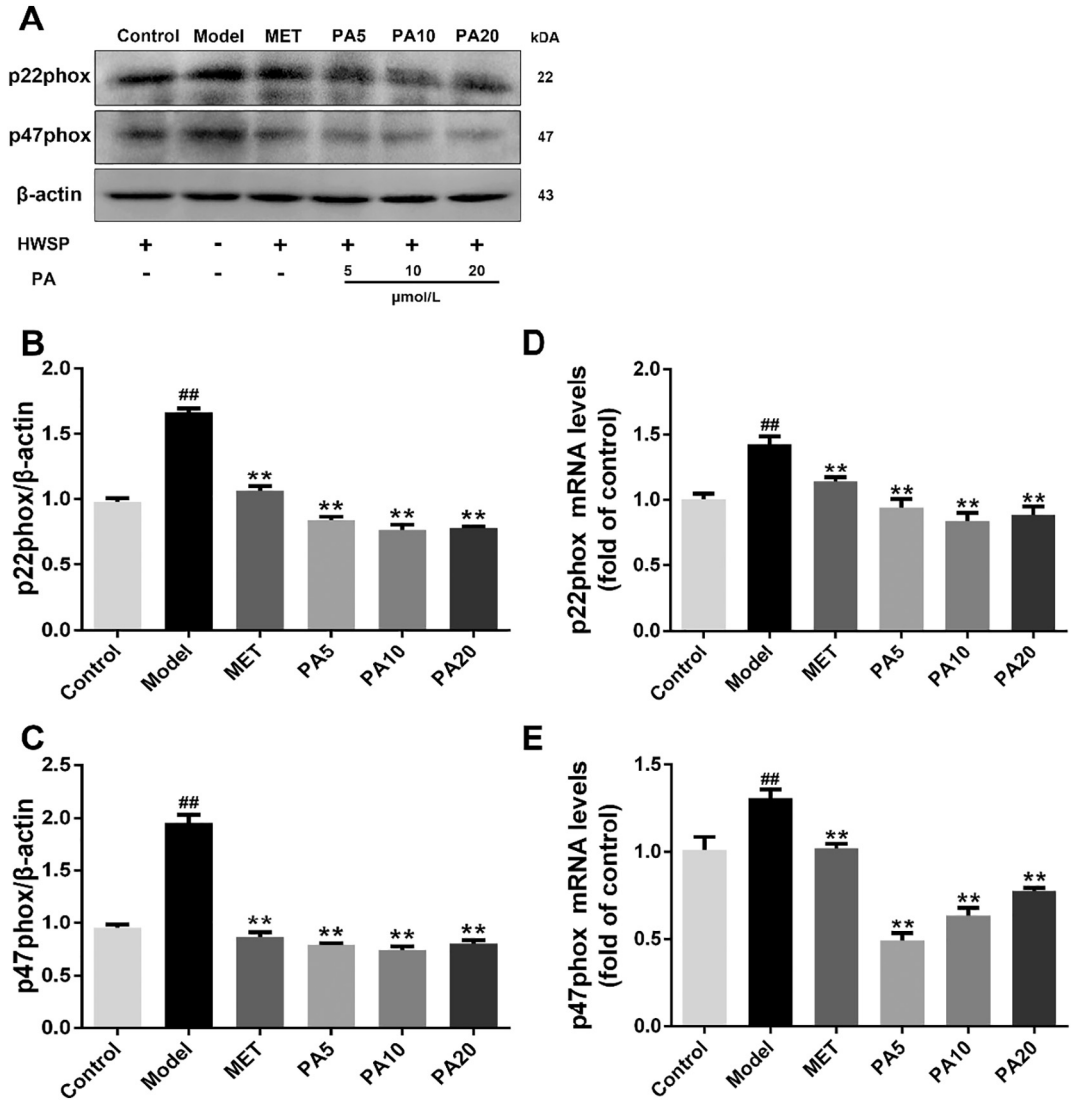


**Fig. 8.** Effect of PA on p22phox/p47phox protein and mRNA expression. Neutrophils were activated by 50 μg/mL HWSP (equal amount of PBS for control group), and treated with MET (0.5 μg/mL) or PA (5, 10, or 20 μmol/L) at 37 °C for 30 min. After treatment, total protein was extracted by RIPA lysis buffer. p22phox and p47phox protein were quantified by Western blot. β-actin was defined as internal reference. RNA was isolated with TRIzol reagent, and qRT-PCR was employed to analyse the gene expression of *p22phox* and *p47phox*. *Gapdh* gene was defined as internal reference. A: p22phox/p47phox bands; B: p22phox protein; C: p47phox protein; D: *p22phox*; E: *p47phox*. Data were presented as the mean ± S.E.M. (n = 8). $^{\#\#}P < 0.01$ vs control, $^{**}P < 0.01$ vs model.

### 3.6. Effect of PA on H. pylori h-nap and sabA gene expression

Two important *H. pylori* genes, *h-nap* and *sabA*, were thought to play vital role in neutrophil recruitment. As shown in Fig. 10, treatment with PA at 10 or 20 μmol/L significantly down-regulated the expression of *h-nap* and *sabA* ($P < 0.05$), while MET markedly up-regulated those genes expression, indicating PA decreased the capacity of *H. pylori* in causing neutrophil recruitment.

## 4. Discussion

*H. pylori* is an ancient microorganism with tenacious vitality that has rooted in the stomach of half the world's population. Some vital factors contribute to the survival of the bacteria in the stomach. One is urease produced from *H. pylori*, which makes it tolerant to the acid surroundings. Another is