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Research Article

The anti-platelet activity of panaxadiol fraction and panaxatriol fraction of Korean Red Ginseng in vitro and ex vivo

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ABSTRACT

Background: The anti-platelet activity of the saponin fraction of Korean Red Ginseng has been widely studied. The saponin fraction consists of the panaxadiol fraction (PDF) and panaxatriol fraction (PTF); however, their anti-platelet activity is yet to be compared. Our study aimed to investigate the potency of anti-platelet activity of PDF and PTF and to elucidate how well they retain their anti-platelet activity via different administration routes.

Methods: For ex vivo studies, Sprague-Dawley rats were orally administered 250 mg/kg PDF and PTF for 7 consecutive days before blood collection via cardiac puncture. Platelet aggregation was conducted after isolation of the washed platelets. For in vitro studies, washed platelets were obtained from Sprague-Dawley rats. Collagen and adenosine diphosphate (ADP) were used to induce platelet aggregation. Collagen was used as an agonist for assaying adenosine triphosphate release, thromboxane B2, serotonin, cyclic adenosine monophosphate, and cyclic guanosine monophosphate (cGMP) release.

Results: When treated ex vivo, PDF not only inhibited ADP and collagen-induced platelet aggregation, but also upregulated cGMP levels and reduced platelet adhesion to fibronectin. Furthermore, it also inhibited Akt phosphorylation induced by collagen treatment. Panaxadiol fraction did not exert any anti-platelet activity in vitro, whereas PTF exhibited potent anti-platelet activity, inhibiting ADP, collagen, and thrombin-induced platelet aggregation, but significantly elevated levels of cGMP.

Conclusion: Our study showed that in vitro and ex vivo PDF and PTF treatments exhibited different potency levels, indicating possible metabolic conversions of ginsenosides, which altered the content of ginsenosides capable of preventing platelet aggregation.

1. Introduction

Platelets are small anucleated cell fragments that are known to prevent further bleeding at the site of injury in its activated state by forming a clot. An array of mechanisms occur when platelets are activated [1]. Some of the agonists that lead to platelet activation include collagen, von Willebrand factor, thrombin, and adenosine diphosphate (ADP), and are known to be activated by lipopolysaccharide and oxidized low-density lipoprotein (oxLDL) [2–5]. Activated platelets form aggregates with other platelets and immune cells, such as T cells and neutrophils, which initiate the formation of an atherosclerotic plaque and microemboli that potentially lead to ischemic stroke [6–8]. Therefore, a treatment that can dampen platelet activation without affecting normal hemostasis would be a therapeutic approach against cardiovascular diseases.

Panax ginseng Meyer is a widely used medicinal herb known to mediate inflammation, oxidative stress, cardiovascular disease [9,10] and the regulation of metabolic syndromes such as high blood pressure, serum triglycerides, and abdominal obesity [11]. *P. ginseng* is an adaptogen that modulates and stabilizes an array of immune response [12]. As we have previously summarized, levels of oxLDL cause the migration of immune cells into the vascular endothelium, causing inflammation, followed by vascular smooth

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muscle cell apoptosis and migration, and releasing agonists for platelet aggregation. We propose that Korean Red Ginseng and ginsenosides have the potential to target each stage of atherosclerosis progression [13].

P. ginseng has been extensively reported for its anti-platelet activity, as summarized previously [14]. However, the different fractions of ginsenosides have variable potency and are dependent on their chemical structure. Rb1 does not have an anti-platelet effect, but after the removal of two glucose molecules (which yields Rg3), it exhibits potent anti-platelet activity [14]. Ginsenosides can be converted via metabolism. Thus, it is important to investigate the potency of the anti-platelet activity of the panaxadiol fraction (PDF) and panaxatriol fraction (PTF) and how well they retain their anti-platelet activity via different routes of administration.

Our study showed that when administered ex vivo, PDF showed better potency against collagen- and ADP-induced platelet aggregation. However, this was not the case when treated in vitro; PDF had no apparent effect, but PTF had a higher potency against platelet aggregation. Thus, our study shows the importance of thoroughly investigating the anti-platelet activity of ginsenosides ex vivo and in vitro because of the metabolic conversion of ginsenosides.

2. Materials & methods

2.1. Reagents

Panaxadiol fraction and PTF were provided by the Korean Ginseng Corporation (Daejeon, Republic of Korea). Acetylsalicylic acid (ASA) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies that were used for western blot analysis were obtained from Cell Signaling Technology (Danvers, MA, USA). Collagen, ADP, and thrombin were obtained from Chrono-log (Havertown, PA, USA).

