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Research Article

Polysaccharides from *Panax ginseng* promote intestinal epithelial cell migration through affecting the Ca^{2+} related regulators

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ABSTRACT

Background and aim: *Panax ginseng*, a key herbal medicine of replenishing Qi and tonifying Spleen, is widely used in the treatment of gastrointestinal diseases in East Asia. In this study, we aim to investigate the potential effects and mechanisms of polysaccharides from *P. ginseng* (PGP) on intestinal mucosal restitution which is one of the crucial repair modalities during the recovery of mucosal injury controlled by the Ca^{2+} signaling.

Methods: Rat model of intestinal mucosal injury was induced by indomethacin. The fractional cell migration was carried out by immunohistochemistry staining with BrdU. The morphological observations on intestinal mucosal injury were also performed. Intestinal epithelial cell (IEC-6) migration in vitro was conducted by scratch method. Western-blot was adopted to determine the expressions of PLC-γ1, Rac1, TRPC1, RhoA and Cav-1. Immunoprecipitation was used to evaluate the levels of Rac1/PLC-γ1, RhoA/TRPC1 and Cav-1/TRPC1.

Results: The results showed that PGP effectively reduced the assessment of intestinal mucosal injury, reversed the inhibition of epithelial cell migration induced by Indomethacin, and increased the level of Ca^{2+} in intestinal mucosa in vivo. Moreover, PGP dramatically promoted IEC-6 cell migration, the expression of Ca^{2+} regulators (PLC-γ1, Rac1, TRPC1, Cav-1 and RhoA) as well as protein complexes (Rac1/PLC-γ1, Cav-1/TRPC1 and RhoA/TRPC1) in vitro.

Conclusion: PGP increases the Ca^{2+} content in intestinal mucosa partly through controlling the regulators of Ca^{2+} mobilization, subsequently promotes intestinal epithelial cell migration, and then prevents intestinal mucosal injury induced by indomethacin.

1. Introduction

Gastrointestinal mucosal injury is one of the common histopathological features of Spleen-deficiency (a classic syndrome-type of Traditional Chinese Medicine, TCM) which is the main TCM syndrome of gastrointestinal disease in clinical practice [1]. After damage, the mucosa goes through an early restitution, a crucial repair process in gastrointestinal tract, presents as a result of resealing the wounds by epithelial cell migration, which is independent of cell proliferation [2]. This rapid reepithelialization acts an essential role in maintaining the structural and functional integrity of gastrointestinal tract, which is highly controlled by multiple cellular factors including cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) [3].

Panax ginseng, a key herbal medicine for replenishing Qi and tonifying Spleen, is widely used in the treatment of gastrointestinal diseases, especially those with Spleen-deficiency syndrome in East Asia [4]. Early clinical research has found that chewing *P. ginseng* alone could effectively relieve upper abdominal pain and accelerate the healing of peptic ulcer [5]. Our previous works showed that *P. ginseng* had prophylactic effect on intestinal mucosal damage caused by indomethacin in rats [6]. Importantly, accelerating the recovery of gastrointestinal diseases like ulcerative colitis and crohn's disease by promoting the repair of mucosal injury has become a strategy of clinical treatment [7,8]. Moreover, epithelial cell migration knows as early restitution, is such an essential repair

Abbreviations: BrdU, 5-bromo-2'-deoxyuridine; Cav-1, Caveolin-1; CCE, CapacitativeCa^{2+} entry; ER, Endoplasmic reticulum; HPGPC, High-performance gel permeation chromatography; IEC-6, Intestinal epithelial cell; IP_3, Inositol (1,4,5)-tresphospate; PGP, Polysaccharides from Panax ginseng; PLC-γ1, Phospholipase C-γ1; Put, Putrescine; SOCs, Store-operated Ca^{2+}channels; TCM, Traditional Chinese Medicine; TRPC1, Canonical transient receptor potential-1; $[Ca^{2+}]_{cyt}$, Cytosolic free Ca^{2+}.

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modality throughout gastrointestinal mucosal injury. Therefore, it is particularly significant to further explore the mechanism of *P. ginseng* on promoting mucosal healing via early restitution. The benefits of *P. ginseng* on gut are due to its primary active ingredient, polysaccharides (PGP), which could be useful for treating gastric ulcer and colitis by its protection on gastrointestinal epithelial barrier and anti-inflammatory activity in rats [4]. Thus, we further investigated the pharmacological effects of *P. ginseng* on intestinal mucosal injury by selecting polysaccharides as the candidate.

Study on intestinal epithelial cell (IEC-6) found that $[Ca^{2+}]_{cyt}$ is a critical regulator of epithelial cell migration during mucosal restitution. The increased $[Ca^{2+}]_{cyt}$ could promote intestinal mucosal restitution both in vitro and in vivo [9]. $[Ca^{2+}]_{cyt}$ relies on the release of Ca^{2+} from intracellular Ca^{2+} store (endoplasmic reticulum, ER), or the extracellular Ca^{2+} entry through Ca^{2+}-permeable channels, which are the crucial mechanisms participate in maintaining a suitable increase in $[Ca^{2+}]_{cyt}$. Phospholipase C-γ1 (PLC-γ1) catalyzes the formation of diglyceride and inositol (1,4,5)-tresphospate (IP_3) which could combine with the IP_3 receptor on ER, and then involves in modulation of $[Ca^{2+}]_{cyt}$ via regulating the release of Ca^{2+} store as well as Ca^{2+} influx [10]. Besides, Rac1, a member of the Rho family of GTPases, could regulate IEC-6 cell migration through altering PLC-γ1-induced Ca^{2+} elevation [11]. As the store-operated Ca^{2+} channel, canonical transient receptor potential-1 (TRPC1) acts an important part in epithelial restitution by controlling $[Ca^{2+}]_{cyt}$ [12]. Furthermore, RhoA and caveolin-1 (Cav-1) could respectively interact with TRPC1, then accelerate the Ca^{2+} influx mediated by TRPC1 [12,13]. Thus, this study aims to assess the effect of PGP on intestinal epithelial restitution, and further explicate the underlying mechanisms during epithelial cell migration via the Ca^{2+} regulation signaling.

