Comparative antiplatelet and antithrombotic effects of red ginseng and fermented red ginseng extracts

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Abstract

Background: Fermentation may alter the bioavailability of certain compounds, which may affect their efficacy and pharmacological responses. This study investigated the antiplatelet effects of red ginseng extract (RGE) and fermented red ginseng extract (FRG).

Methods: A rodent model was used to evaluate the antiplatelet and antithrombotic effects of the extracts. Rats were orally fed with human equivalent doses of the extracts for 1 week and examined for various signaling pathways using standard in vivo and ex vivo techniques. Light transmission aggregometry was performed, and calcium mobilization, dense granule secretion, integrin αIIbβ3-mediated signaling molecules, cyclic nucleotide signaling events, and various protein molecules were evaluated ex vivo in collagen-stimulated washed platelets. Furthermore, antithrombotic properties were evaluated using a standard acute pulmonary thromboembolism model, and the effects on hemostasis were investigated using rat and mice models.

Results: Both RGE and FRG significantly inhibited platelet aggregation, calcium mobilization, and dense granule secretion along with integrin-mediated fibrinogen binding and fibrinogen adhesion. cAMP levels were found to be elevated in RGE-treated rat platelets. Ginseng extracts did not exert any effect on prothrombin time and activated partial thromboplastin time. RGE-treated mice showed significantly better survival under thrombosis than FRG-treated mice, with no effects on hemostasis, whereas FRG-treated mice exhibited a slight increment in bleeding time.

Conclusion: Both extracts, especially RGE, are remarkable supplements to maintain cardiovascular health and are potential candidates for the treatment and prevention of platelet-related cardiovascular disorders.

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1. Introduction

Cardiovascular disease (CVD) is considered as one of the leading causes of death globally. Studies have reported that in 2013, coronary heart disease caused almost one in seven deaths, and heart failure caused one in nine deaths in the United States [1,2]. In Europe, CVD was reported to cause 42% of deaths in men and 52% of deaths in women [3]. CVD includes a number of diseases and conditions such as atherosclerosis, coronary heart disease, heart attack, hypertension, and stroke. Although several risk factors are involved in the pathophysiology of CVD, hyperactive platelets are considered as the major culprits as they cause the hemostatic plug and stenosis of vasculature, which may also lead to ischemic stroke. Under normal physiological conditions, platelets play an important role in maintaining hemostasis; however, aberrant activation or hyper-activation of platelets may cause thrombotic complications that contribute to the development of thrombosis or other platelet-related CVD [4,5]. Pharmacological suppression of platelet activation has been confirmed to be effective in the treatment and prevention of CVD; however, available treatment options are often hampered by side effects and complications. For instance, aspirin is a remarkable antiplatelet agent but may cause severe gastric ulcers or prolonged bleeding, and clopidogrel sometimes causes aplastic anemia and thrombocytopenic purpura [6]; furthermore, a significant proportion of population is resistant to these most commonly used antiplatelet agents [7,8]. Therefore, this situation necessitates the development of safer and efficacious therapeutic regimens with...
no or minimum side effects. In this context, ethnomedical application could be one of the best approaches to prevent CVD and CVD-related complications [9]. Studies have reported that natural herbal compounds and the Mediterranean diet might possess antiatherosclerotic and antithrombotic properties owing to the presence of bioactive compounds affecting platelet function, thereby reducing the future risk for thrombosis [10–12].

We had previously evaluated the comparative immunostimulatory effects of red ginseng extract (RGE) and fermented red ginseng extract (FRG) in murine and rodent models [13]. The present study was designed to evaluate the potential comparative antiplatelet and antithrombotic effects of RGE and FRG using murine and rodent models, and the results showed that both RGE and FRG possess potent antiplatelet activities, with RGE being more potent due to the difference in their change in the constituent profiling produced as a result of fermentation.

2. Materials and methods

2.1. RGE and FRG procurement

RGE (Rg1 + Rb1 + Rg3 = 7.58 mg/g) and FRG (Rg1 + Rb1 + Rg3 = 7.42 mg/g) were purchased from the local market and analyzed for ginsenoside contents according to previously reported studies by HPLC analysis as shown in Table S1.