2.2. Treatment regime of animals

Five–week old male Sprague-Dawley rats were obtained from Orient Bio (Seongnam-si, Gyeonggi-do, Republic of Korea) and acclimatized for 7 days in a pathogen-free environment (12-h light/dark cycle; 23 ± 2°C; humidity of 50 ± 10%). After 1-week of acclimatization, animals were treated with 250 mg/kg PDF, PTF, or 100 mg/kg ASA for 7 days (once daily, orally), in groups of 5. The rats were handled according to the Institutional Animal Care and Use Committee of Kyungpook National University (approval number: KNU2020-0062).

2.3. Blood collection and preparation of washed rat platelets

Blood was collected via cardiac puncture from 6–week old Sprague-Dawley rats using acid citrate dextrose as an anticoagulant. The blood was then centrifuged at 1,000 rpm for 7 min. The platelet rich plasma (PRP) was separated and centrifuged at 2,000 rpm for another 7 min. The supernatant was then discarded and the pellet was resuspended in Tyrode's buffer (137mM NaCl, 12mM $NaHCO_3$, 5.5mM glucose, 2mM KCl, 1mM $MgCl_2$, 0.3mM $NaHPO_4$, pH 7.4) to obtain washed platelets.

2.4. Light transmission aggregometry

Platelet aggregation was measured by light transmission aggregometry using an aggregometer (Chrono-Log, Havertown, PA, USA). Platelets were briefly incubated with 1 mM $CaCl_2$ (in vitro studies), without $CaCl_2$ (ex vivo studies), with or without PDF and PTF (in vitro studies). For ex vivo, platelets were activated with 0.625 μg/mL collagen or 2.5 μM ADP and incubated at 37°C for another 5 min. For in vitro studies, the concentrations of collagen used was 2.5 μg/mL, ADP was 10 μM, and 0.1 U/mL for thrombin. Panaxadiol fraction was treated at 1000 μg/mL while PTF was treated 250–1000 μg/mL. Doses for saponin fraction and non-saponin fraction were 100–400 μg/mL and 1000–2000 μg/mL, respectively.

2.5. Adenosine triphosphate release assay

The platelets were subjected to aggregation, as mentioned above, and the reaction was immediately quenched in ice. Centrifugation was performed to separate supernatants. The supernatant was then subjected to an adenosine triphosphate (ATP) assay kit according to the manufacturer's instructions (Biomedical Research Service Center, University of Buffalo, NY, United States).

2.6. $[Ca^{2+}]_i$ mobilization assay

Blood was collected via cardiac puncture as described above. The PRP was then separated and incubated with Fura-2AM at 37°C for 1 h. Platelets were then washed with Tyrode's buffer, washed platelets were stimulated with collagen to induce platelet aggregation, and calcium ion mobilization was detected using a spectrofluorometer (F-2500; Hitachi, Chiyoda, TYO, Japan).

2.7. Western blot analysis

Platelets were subjected to aggregation as described above. The reaction was quenched on ice, and a protein extraction solution was added to the platelets (Pro-Prep; iNtRON, Seongnam-si, Gyeonggi-do, Republic of Korea). Proteins were separated via centrifugation, and their concentration measured using the Bradford method (Pro-Measure; iNtRON). Proteins were separated by 10% SDS-PAGE and transferred onto PVDF membranes, followed by blocking using skim milk. The membranes were then incubated with the respective target primary antibodies (1:1,000) overnight at 4°C, followed by incubation with the secondary antibody (1:3,000) for 90 min. The membranes were developed in a gel developer using enhanced chemiluminescence solution.

2.8. Enzyme-linked immunosorbent assay (ELISA)

Secretion of serotonin, cyclic adenosine monophosphate (cAMP), and cyclic guanosine monophosphate (cGMP) was detected using ELISA kits (Cayman Chemicals, Ann Arbor, MI, USA), and thromboxane B2 secretion was detected using a kit acquired from LDN (Nordhron, Germany). Platelet aggregometry was conducted as described above, and the respective levels of cAMP, cGMP, TXB2, and serotonin were detected using a microplate reader (Versamax, Molecular Devices, CA, USA).

2.9. Fibronectin adhesion assay

Platelet adhesion was investigated using a fibronectin adhesion assay kit acquired from Cell Biolabs (San Diego, CA, USA), according to the manufacturer's instructions. Platelets were briefly added to the wells and incubated for 90 min. This was followed by crystal violet staining. Adherent cells were lysed, and the lysate was measured at 540 nm using a microplate reader (Versamax).