flagellar-based motility, which allows it penetrates through the mucus and adheres to gastric epithelial cells. The special pathogen-associated molecular patterns of *H. pylori* have evolved to evade detection by the innate immune recognition. Besides, the toxins (such as VacA and CagA) released from *H. pylori* disturb the normal apoptosis system of gastric epithelial cell and increase the expression of inflammatory factors. Such the persistent colonization would lead to chronic gastritis and other diseases [22,23].

Triple therapy has provided convince therapeutic effects; however, it has offered no relief on the development of some gastric diseases caused by *H. pylori*, such as metachronous gastric cancer, in spite successful bacterial eradication, thereby indicating that some alternative or additional drugs may be used for the co-existing diseases [24]. In the acute stage of *H. pylori* infection, the neutrophils, which is the natural immune cell in humans, is massively recruited to the gastric mucosa; this is a characteristic feature of *H. pylori*-induced acute inflammation [25]. In our in vivo experiment, the air pouch model was especially selected to investigate the effects of PA on *H. pylori*-induced neutrophil infiltration because the exudate in the pouch mainly consisted of neutrophils. Establishing an acute gastritis animal model using *H. pylori* is difficult; therefore, we used the air pouch rat model because it is a simple and adoptable animal model for drug research and development. Results in Fig. 1 describe the inhibition of exudate formation and neutrophil recruitment by PA.