2.2. Animals and dosage

Male Sprague–Dawley rats aged 7 weeks and weighing 250–260 g and C57BL/6 mice aged 7 weeks and weighing 20–22 g were purchased from Orient Co. (Seoul, Korea). The rats and mice were acclimatized for 1 week in an air-conditioned room with a 12 h/12 h light/dark cycle at a temperature and humidity of 22°C ± 2°C and 55% ± 10%, respectively. Animal experiments were conducted according to IACUC guidelines, and the protocols were approved by the Ethics Committee of the Kyungpook National University, Daegu, Republic of Korea (Permit number: 2020-0062).

After 1 week of acclimatization, rats were randomly divided into six groups as follows: control (vehicle-treated group), RGE (317 mg/kg), 2RGE (634 mg/kg), FRG (338 mg/kg), 2FRG (676 mg/kg), and ASA (100 mg/kg). Treatments were administered orally once a day for 1 week.

2.3. Platelet aggregation assay

The antiplatelet activity of ginseng extracts was evaluated using standard techniques. Light transmission aggregometry was conducted to evaluate the effects of ginseng extracts on agonist-stimulated platelet aggregation using a Chrono-Log aggregometer as previously described [14]. Briefly, washed platelets obtained from various treatment groups were incubated for 1 min at 37°C. Then, the mixture was stimulated with an agonist (ADP, collagen, or thrombin) and incubated for 5 min with continuous stirring.

2.4. Survival rate study

A collagen- and epinephrine-induced acute pulmonary thromboembolism experiment was conducted in mice as previously described [15]. Briefly, mice were administered saline, RGE (634.1 mg/kg), 2RGE (1.26 g/kg), FRG (676 mg/kg), 2FRG (1.35 g/kg), and ASA (100 mg/kg) once a day for 3 days (10 mice in each group). Then, 1 h after the final injection, the mice were challenged with 0.1 mL of a mixture containing collagen (500 μg/mL) and epinephrine (60 μg/mL) by smooth injection into one of the tail veins. The mortality of mice in each group was monitored for 10 min, and the data are presented as the percentage of surviving animals in each treatment group. At the end of each experiment, surviving animals were euthanized with an overdose of anesthesia.

2.5. Lung histology

Two minutes after the thrombotic challenge, mice were quickly euthanized with an overdose of anesthesia, and the lungs were perfused with a fixing solution (10% formalin buffered with calcium carbonate). The trachea was ligated and harvested together with the lungs, and the organs were soaked in cold saline and fixed with 10% formalin for 24 h. The lungs were then embedded in paraffin, and 5- to 6-μm-thick sections were cut and stained with hematoxylin and eosin for thrombus identification. The samples were observed under a light microscope (Axio Lab A1; Carl Zeiss Microlmaging, Jena, Germany) by a histopathologist blinded to the treatment, and a minimum of 10 fields were observed in each specimen.

2.6. Mice tail bleeding assay

Male mice were administered saline, RGE (634.1 mg/kg), 2RGE (1.26 g/kg), FRG (676 mg/kg), 2FRG (1.35 g/kg), and ASA (100 mg/kg) once a day for 7 days (five mice in each group). After 1 h of the final administration, mice were anesthetized, and the tail bleeding assay was conducted as previously described [16]. Briefly, the tail was prewarmed for 2–3 min in a normal saline solution at 37°C. Then, the tail was precisely transected 3–5 mm from the tip to induce bleeding. The distal portion of the tail was vertically immersed in a normal saline solution at 37°C. The bleeding time was recorded as the time between the start of transection to the cessation of bleeding.

Detailed description on; chemicals sources, washed rat platelet preparation, calcium mobilization, ATP assay, ELISA’s (serotonin, TXB2, cGMP and cAMP assay’s), flow cytometry, fibrinogen adhesion assay, immunoblotting, PT/aPTT measurement, blood biochemical analysis and statistical analysis, is included in Supplementary material.