2.10. Statistical analysis

Statistical significance was analyzed using GraphPad Prism version 7.00 (San Diego, CA, USA) with Dunnett's post-hoc test.

*P < 0.05, **P < 0.01, ***P < 0.001 were considered significant and data were presented as mean ± SD.

3. Results

3.1. Panaxadiol fraction exhibits stronger anti-platelet activity than panaxatriol fraction ex vivo

Collagen and ADP-induced platelet aggregation were significantly inhibited by PDF, whereas PTF did not show a significant change in platelet aggregation (Fig. 1A–D). To confirm the inhibitory pathway, we analyzed ATP, TXB2, and serotonin release. We found that PDF reduced the release of ATP and serotonin but not TXB2. Panaxatriol fraction, in contrast, only inhibited ATP release, but was less effective than PDF (Fig. 1E–G). There were no changes in cAMP levels among the groups, and we found that PDF increased cGMP levels and inhibited fibronectin adhesion. Panaxatriol fraction showed a visible reduction in fibronectin adhesion, but this was not statistically significant. Acetylsalicylic acid was used as the positive control (Fig. 2A–C).

3.2. Panaxadiol fraction acts via the phospho-Akt pathway to inhibit platelet aggregation ex vivo

Western blot analysis was conducted to confirm the pathway of action of both PDF and PTF. We found that PDF strongly inhibited p-Akt expression, whereas PTF showed a decrease in p-Akt and p-JNK expression (Fig. 3A–F). This may indicate that both fractions act via different pathways.

3.3. Panaxatriol fraction shows stronger anti-platelet activity in vitro

When platelets were isolated and treated in vitro, we found that PTF had stronger anti-platelet activity; PTF inhibited collagen-, ADP-, and thrombin-induced platelet aggregation, mobilization of calcium ions, and significantly increased levels of cGMP (Fig. 4A–D, G). Conversely, PDF only showed fairly low inhibition of thrombin-induced platelet aggregation and release of serotonin (Fig. 4C, E). Panaxatriol fraction treatment in vitro only inhibited serotonin and TXB2 release slightly (Fig. 4E and F). The cAMP levels were not altered among the groups (Fig. 4H). To confirm this finding, we assessed platelet aggregation in vitro with saponin and non-saponin fractions. We showed that the saponin fraction, but not non-saponin fraction, had the ability to inhibit collagen-induced platelet aggregation even at high doses (Fig. 4I). As PDF and PTF are part of the saponin fraction, it is probable that the PTF of the saponin fraction actively acts in vitro to inhibit platelet aggregation.

4. Discussion

A study on the comparison of PDF and PTF in an anti-obesity study showed that PD-type saponins contained the active ginsenosides that attenuated obesity [15]. To date, there were no studies comparing PDF and PTF on anti-platelet activity. From our ex vivo findings, PDF has much higher potency than PTF, However, our findings were opposite when treated in vitro in washed rat platelets. Interestingly, different administration routes of PDF and PTF have different efficacies, leading us to hypothesize that the