Its specific mechanism is unclear, but neutrophil recruitment is

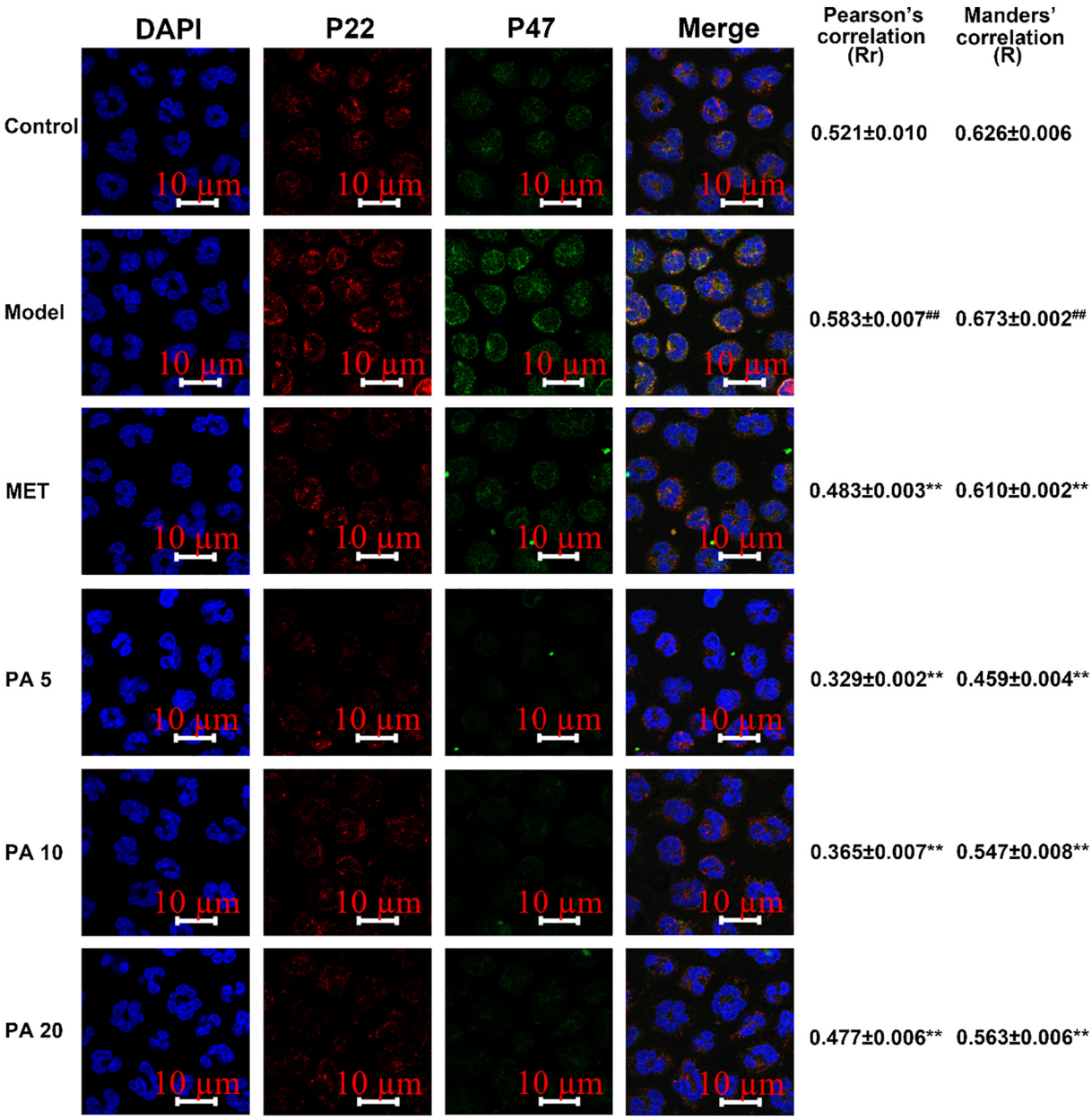


**Fig. 9.** Effect of PA on p22phox/p47phox protein photographed by laser confocal. Neutrophils were activated by 50 μg/mL HWSP (equal amount of PBS for control group), and treated with MET (0.5 μg/mL) or PA (5, 10, or 20 μmol/L) at 37 °C for 30 min. After treatment, cells were resuspended by PBS, dropped to an adhesion microscope slide and fixed with 4% paraformaldehyde at room temperature. After incubation with P22phox/P47phox primary antibodies and Alexa Fluor 488/555-conjugated corresponding secondary antibodies, pictures were acquired by confocal. Analysis was performed using the image pro plus software by calculating the Pearson's correlation coefficient and Manders' overlap coefficient. $^{\#\#}P < 0.01$ vs control, $^{*}P < 0.05$, $^{**}P < 0.01$ vs model.