2. Materials and methods

2.1. Herbs and regents

Panax ginseng Meyer was provided by Hebei Chufeng Chinese Medicine Decoction Pieces Co.Ltd (Lot.NO.B704201). Indomethacin (Lot.056M4036V0) and Putrescine (Put, Cat.No.51799) were purchased from Sigma (MO, U.S.A); DEAE-52 cellulose filler (Cat.-NO.C8930) was purchased from Solarbio (Beijing, China). Australian fetal bovine serum (REF10099-141, Lot.No.1846382) were purchased from Gibco (Shanghai, China). Calcium colorimetric assay kit (Cat.NO.S1063S) and Protein A+G Agarose (Fast Flow, for IP, Cat.No.P2055-50 mL) was obtained from Shanghai Beyotime Biotechnology (Shanghai, China). Protein G Plus/Protein A Agarose Suspension (Cat.No.IP05-1.5 mL) were obtained form Millipore (Shanghai, China). Rabbit monoclonal anti-RhoA antibody (Cat.-No.ab187027), Rabbit monoclonal anti-TRPC1 antibody (Cat.-No.ab51255), Mouse monoclonal anti-Rac1 antibody (Cat.No. ab33186), Rabbit monoclonal anti-PLC-γ1 antibody (Cat.-No.ab76155), Rabbit monoclonal anti-GAPDH antibody (Cat.-No.ab181602), Goat Anti-Mouse IgG H&L (HRP, Cat.No.ab6789) and Goat Anti-Rabbit IgG H&L (HRP, Cat.No.ab6721) were purchased from Abcam (MA,U.S.A). Rabbit monoclonal anti-Cav-1 antibody (Cat.No.CST#3267) was obtained from CST Corporation (MA, U.S.A). Mouse monoclonal anti-BrdU antibody was provided by Santa Cruz Biotechnology, INC (TX, U.S.A).

2.2. Cell

IEC-6 cells were purchased from the American Type Culture Collection (Ca.No. CRL-1592, Lot.NO.70009131).

2.3. Animal

Male Sprague-Dawley rats (180–220 g) were obtained from Guangdong Medical Experimental Animal Center [SCXK(Guangdong) 2018-0002, NO.44007200072394]. Animal had free access to water and food, and were maintained in SPF laboratory animal room provided by Guangzhou University of Chinese Medicine. The animal experimental protocol was approved by The Committee of Animal Ethics, Guangzhou University of Chinese Medicine.

2.4. Extraction, separation and purification of P. ginseng polysaccharides

According to our previous work [14], *P. ginseng was* powdered, soaked for 2 h in 12-times amount of pure water, boiled for another 2 h, and filtered, then these processes were repeated once again. After being collected and concentrated, the decoction was precipitated with 95% ethanol overnight at 4 °C, which was repeated for 3 times. Then, the precipitation was dissolved, centrifugated and freeze-dried to produce *P. ginseng* polysaccharides1 (PGP1). PGP1 was deproteinized by shaking vigorously with Sevag reagent (chloroform and *n*-butyl alcohol) to obtain PGP2 which was applied in vivo. PGP2 was dissolved and load into a DEAE-52 cellulose chromatography column which was eluted with ultrapure water at a flow rate of 1.7 mL/min. Subsequently, the eluent was gathered and freeze-dried to make PGP3 which was used in vitro.

2.5. Properties and homogeneity of PGP

The total carbohydrate of PGP was tested through Phenol-sulfuric acid assay with D-glucose as the standard. According to Lowry's method [15], the determination of protein content of PGP2 was conducted by using bovine serum albumin as the standard. The homogeneity of PGP3 were evaluated by high-performance gel permeation chromatography (HPGPC) on a Waters G 4000 PWXL column (MA, U.S.A) as described previously [14].

2.6. Relative molecular weight and monosaccharide composition of PGP3

The molecular weight of PGP3 were also determined by using HPGPC with glucan as the standard, while the monosaccharide composition of PGP3 were analyzed with GC-MS. These two experiments were completed by China National Analytical Center, Guangzhou.

2.7. Experimental protocol in vivo

According to Okayama' method of modeling with some modifies [16], 32 Rats were equally and randomly divided into 4 groups: Control group, indomethacin group, PGP (0.253 g/kg) group, PGP (0.759 g/kg) group. After being adaptively fed for 5 days, the model of intestinal mucosal injury was replicated and administered. All rats were treated by gavage (1 mL/100 g) per day, rats in PGP group were treated with PGP2 (0.253 g/kg or 0.759 g/kg, dissolved in pure water) by intragastric gavage, while rats in other group were given pure water. One hour after intragastric gavage, rats in model group and PGP group were subcutaneously injected with indomethacin (5 mg/kg, dissolved in $NaHCO_3$ solution), rats in control group were treated with same amount of saline once daily for 4 days. 24 h after the last treatment, the animals were sacrificed, the ulcers and perforations of small intestine were observed, and the small intestinal tissue samples were collected.

2.7.1. Histological assessment of intestinal mucosal injury

Paraffin section of intestinal mucosa were stained with Hematoxylin and Eosin, and were assessed to determine the degree of pathological changes by pathology practitioner who was not aware of the experimental grouping. The pathological changes were divided into 6 grades on basis of Chiu's method with some modifies, which had been described in our previous research [14].