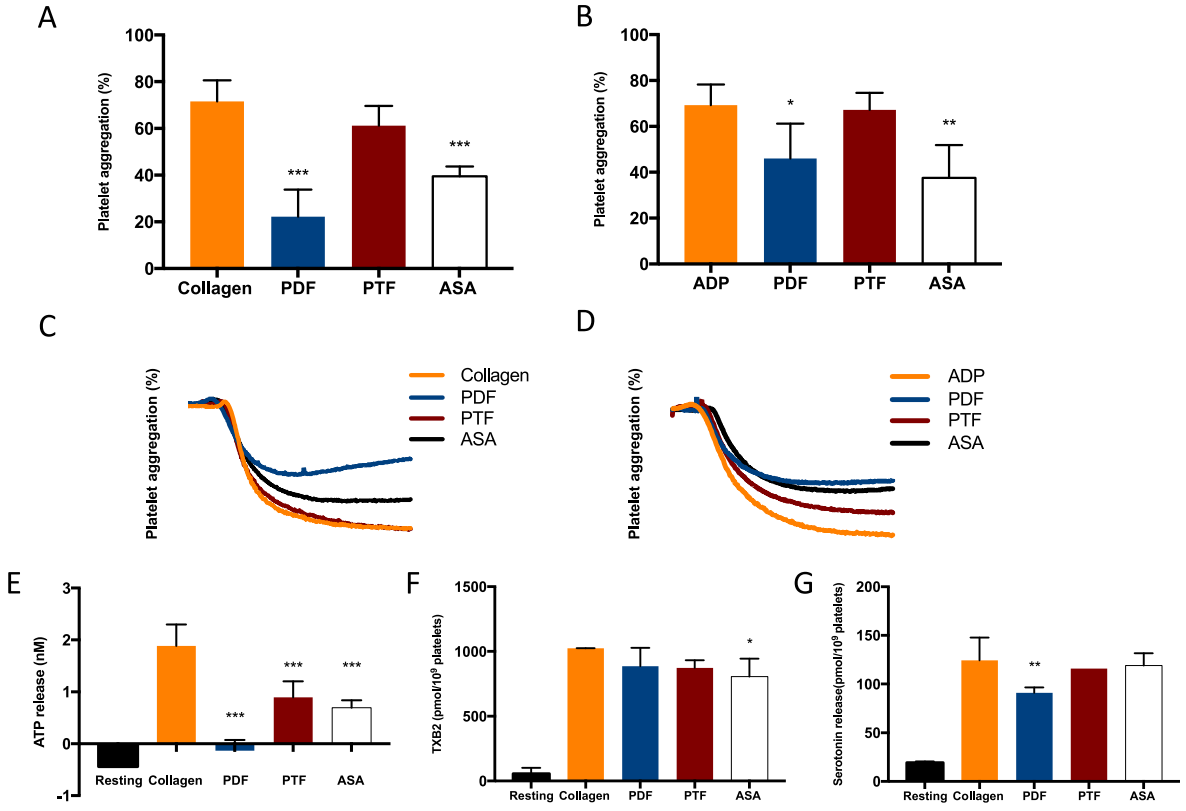

Fig. 1. Panaxadiol fraction inhibits collagen and ADP-induced platelet aggregation ex vivo. Platelet aggregation induced by collagen was suppressed by PDF (A) and represented by curves indicating platelet aggregation (C). Adenosine diphosphate-induced platelet aggregation was inhibited by PDF (B) and the representative curves were shown in (D). Platelet aggregation was induced using collagen and the supernatant was collected and subjected to ATP assay (E), thromboxane B2 level detection (F), and serotonin level detection (G). *P < 0.05, **P < 0.01, ***P < 0.001 were considered significant and data were presented as mean ± SD.