commonly considered as a result of the signal conduction of endogenous and bacterial chemoattractant signals [26]. Moreover, the immune and epithelial cells contribute to the immunoreaction caused by *H. pylori* infection. Several pro-inflammatory factors, including IL-8, IL-1β and TNF-α are the main promoters [27]. These factors trigger macrophages and mononuclear cells, both of which are immune cells that could generate IL-8 and cause neutrophil infiltration and inflammation aggravation. Significant changes in pro-inflammatory factors have been observed in numerous *H. pylori* infection studies; however, the interaction among them have not been illustrated clearly [28–30]. In this study, after treatment with *H. pylori*, significant increases in the levels of IL-8, IL-1β and TNF-α in the exudate were observed, similar to results from previous research. Moreover, the effects of PA on TNF-α and IL-8 regulation were confirmed. Based on in vivo results, we confirmed the effects of PA in reducing neutrophil recruitment, and the mechanisms were associated with the reduction of inflammatory factor release.

In the in vitro experiment, neutrophils were isolated to explore the effects of PA on neutrophil activation, and HWSP was used as the stimulant to activate neutrophils. HWSP and its related purified proteins have been commonly adopted to explore the influence and mechanism of *H. pylori* on immune responses [31–33]. In order to obtain a reasonable HWSP concentration for further experiments, the isolated neutrophils were treated with different HWSP concentrations, with ROS level as indicator (6.25–100 μg/mL HWSP was used, and results are shown in Supplementary material). For neutrophil activation, we focused on ROS generation. Generally, neutrophils work under a rigorous system involving internalization, phagosome-granule fusion and toxic ROS targeting. *H. pylori* would be phagocytosed by neutrophils, and the phagosomes were formed subsequently. Then, the intracellular ROS

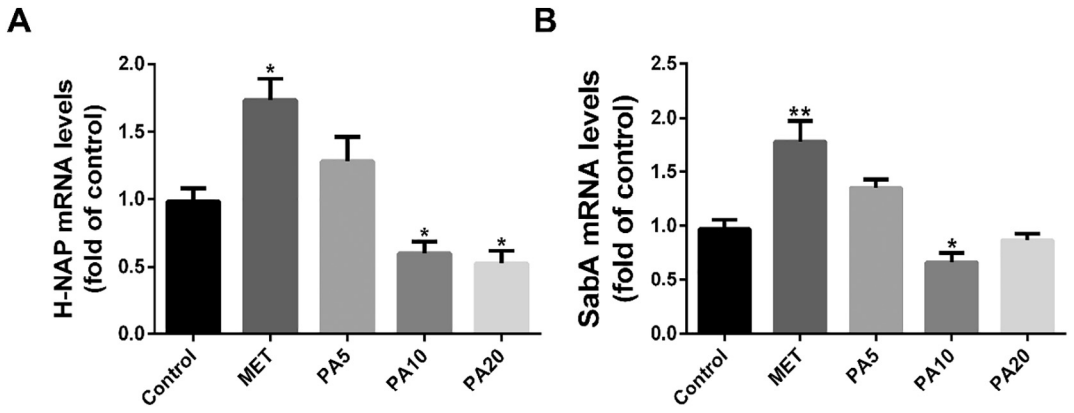


**Fig. 10.** Effect of PA on *H. pylori h-nap* and *sabA* gene expression. *H. pylori* was treated with PA (5, 10, or 20 μmol/L), or MET (0.5 μg/mL) at 37 °C for 1 h in a tri-gas incubator. After treatment, *H. pylori* was collected to isolate RNA, and qRT-PCR was employed to analyse *h-nap* and *sabA* gene expression. 16 s gene was defined as internal reference. A: *h-nap*; B: *sabA*. Data were presented as the mean ± S.E.M. (n = 8). $^{*}P < 0.05$, $^{**}P < 0.01$ vs control.

was recruited to the phagosomes and attacked the bacteria [34]. Intracellular and extracellular ROS were deemed to be activated and produced in different mechanisms. Mitochondria were the major organelle producing intracellular ROS, while extracellular ROS was generated at the external surface of cytomembrane [35]. Research of intracellular ROS generation was insufficient and limited. As shown in Fig. 6, it seems that PA didn't disrupt the generation of intracellular ROS while more evidence in the coming challenging research should be collected.