3. Results

3.1. RGE and FRG inhibit agonist-stimulated ex vivo platelet aggregation

Light transmission aggregometry is a standard protocol used for evaluating the antiplatelet activity of certain compounds. Our initial screening revealed that washed platelets obtained from both RGE- and FRG-treated groups significantly inhibited platelet aggregation induced by different agonists (collagen, ADP, and thrombin), whereas increasing the agonist concentration of collagen from 2.5 to 5 μg/mL resulted in dose-dependent inhibitory effects on platelet aggregation. RGE exhibited more potent inhibition (p < 0.001) than FRG (p < 0.05), indicating that RGE is more effective in inhibiting the collagen-induced platelet aggregation (Fig. 1A–B). Platelets obtained from the ASA-treated group (taken as positive control) also significantly inhibited platelet aggregation induced by several agonist.
3.2. RGE and FRG attenuate calcium mobilization, thromboxane secretion, and dense granule secretion

When platelets come in contact with any potentiating agonist, they trigger a signaling cascade that results in granule secretions, thereby priming the activation and aggregation of platelets. These granules are primarily of two types, alpha and dense. Dense granules include ATP, ADP, serotonin, and Ca cations [17]. Furthermore, triggering calcium mobilization plays a key role in platelet activation [18]. It is known that inhibiting the secretion of these granules may halt the process of platelet activation [19]. Our results showed that RGE and FRG dose-dependently and significantly attenuated the secretion of platelet-dense granules (serotonin and ATP release) along with calcium mobilization and thromboxane secretion (Fig. 2A–D).

3.3. RGE and FRG regulate integrin $\alpha_{IIb}\beta_3$ signaling

A conformational change in the surface integrins, especially $\alpha_{IIb}\beta_3$ of platelets, is considered as a marker of inside-out signaling, which further enhances its affinity to bind with fibrinogen, consequently adding up to platelet aggregation and thrombus formation. Subsequently, when surface integrins are activated, they transduce signals into the cell that cause structural modification and platelet shape changes, thereby allowing the platelets to spread and adhere to the vascular epithelium (also termed as early outside-in signaling) and in later phases thrombus formation and clot retraction [20]. Our results showed that both RGE and FRG strongly inhibited fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ (Fig. 2E–F) and platelet spreading and adhesion to immobilized fibrinogen surface (Fig. 2G) in a significant and dose-dependent manner.

RGE and FRG attenuate activation of COX-I and phosphorylation of MAPK and PI3K/Akt in GPVI signaling pathway

COX-I aids the conversion of arachidonic acid into thromboxane A2, which is a potent aggregation agent, and its inhibition hinders platelet activation and aggregation [21]. Molecules of the MAPK pathway are known to be involved in platelet activation [22]. Src family kinases and PLC$\gamma_2$ are known to regulate platelet activation and thrombus formation [19], and they have also been reported to be involved in clot retraction [23]. The PI3K/Akt pathway is downstream to Src, and PI3K also interacts with PLC$\gamma_2$, known to contribute to platelet granule secretion and aggregation [19]. Our results clearly showed that both RGE and FRG inhibited the activation of COX-I (Fig. 3A&D) and inhibited the phosphorylation of MAPK (p38 and ERK) (Fig. 3B&E), Src, PLC$\gamma_2$, and PI3K/Akt (Fig. 3C&F) in a significant dose-dependent manner, thus indicating the possible inhibitory mechanism of RGE and FRG on platelet activation.
3.4. Effect of RGE and FRG on cyclic nucleotide signaling

Cyclic-AMP and cyclic-GMP are second messengers that regulate multiple pathways and block several other signaling molecules in platelets involved in granule secretion or integrin stimulation; therefore, increasing the levels of cAMP or cGMP inhibits platelet activation and aggregation [24]. Second, VASPSer-157 is a substrate of cAMP-dependent protein kinase A, and its activation is known to inhibit agonist-stimulated platelet aggregation [25]. Our results demonstrated that RGE specifically increased cAMP levels (Fig. 4A–B) and enhanced VASPSer-157 expression in RGE-treated platelets (Fig. 4C–D), thereby indicating its possible inhibitory mechanism on platelet activation.