2.7.2. Determination of Ca^{2+} content in small intestinal mucosa

The intestinal samples were weighed, homogenized and lysed on ice, then the Ca^{2+} content of lysate was assessed by measuring the absorbance at 575 nm with calcium solution as a standard.

2.7.3. Immunohistochemistry staining

18 h before sacrificed, rats were intraperitoneally injected with 5-bromo-2′-deoxyuridine (BrdU, 50 mg/kg, dissolved in saline solution). The labeling with BrdU immunohistochemistry staining was performed in accordance with the previous study [17].

2.7.4. Estimation of cell migration

According to Silver's method [18], the length of 10 complete villus and crypts from per animal were measured, in order to provide an overall mean length of villus and crypts for each groups($\overline{L}_a$). Moreover, the maximum distance (L_b) of BrdU-positive cells along the villus-crypts axis in 10 villus and crypts per animal were also collected. Then, the fractional migration distance of mucosal epithelial cell in each animals was carried out on the basis of this formula $\triangle L = L_b / \overline{L}_a$.

2.8. Experimental protocol in vitro

2.8.1. Cell culture

IEC-6 cells were maintained in DMEM containing 5% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin, which were incubated in a 95% air −5% CO_2 humid atmosphere at 37 °C.

2.8.2. Cell migration assays

Cells were scratched by micro-pipette tip to generate areas available for migration, and were treated with control, putrescine (Put, positive control) and PGP3. Then, the area of cell migration were photographed at 0 h (S_0) and 12 h (S_{12}) after treatment. Cell migration were calculated as the ratio of (S_0−S_{12}) to $\overline{S}_0$.

2.8.3. Western-blot analysis

Semi-quantitative analysis of proteins TRPC1, RhoA, Cav-1, PLC-γ1, Rac1 and GAPDH expression were analyzed by Image Lab software. GAPDH was used as a loading control.

2.8.4. Immunoprecipitation assays

Equal amounts (400 μg) of protein in lysates from different groups were incubated with specific antibodies (anti-TRPC1 or anti-PLC-γ1, 1:100) or control IgG overnight at 4 °C, then were precipitated by Protein G Plus/Protein A Agarose Suspension for 3 h at 4 °C. The agarose was washed with cold PBS buffer for 5 times and denatured in 1X SDS-PAGE loading buffer. For immunoblotting, samples were also analyzed by western-blot.

2.9. Statistical analysis

Statistical analysis was conducted by using SPSS 19.0 software. Measurement data of non-normal distribution was expressed as $\overline{R}$ (P25, P50, P75), and analyzed by Kruskal-Wallis H tests and Nemenyi test. Measurement data of normal distribution was performed as means ± SD, and the differences in mean was determined by ANOVA. Then, the multiple comparisons were analyzed by using S−N−K method or Dunnett's T3 method. Statistical significance was set at $P < 0.05$.

3. Results

3.1. The extraction and properties of PGP

After extraction, the yields of PGP1, PGP2 and PGP3 from *P. ginseng* were 6.50%, 5.06% and 2.70% respectively. The result of phenol-sulfuric acid assay revealed that the total carbohydrate of PGP1, PGP2 and PGP3 were 65.25%, 77.38% and 97.21% respectively. Furthermore, the protein content of PGP2 was 1.70%, suggesting that proteins in polysaccharides had been almost removed. HPGPC analysis showed that PGP3 had two major peak around 14.45 min and 20.70 min (Fig. 1), which means PGP3 wasn't homogeneous polysaccharides. Moreover, the average molecular weight of PGP3 was $4.64*10^6$ Da, and the monosaccharide composition of PGP3 was showed in supplementary Table 1, in which the amount of Glc was most abundant.

3.2. Effect of PGP on intestinal mucosal injury in vivo

3.2.1. Visual observation and score of intestinal mucosa

There was no macroscopic damage on intestinal mucosa in Control group, but indomethacin (5 mg/kg) induced distinct injuries presented as large numbers of intestinal ulcers, perforations as well as longer total length of intestinal ulcers, which could be alleviated by the treatment with PGP (Fig. 2A and B). The average number and total length of intestinal ulcers in group of indomethacin were 27.43 ± 15.91 and 66.21± 29.01 mm, respectively (Fig. 2B). The treatment with PGP 0.253 g/kg and 0.759 g/kg respectively reduced the average number of intestinal ulcers by 64.75% and 76.05%, and reduced the total length of intestinal ulcers by 68.78% and 79.72% ($P < 0.05$, Fig. 2B). Besides, the medium number of intestinal perforations were 3, 0 and 0 in group of indomethacin, PGP 0.253 g/kg and PGP 0.759 g/kg respectively, treatment with PGP 0.759 g/kg could reduce intestinal perforations ($P < 0.05$).

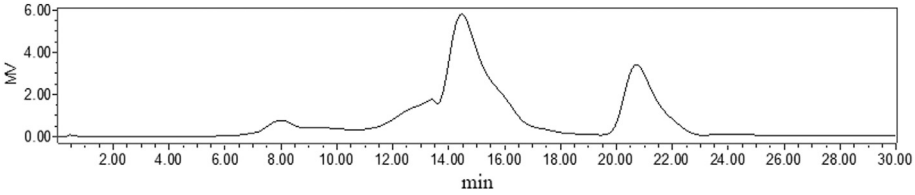

Fig. 1. HPGPC analysis of PGP3.