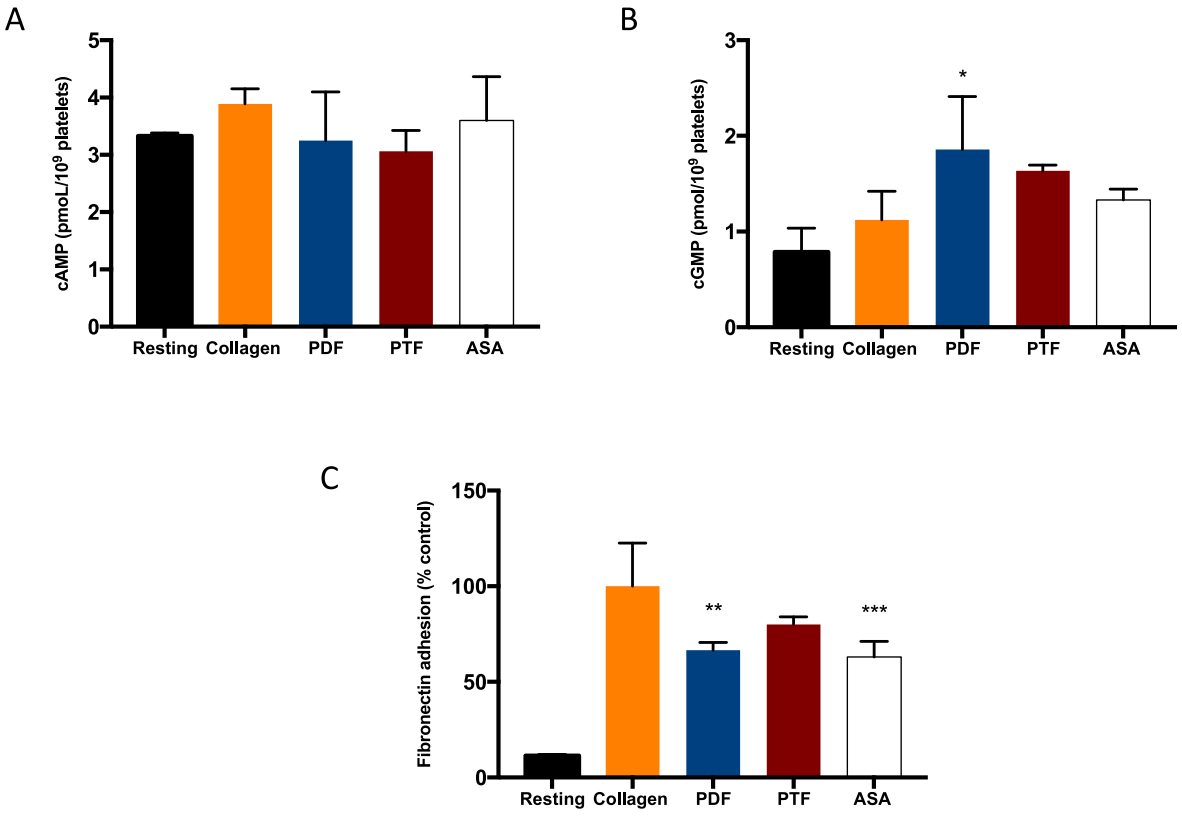

Fig. 2. Panaxadiol fraction increased expression of cGMP to prevent platelet aggregation with ex vivo treatment. Platelet aggregation was induced using collagen and the supernatant was collected for cAMP (A) and cGMP (B) detection. Platelets that were collected from animals that were treated with PDF, PTF, and ASA were added to fibronectin-coated plates to investigate fibronectin adhesion (C). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ were considered significant and data were presented as mean ± SD.

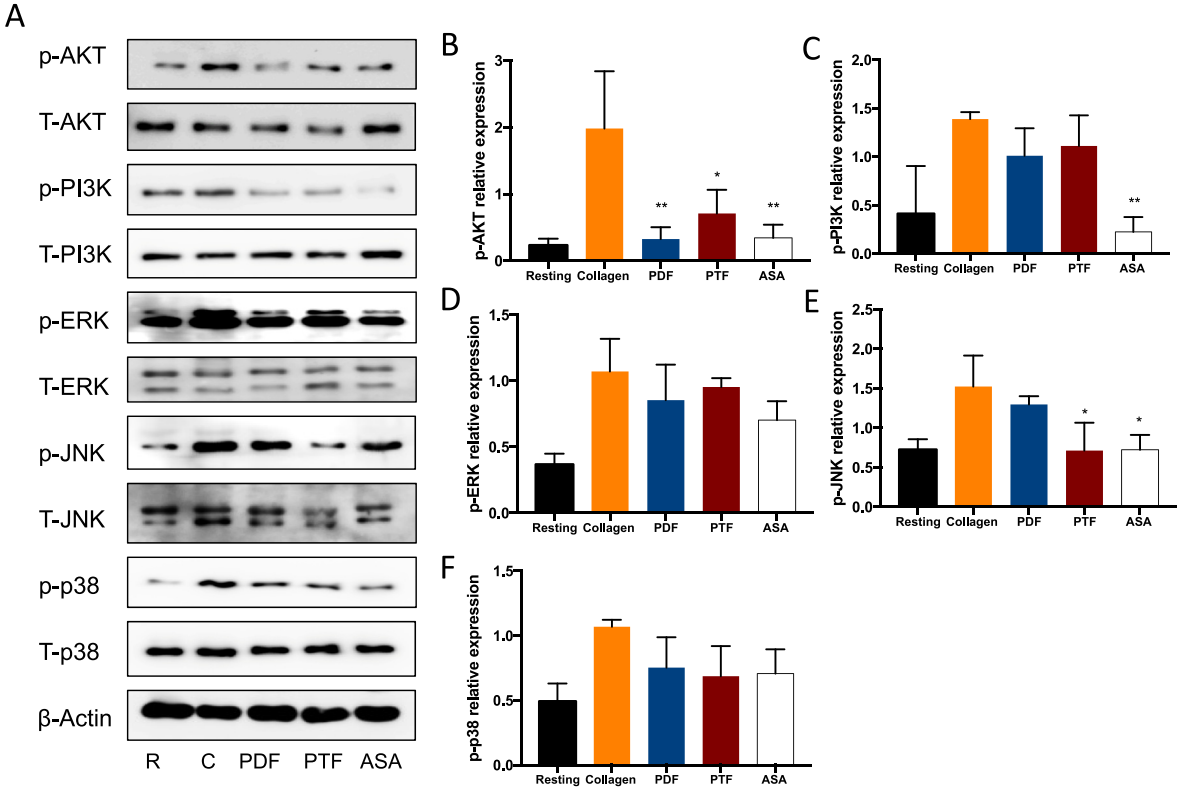

Fig. 3. Panaxadiol fraction effectively suppressed expression of phospho-Akt ex vivo. Western blot analysis was conducted to investigate the protein expression of proteins in the PI3K/Akt and mitogen-activated protein kinase (MAPK) pathway. Representative blot were shown in (A). Western blot was conducted in triplicate and the blots were quantified using ImageJ. The relative expressions of p-Akt (B), p-PI3K (C), p-ERK (D), p-JNK (E) and p-p38 (F) were shown. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ were considered significant and data were presented as mean ± SD. R; resting, C; collagen.