Although it is still an arduous job to clearly clarify the relation among *H. pylori*, neutrophils and the host, some researchers have reported that ROS overproduction cannot eradicate *H. pylori*, but becomes the major damaging factor to gastric mucosa in most cases of neutrophil-related inflammation [36–38]. Results in Fig. 7 show that 50 μg/mL HWSP significantly increased extracellular ROS, and treatment with PA reduce the amount of ROS, which was considered contributing to remit the ROS-induced inflammation.

NADPH phagocyte oxidase, which consists of gp91phox, p47phox, p67phox, p22phox and RAC2 unit, is the essential protein complex in generating ROS [39]. This complex is expressed in both polymorphonuclear phagocytes and mononuclear phagocytes; however, ROS production is higher in neutrophils than in macrophages. In the resting state, phox units are stable and separated. Once neutrophils are activated, phox units are assembled, and matured NADPH phagocyte oxidase would generate toxic ROS [40]. Our results revealed that HWSP can up-regulate the expression levels of p22phox/p47phox mRNA and protein in the isolated rat neutrophil, whereas PA can inhibit their expression levels. Cytosolic regulatory proteins (a heterotrimer consisting of p47phox, p40phox and p67phox) combing with flavocytochrome b558 (consisting of p22phox and gp91phox) was a critical step in ROS generation [41]; thus, Pearson's correlation coefficient and Manders' overlap coefficient of p22phox/p47phox were calculated to quantify the colocalization [21]. Data in Fig. 8 indicate that HWSP enhanced the binding degrees, whereas PA treatment weakened the assemblage. Inhibiting the conformation changes or the phosphorylation of p47phox could be another related mechanism, and further experiments to prove this hypothesis and mechanism should be done.

*H. pylori* toxic factors, namely, H-nap and SabA, are two important toxic factors that activate neutrophils as bacterial chemoattractant signals [10,42]. The available literature show that purified H-nap activates neutrophils through the pertussis toxin-sensitive pathway, subsequently inducing extracellular regulated kinase and p38-mitogen-activated protein kinase [43]. SabA was initially thought to be the adhesive factor of *H. pylori*; however, *H. pylori* strains without *sabA* lose their neutrophil-activating capacity, thereby indicating that binding to neutrophil receptors is one of the key steps in inducing neutrophil-ROS generation [11,42]. As presented in Fig. 9, down-regulation of *H. pylori h-nap* and *sabA* gene expression levels were observed after PA treatment, thus revealing that the capacity of *H. pylori* in activating neutrophil is restricted by PA.

PA, in addition to its anti-*H. pylori* and anti-ulcer activities, inhibited neutrophil infiltration and ROS generation induced by *H. pylori*, which would be the promote activity in the treatment of *H. pylori* related gastritis in vivo. PA showed immense therapeutic potential against *H. pylori* infection and was highly effective in eliminating *H. pylori* from infected mice, as well as in restoring *H. pylori*-induced acute gastritis. This study provides novel insights into the polyvalent therapeutic effects of PA against *H. pylori* infection.

## 5. Conclusions

In summary, the studies demonstrated that PA can inhibit the recruitment of neutrophils in rat air pouch model induced by *H. pylori*, and the underlying mechanisms were closely associated with the reduction of TNF-α and IL-8 levels. Besides, PA can inhibit the generation of extracellular ROS produced from neutrophils stimulated by HWSP, which contributes to alleviating oxidative damage. Inhibiting the production and combination of P22/P47phox subunits was the probably relevant mechanism. Besides, the potential ability of PA on down-regulation of *h-nap* and *sabA* gene expressions was considered to be a different mechanism in the therapeutic effect.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2018.12.044.

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