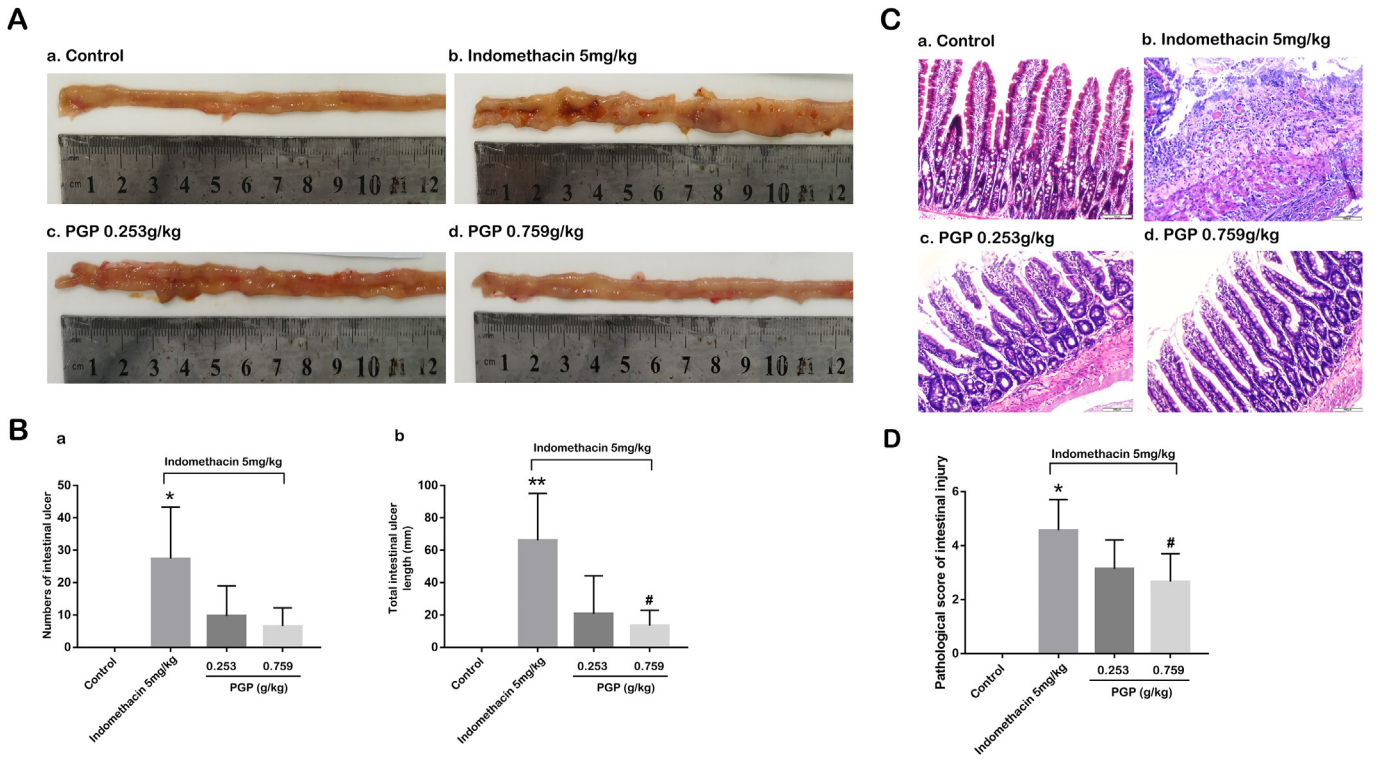

Fig. 2. Effects of PGP on indomethacin-induced intestinal mucosal injury in rats. (A) Macroscopic appearance of indomethacin-induced intestinal mucosa injury (B) Histograms presented the average numbers (a) and total length (b) of intestinal ulcers from macroscopic observation. (C) Histopathological changes of intestinal mucosa (× 100). (D) Histogram showed the histopathological score of intestinal mucosal injury. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared to Control group; $^{\#}P < 0.05$, as compared to Indomethacin group.

3.2.2. Pathological grading of intestinal mucosa

There was also no injured intestinal mucosa in Control group has been observed under light microscopy. However, after indomethacin intervention, the intestinal mucosal injury was induced like degenerated and necrotic epithelium, shed villi, dislocated lamina propria, and ulcers, which was abated by the treatment of PGP. In addition, the pathological score of intestinal mucosal injury in PGP 0.759 g/kg group was significantly less than that in Indomethacin group ($P < 0.05$, Fig. 2).

3.3. Effect of PGP on cell migration in vivo

Intestinal epithelial cell migration is a vital repair process during mucosal restitution, thus we further detected the cell migration by labeling with BrdU in rats. Comparing with the Control group, the administration of indomethacin significantly depressed the duodenal cell migration ($P < 0.05$, Fig. 3A and B). However, treatment with PGP (0.253 and 0.759 g/kg) could reverse the depression of cell migration induced by indomethacin ($P < 0.05$, Fig. 3A and B), and almost restore to normal values.

3.4. Effect of PGP on the content of Ca^{2+} from small intestinal mucosa

Since Ca^{2+} is a crucial regulator on intestinal epithelial cell migration, we further confirmed the content of Ca^{2+} in intestinal mucosa by colorimetric determination. After indomethacin intervention, the content of Ca^{2+} in the small intestinal mucosa was slightly decreasing ($P > 0.05$, Fig. 3C). While, the content of Ca^{2+} in PGP 0.759 g/kg group was significantly increased as compare to Indomethacin group ($P < 0.05$, Fig. 3C).

3.5. Effect of PGP on IEC-6 cells migration in vitro

IEC-6 cell migration after scratch damage was performed in Fig. 4. PGP (20, 40, 80 and 160 mg/L) remarkably promoted cell migration ($P < 0.05$ or $P < 0.01$), and the migration rates had increased to 114.6%, 117.5%, 125.6% and 129.8% of the Control group, which was similar as the intervention of putrescine (5 μmol/L).