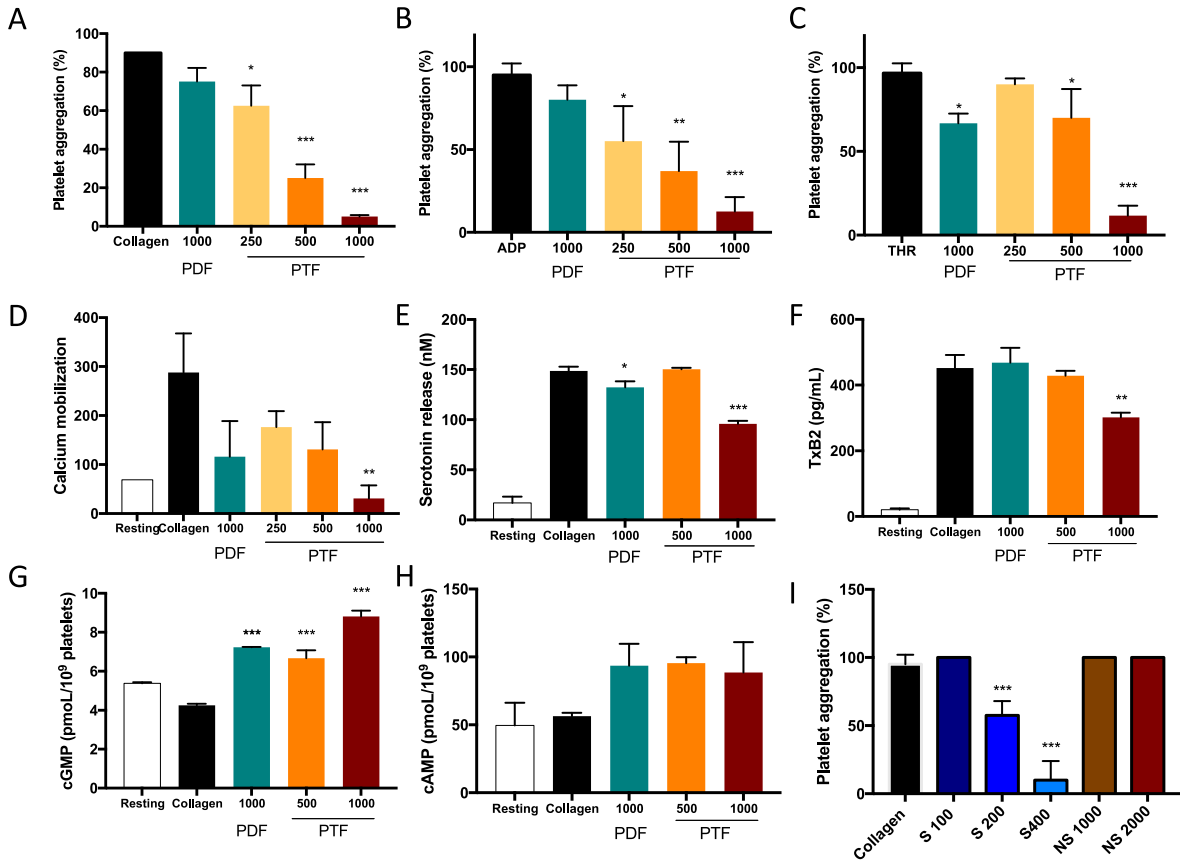

Fig. 4. Panaxatriol fraction exhibits anti-platelet activity in vitro. Platelets were treated with PDF or PTF and analyzed using a platelet aggregometer. Aggregation was induced with collagen (A), ADP (B), and thrombin (C). Calcium mobilization (D), serotonin secretion (E), and levels of TXB2 (F) were investigated in platelets treated with PDF and PTF. Platelet aggregation was induced by collagen. The supernatants were collected and levels of cGMP (G) and cAMP (H) were analyzed using ELISA. The saponin (S) and non-saponin (NS) fractions of *Panax ginseng* was analyzed in vitro against collagen-induced platelet aggregation (I). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ were considered significant and data were presented as mean ± SD.

metabolism of PDF and PTF may lead to increased or decreased efficacy against platelet aggregation. A previous study found that PDF consists of ginsenosides Rb1, Rc, Rb2, Rb3, and Rd, whereas PTF contains Rg1 and Re [16]. These ginsenosides may undergo metabolic changes when metabolized (by gut microbiota or the liver).