3.6. Effect of PGP on expression of Ca^{2+} regulatory proteins and complexes

To elucidate the potential mechanism of PGP on accelerating intestinal epithelial cell migration, we further detected the expression of Ca^{2+} related regulators. As showed in Fig. 5, treatment with PGP (20, 40, 80 and 160 mg/L) as well as putrescine (5 μmol/L) overtly increased RhoA, TRPC1, Cav-1, Rac1 and PLC-γ1 proteins expression, in comparison with Control ($P < 0.05$ or $P < 0.01$), suggesting that PGP could improve Ca^{2+} regulatory proteins expression during cell migration after wounding. Protein complexes such as RhoA/TRPC1, Cav-1/TRPC1 and Rac1/PLC-γ1 are important regulators in facilitating Ca^{2+} influx in order to trigger and maintain intestinal epithelial cell migration after mucosal injury. Thus, we had tested the expression of these protein complexes during cell migration. As presented in Fig. 5 (G, H and I), control IgG immunoprecipitates of lysates from IEC-6 after wounding contained a few non-specifically co-immunoprecipitated target protein (RhoA or Cav-1). Importantly, PGP (80 and

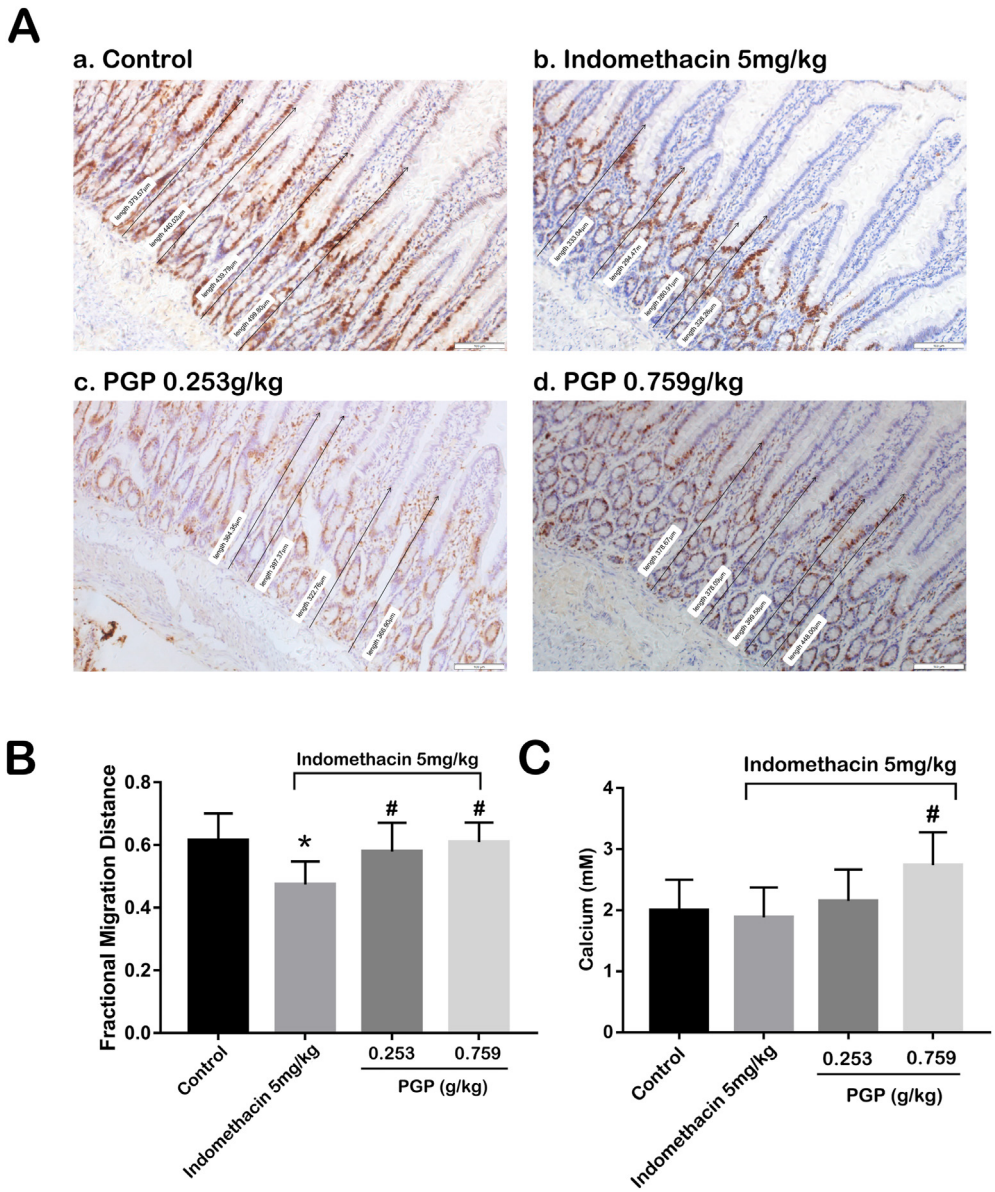

Fig. 3. Effect of PGP on the duodenal cell migration in rats. (A) Immunohistochemical diagram of duodenal mucosa, in which the black arrow marked the distance of cell migration determined by the farthest distance of BrdU positive epithelial cells along the villus-crypt axis (× 100). (B) Histograms showed the relative means of migration distance of epithelial cells from different groups. (C)Histograms presented the means of Ca^{2+} content in intestinal mucosa detected by colorimetric method. *$P < 0.05$, as compared to Control group. #$P < 0.05$, as compared to Indomethacin group.

160 mg/L) as well as putrescine (5 μmol/L) intervention elevated the levels of RhoA/TRPC1, Cav-1/TRPC1 and Rac1/PLC-γ1 association, in comparison with Control group.

4. Discussion

The monolayer epithelial cells covering the gastrointestinal tract maintain a physical and functional barrier to luminal pathogens. Gastrointestinal mucosal injuries such as hyperemia, erosion and ulcer visible under endoscopy are common pathological features in the syndrome of Spleen-deficiency as well as gastrointestinal diseases like peptic ulcer, NSAIDS-related gastrointestinal diseases and inflammatory bowel disease [19]. In response to gastrointestinal mucosal damage, the injured cells are sloughed, meanwhile the residual viable cells migrate to reseal denuded areas, a process that is independent of cell proliferation and highly regulated by $[Ca^{2+}]_{cyt}$ [3]. The increasing $[Ca^{2+}]_{cyt}$ in IEC-6 cell is closed related to the acceleration of cell migration and mucosal restitution. Therefore, promoting the repair of mucosal injury by regulating $[Ca^{2+}]_{cyt}$ level may be a potential treatment of gastrointestinal diseases. What's more, as a representative herb of replenishing Qi and tonifying Spleen, *P. ginseng* may be a considerable candidate in treating gastrointestinal mucosal injury. Our previous work showed that intestinal mucosal injury in rats could be prevented and treated by *P. ginseng* in which polysaccharides were the major active and most abundant ingredient [6]. Here, we further elucidated the mechanism of PGP in promoting the gastrointestinal mucosal restitution, and found that PGP prevented intestinal mucosal injury partly through controlling the regulators of Ca^{2+} mobilization in order to increase the Ca^{2+} content, followed with the promotion of intestinal epithelial cell migration.

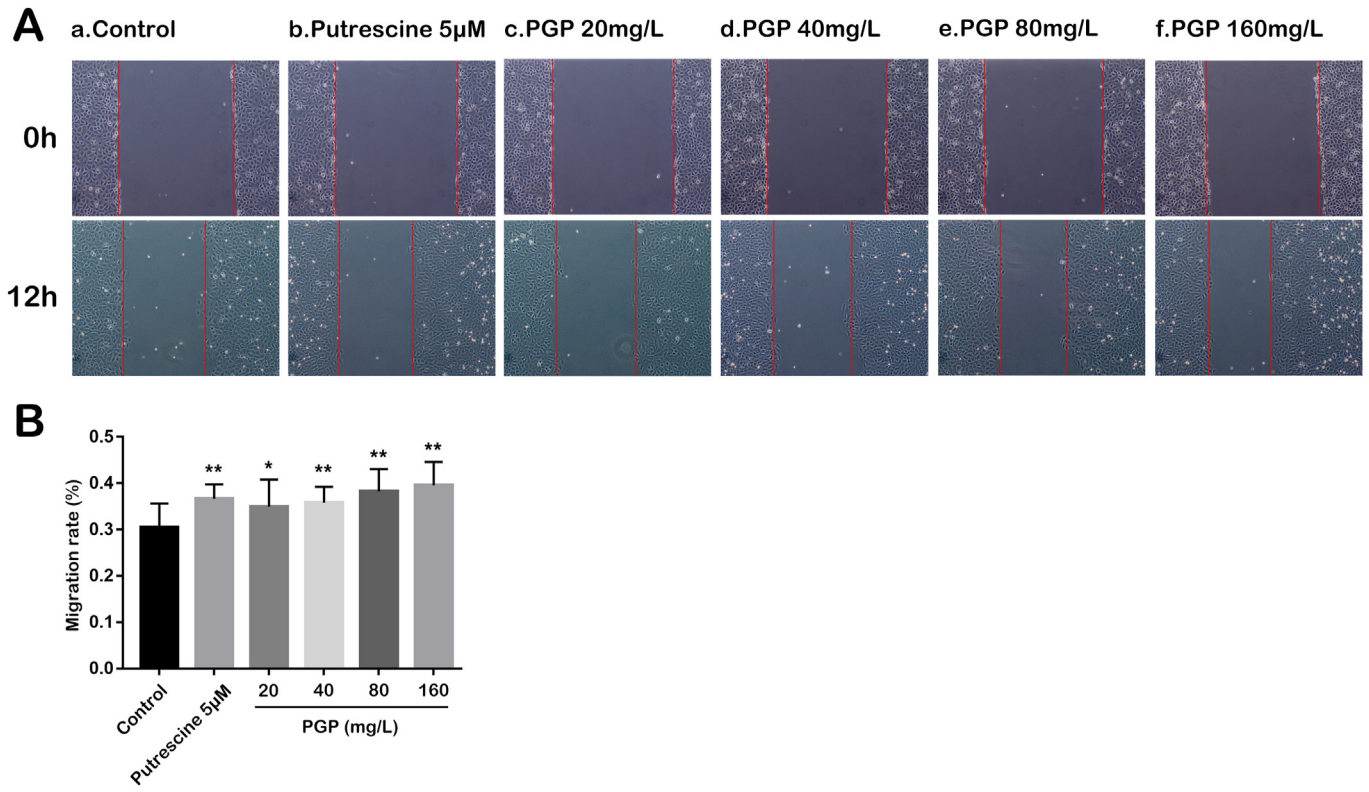

Fig. 4. Effect of PGP on IEC-6 cell migration in vitro. (A) Microscopic images of IEC-6 cell migration before (0 h) and after (12 h) scratching with different treatment. (B) The changes of cell migration were defined as the ratio of the covered wound area to the initial scratched area (× 100). *$P < 0.05$, **$P < 0.01$, as compared to Control group.