The main ginsenosides in PDF are Rc, Rb1, and Rd [14]. In rats, it is proposed that orally administered ginsenoside Rc will be metabolized and converted into ginsenoside Mc, followed by conversion into compound K [17]. In human intestinal bacteria, ginsenoside Rc is metabolized and converted to ginsenosides Rd and Mb. Ginsenoside Rd was further metabolized into F2, compound K, and finally 20(S)-Protopanaxadiol (PPD). Furthermore, ginsenoside Mb is metabolized to ginsenoside Mc and then to compound K. This also leads to PPD formation, as previously proposed [18].

Other than ginsenoside Rc, Rb1 is also present in PDF [14]. Based on previous rat studies, Rb1 is converted to Rd, F2, compound K, and subsequently PPD (as detected in the plasma) when administered orally [19]. This finding is consistent with the results of another study conducted by Chen et al (2008), who proposed a more detailed metabolic pathway of Rb1 in rats, Rb1 yields Rd and Gypenoside XVII. Both Rd and gypenoside can be transformed into F2, which becomes compound K, followed by hydroxydammar-3-one-20(S)—O-β-d-glucopyranoside. In addition, Rd can be transformed into Rg3 [20]. Rg3 has been reported to exhibit potent anti-platelet activity [21]. This may provide an explanation for the variable efficacy of orally administered PDF in animals compared to in vitro platelet treatment. Another study on the metabolism of ginsenoside Rd in rats showed that Rd was converted to Rb1, Rg3 and Rh2 [22]. These metabolic conversions show that PDF has increased anti-platelet activity when treated ex vivo, most likely due to its metabolic conversion to Rg3.

PTF is composed of ginsenosides Rg1, Re, and Rf [14]. In rats, Wang et al (2001) found that Rg1 is metabolized into Rh1 or F1 and finally converted into 20(S)-protopanaxatriol (PPT). They also proposed that in human intestinal bacteria in vitro, Rg1 is converted to Rh1 and then to PPT [23]. In addition, a study by Bae et al (2005) proposed a metabolic pathway for ginsenoside Re in the human intestinal flora. Re that is converted into Rg1 and Rg2. Rg1 undergoes a conversion similar to that mentioned above, whereas Rg2 is metabolized into Rh1 and subsequently PPT [24]. This suggests that PTF is metabolized into PPT ex vivo, and most of the potent anti-platelet ginsenosides are metabolized into other less potent ginsenosides. The proposed metabolic conversions of ginsenosides by the above literature were summarized in Fig. 5.

In our current study, we found that the increase of cGMP was observed regardless of administration route in PDF, where Rb1, Rc and Rd were present pre-conversion. In a previous study, Rb1 was reported to improve homocysteine-induced impairment of endothelial nitric oxide synthase (eNOS) in porcine coronary arteries [25]. Other than that ginsenoside Rb1 was also reported to increase

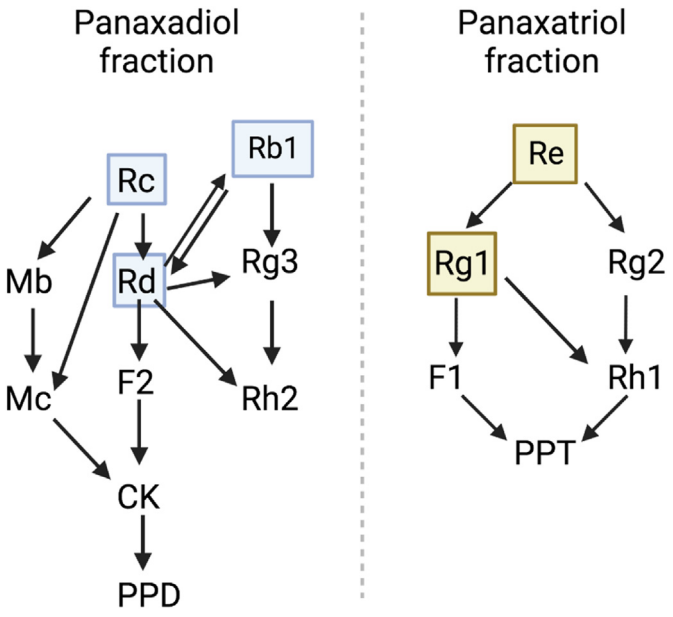

Fig. 5. Proposed metabolic conversion of ginsenosides in PDF and PTF based on previous literature. Ginsenosides Rd. Rb1 and Rc are found in PDF, while Re and Rg1 are found in PTF (indicated by square boxes) before metabolic conversion. CK; compound K, PPD; protopanaxadiol, PPT; protopanaxatriol.