Epithelial cell migration is such an essential repair modality throughout gastrointestinal mucosal injury, and maintains the gut mucosal integrity against undesirable luminal antigens. Moreover, promoting cell migration is also an important process for the herbal medicine of replenishing Qi and tonifying Spleen in treating intestinal mucosal injury [20]. Therefore, we firstly observed the effects of PGP on intestinal mucosal injury as well as epithelial cell migration in vivo. The results showed that PGP significantly reduced the assessment of both visual observation and pathological grading of mucosal injury, and decreased the perforations induced by indomethacin, indicating that PGP could effectively prevent gut mucosal injury. Experiment in vivo has confirmed that delay healing of gastrointestinal mucosal injury by NSAIDs like indomethacin is associated with the inhibition of cell migration, which has also been detected and verified in IEC-6 cell migration model [18]. In this study, we found that administration of indomethacin could depress the epithelial cell migration, which was reversed by the treatment with PGP, suggesting that PGP promoted gut mucosal restitution partly via accelerating epithelial cell migration. Since, the Ca^{2+} is a vital regulator during intestinal epithelial cell migration, we further carried out the detection of Ca^{2+} content in intestinal mucosa, and found that PGP could increase the content of Ca^{2+} in intestinal mucosa, which may be the potential mechanism in promoting cell migration by PGP.

An increasing $[Ca^{2+}]_{cyt}$ is a trigger for the initiation of intestinal epithelial cell migration, and accelerates mucosal restitution, and vice versa [2]. Our previous researches found that the medicine of replenishing Qi and tonifying Spleen like Sijunzi decoction and *Astragalus mongholicus* could enhance $[Ca^{2+}]_{cyt}$ and promote IEC-6 cell migration [21,22]. This current study found that PGP could increase the content of Ca^{2+} in intestinal mucosa. Therefore, we further elucidated the mechanism of PGP in promoting mucosal restitution through Ca^{2+} signaling. The increasing $[Ca^{2+}]_{cyt}$ depends on the release from internal Ca^{2+} stores and extracellular Ca^{2+} entry, which are complicated and vital mechanism for maintaining an appropriate $[Ca^{2+}]_{cyt}$. PLC-γ1-Ca^{2+} signaling serves as a critical regulator of intracellular Ca^{2+} store mobilization as well as Ca^{2+} homeostasis during mucosal restitution. PLC-γ1 catalyzes the formation of IP_3 and diacylglycerol. As a Ca^{2+}-mobilizing messenger, IP_3 leads to the Ca^{2+} release from IP_3-sensitive ER through binding to IP_3 receptor in plasma membrane [10]. What's more, this depletion of Ca^{2+} store could further induce Ca^{2+} influx. During intestinal mucosal restitution, stimulating PLC-γ1 could increase the level of IP_3 following with the up-regulation of $[Ca^{2+}]_{cyt}$ and the promotion of cell migration. On the contrary, decreasing PLC-γ1 expression would reduce both IP_3 and $[Ca^{2+}]_{cyt}$, and subsequently suppress cell migration. In current study, we found that PGP notably increased the PLC-γ1 expression and accelerated IEC-6 cell migration. Moreover, Rac1 functions as a molecular switch, which participates in the regulation of IEC cell migration through altering PLC-γ1 related Ca^{2+} signaling [11]. Specifically, Rac1 directly interacts with PLC-γ1 and forms the complex of Rac1/PLC-γ1 after wounding, then promotes PLC-γ1 induced elevated $[Ca^{2+}]_{cyt}$ [11]. The result from this current research presented that not only did PGP enhance Rac1 and PLC-γ1 expression, but it also promoted the interaction between Rac1 and PLC-γ1 after injury.

Ca^{2+} influx owing to the depletion of Ca^{2+} store is referred to as capacitative Ca^{2+} entry (CCE) which is mediated via Ca^{2+}-permeable ion channel known as store-operated Ca^{2+} channels (SOCs), contributing to a suitable enhancement of $[Ca^{2+}]_{cyt}$. TRPC1 served as the component of SOCs for modulating CCE and $[Ca^{2+}]_{cyt}$, and regulating IEC-6 cell migration [23]. Induced TRPC1 expression could enhance CCE and cell migration after injury [23]. We