cGMP and nitric oxide (NO) concentration in mouse corpus cavernosum after a 3-week intraperitoneal treatment in mouse [26]. This may explain the in vitro increment of cGMP of PDF. After metabolic conversion, compound K, Rh2, Rg3, PPD may be present. A previous study showed that Rg3 elevated levels of cAMP while inhibiting thrombin-induced platelet aggregation [27]. However, we did not observe this increment probably due to the conversion of Rg3 into Rh2 and there were no reports on the role of Rh2 in cGMP enhancement. In PTF, ginsenoside Re was converted after metabolism. Ginsenoside Re was reported to inhibit platelet-derived growth factor-BB-induced vascular smooth muscle cell proliferation via the activation of the eNOS/NO/cGMP pathway [28] and increased sperm capacitation via the NO/cGMP/PKG pathway in a separate study [29]. However there were no reports on PPT and the increment of cGMP (product of the proposed metabolic conversion of ginsenosides in the PTF). These findings provide insight on the trend of cGMP with in vitro and ex vivo treatment of PTF and PDF, which further supports our hypothesis on ginsenoside metabolism in the gut.

Dietary phytochemicals should be bioavailable for it to be readily absorbed and available at the site of action. Anthocyanins, water-soluble flavonoids found in various fruits and vegetables, are metabolized by the digestive system. In the form of anthocyanin-glycoside, it can be readily absorbed in the stomach, where it undergoes methylation, glucuronidation, and sulfation in the liver, which is then sent into systemic circulation. The remaining anthocyanin-glycosides that are absorbed in the small intestine are converted into hemiketal, quinonoidal, and chalcone forms that are absorbed in the jejunum, as proposed in a previous review [30]. One study has proposed an intestinal model for the study of uptake, metabolism, and bioactive compounds that utilizes a two-dimensional and three-dimensional enteroid panel or an intestine-on-a-chip model [31]. The uptake and transport of metabolites can be detected via fluorescence, as previously reported for the detection of flavonoids [32] and LC-MS for polyphenols [33]. This method can be used to validate the metabolism of PDF and PTF.

The limitation of our study is that these metabolic conversions were hypothesized based only on previous studies consisting of human and rat models. Hence, the validity of our hypothesis is yet to be scientifically evaluated. However, our study showed a difference in potency following different administration routes. In conclusion, it is important to investigate the potency of ginsenosides with in vitro and ex vivo treatments, as ginsenosides are metabolized by gut microbiota before they enter the blood stream to exhibit anti-platelet activity. In our study, we found that the potencies of both PTF and PDF were different in vitro and ex vivo. Referring to previous literature, we found that ex vivo treatment converts ginsenosides in the PDF fraction into more potent ginsenosides, whereas existing ginsenosides that were potent, were metabolized into less potent ginsenosides. Future studies should be conducted to confirm the efficacy of various ginsenosides and fractions that are potent in ginseng extracts when administered orally and the detailed mechanism of PDF and PTF in vitro and ex vivo that may involve the cGMP/VASP pathway.

Authorship statement

All persons who meet authorship criteria are listed as authors, and all authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript. Furthermore, each author certifies that this material or similar material has not been and will not be submitted to or published in any other publication before its appearance in the *Journal of Ginseng Research*.

Please indicate the specific contributions made by each author (list the authors' initials followed by their surnames, e.g., Y.L. Cheung). The name of each author must appear at least once in each of the three categories below.

Conception and design of study: MH RHEE , YY Lee, D Kwak, ;acquisition of data: YY Lee, MS SEO, MG SEO, Y Oh ;analysis and/or interpretation of data: JE HAN, SD PARK, JK PARK, KT KIM, ,MG SEO,

Drafting the manuscript: D KWAK, SJ PARK; revising the manuscript critically for important intellectual content: MS SEO, D KWAK, Y Oh, MH RHEE.

Approval of the version of the manuscript to be published (the names of all authors must be listed):YY LEE, Y OH, MS SEO, MG SEO, JE HAN, KT KIM, JK PARK, SD KIM, SJ PARK, D KWAK, , MH RHEE,

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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