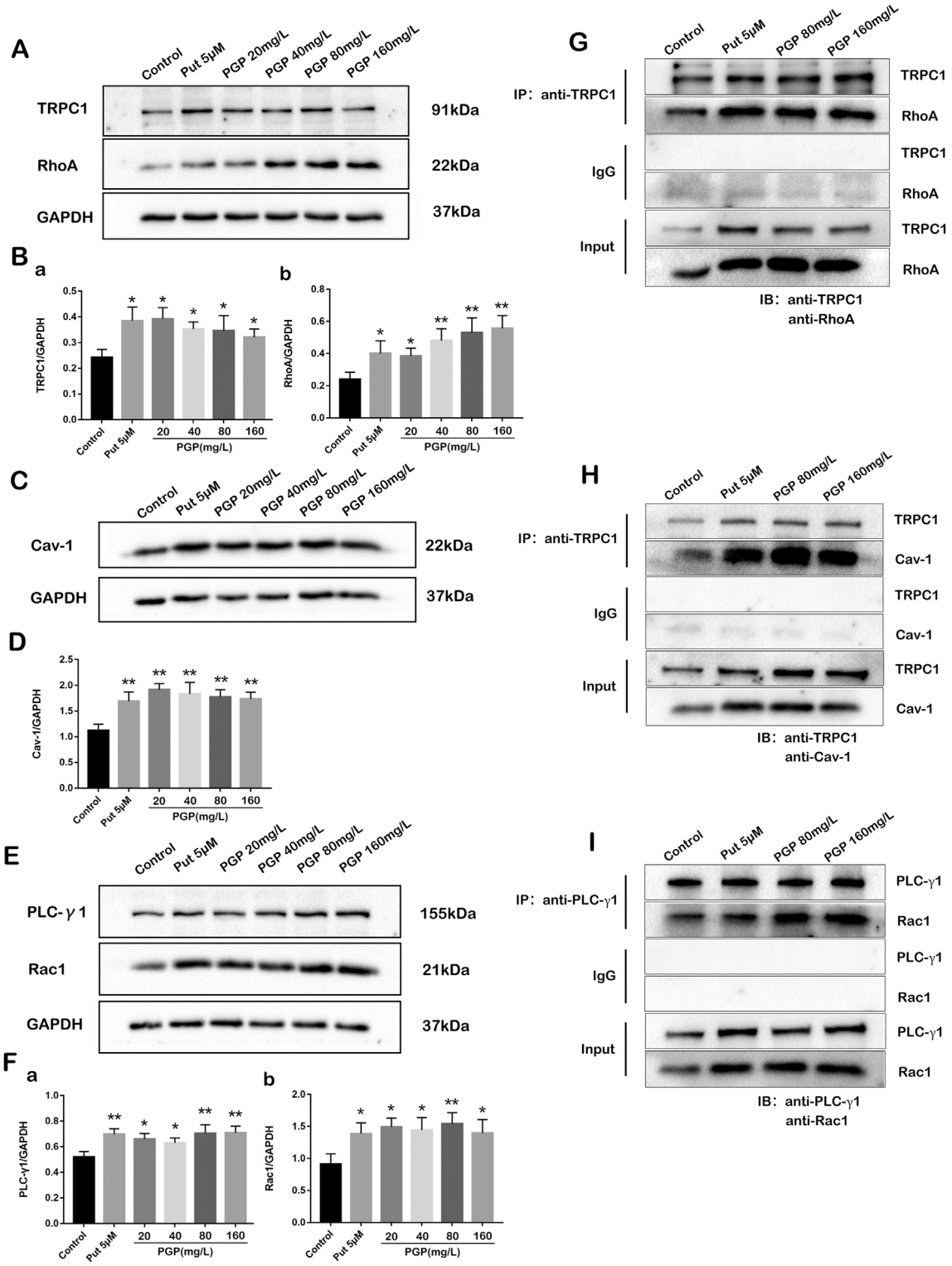

Fig. 5. Effect of PGP on expression of Ca^{2+} regulatory proteins and complexes during IEC-6 cell migration. (A.C.E) The protein bands of RhoA, TRPC1, Cav-1, Rac1, PLC-γ1 and GAPDH were showed by western blotting, and (B.D.F) semi-quantitative analysis of these proteins were normalized by corresponding GAPDH bands. (G.H.I) Co-immunoprecipitation with anti-TRPC1 or anti- PLC-γ1 were performed, and RhoA, Cav-1, TRPC1, Rac1, PLC-γ1 proteins were visualized by western blotting. $*P < 0.05$,$**P < 0.01$, as compared to Control group.

previously found that the polysaccharides from the herbs of replenishing Qi and tonifying Spleen could increase the expression of TRPC1 and $[Ca^{2+}]_{cyt}$ in IEC-6 cell after injury [24]. Thus, we further elucidated the PGP, a representative herb of replenishing Qi and tonifying Spleen, in regulating cell migration via TRPC1-mediated Ca^{2+} signaling. TRPC1 is predominantly localized within caveolae microdomains in cell membrane, while Cav-1 is a scaffolding protein expressed within caveolae, which is associated with the regulation of Ca^{2+} signaling [25]. In fact, Cav-1 is able to interact with Ca^{2+}-permeable channel, and it's binding to both the COOH

and NH_2 terminal of TRPC1 is indispensable for the distribution of TRPC1 within caveolae [26]. Follow-up study has found that Cav-1 physically interacts with TRPC1 in order to form Cav-1/TPRC1 complex, and further promotes TRPC1-mediated Ca^{2+} signaling after mucosal injury [13]. Our study showed that treatment with PGP increased the levels of both Cav-1 and TRPC1, and then enhanced the interaction between Cav-1 and TRPC1. RhoA, another member of small GTP-binding protein, also participates in intracelluler Ca^{2+} homeostasis. It activates the interaction of TRPC1 and IP_3R at the plasma membrane, then induces Ca^{2+} entry in endothelial cells [12]. Moreover, RhoA could directly interact with and activate TRPC1, and then promote IEC-6 cell migration during restitution via inducing Ca^{2+} signaling [12]. Therefore, we also explored the mechanism of PGP stimulating IEC-6 cell migration by RhoA/TRPC1-mediated Ca^{2+} signaling. Our results indicated that intervention of PGP improved the expression of both RhoA and RhoA/TRPC1 complex. These findings suggesting that PGP could significantly stimulate intestinal epithelial cell migration, through regulating the levels of Ca^{2+} regulators (PLC-γ1, Rac1, TRPC1, Cav-1 and RhoA) as well as protein complexes (Rac1/PLC-γ1, Cav-1/TRPC1 and RhoA/TRPC1), and then prevent gut mucosal injury.

Finally, we hadn't further observation on the morphological properties of PGP, and didn't label Ca^{2+} with fluorescence to provide an intuitive change of $[Ca^{2+}]_{cyt}$, these were limitations of the present research. We also tried to explore the mechanism by which PGP promoted the binding of protein complex (Ca^{2+} regulator). For example, by using NSC-23766 (an inhibitor of Rac1 activity) as an intervention, we found that PGP probably promoted the interaction between Rac1 and PLC-γ1 by upregulating the activity of Rac1 (i.e., GTP-Rac1), which may be the part of future works.

5. Conclusion

In summary, PGP increases the Ca^{2+} content in intestinal mucosa partly through controlling the regulators of Ca^{2+} mobilization including both Ca^{2+} release and influx, subsequently promotes intestinal epithelial cell migration in vitro and vivo, and then prevents intestinal mucosal injury induced by indomethacin.

Declaration of competing interest

All authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2022.05.010.

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