3.5. RGE and FRG inhibit thrombosis without affecting hemostasis

Antiplatelet agents often cause side effects or complications on hemostatic functions, such as thrombocytopenia, increased bleeding time, impaired prothrombin time (PT), and activated partial thromboplastin time [6]. Our results revealed that administration of RGE and FRG had no effect on PT and aPTT (Fig. 5A–B). A slight increase in bleeding time was observed at a higher dosage in FRG-treated rats (p < 0.05) but not in RGE-treated rats at both dosages (Fig. 5C).

Collagen-epinephrine-induced acute pulmonary thromboembolism in mice is an established model to evaluate antithrombotic agents [26]. It has been reported that the microvasculature of lungs gets clogged with platelet-rich thrombus Shortly after the intra-venous injection of collagen and epinephrine mixture, leading to lethal fatality [27]. Platelet-rich thrombus can be observed histologically in the microvasculature of lungs [28]. Our results demonstrated that both RGE- and FRG-treated mice significantly survived the thrombosis compared with control group mice, with a greater percentage of survival rate in the RGE-treated group (Fig. 5D). The histological results showed numerous occluded blood vessels with platelet thrombus in the control group, whereas RGE-, FRG-, and ASA-treated mice showed lesser number of occluded blood vessels compared with the control group (Fig. 5E). These results indicate that both RGE and FRG inhibit platelet functions and thrombus formation without affecting hemostasis, considering that a higher dosage of FRG moderately increased the bleeding time.

3.6. Blood analysis

As shown in Table S2, there was no significant difference in most of the blood cell counts; however, there was a slight but significant decrease in platelet count in the ASA-treated group (p < 0.05) compared with the control group, which is a common observation in ASA treatment. There was no significant difference compared with the control group.

4. Discussion

Worldwide, an increasing risk for cardiovascular ailments has been a significant concern, especially in the western world. CVD is considered as one of the leading causes of death, and researchers have been attempting to find better curative and preventive measures. Platelets are the primary etiological agents behind these thrombosis-related cardiovascular disorders. When platelets come in contact with any potentiating factor or agonist such as collagen, ADP, or thrombin, they undergo a huge signal transduction process that consequently leads to platelet activation and aggregation and thrombus formation. These signaling events could be hampered or blocked by the pharmacological suppression of platelets. Although a number of pharmacological drugs are available for the treatment and prevention of CVD, their use in often impeded due to side effects and complications, including resistance to these common drugs in some populations [9–8]. Better options to prevent these ailments are the Mediterranean diet and herbal formulations that have minimum side effects and complications and increased nutrient bioavailability and impart their maximum pharmacological and clinical effects [12,29,30]. Fermented formulations are also considered as an excellent option with enhanced nutrient profile and increased bioavailability and digestibility [11,31]. Ginseng is known to possess cardioprotective effects, including antiplatelet and antithrombotic properties [11,12,14].

In the present study, we evaluated the comparative antiplatelet and antithrombotic effects of RGE and FRG using murine and rodent models. Our results initially showed that both RGE and FRG inhibited platelet aggregation induced by several agonists, and increasing the concentration of agonist (collagen) helped in differentiating the comparative effects of RGE and FRG, where RGE exhibited better inhibitory effects on platelet aggregation in a dose-dependent manner. The secretion of dense granules, including ATP and serotonin, was impaired in a dose-dependent manner, with better inhibitory percentage in RGE-treated rats. Similarly, intracellular calcium ion mobilization, which is a critical regulator of several platelet functions, was also suppressed, thereby inhibiting platelet aggregation. Lucotti et al. [21], has reported that COX-I helps in the conversion of arachidonic acid into thromboxane A, which is a potent aggregating factor that enhances platelet activation and aggregation. COX-1 is constitutively expressed in platelets. COX-1 couples with TXA2 synthase (TXAS) to generate prothrombotic TXA2 upon procoagulant stimuli (e.g., collagen, thrombin, and ADP). Previously [12], we have summarized the inhibitory effects of various ginseng compounds (Rg3, Rg3, and Rk1) on COX-1 and thromboxane-A generation pathway. Thromboxane A is unstable, and therefore we evaluated the production of thromboxane B2, which was found to be inhibited by both RGE and FRG in a dose-dependent manner, with comparatively better inhibition among the RGE-treated groups. Correspondingly, COX-I expression was also diminished in the RGE- and FRG-treated groups compared with control group rats.
Surface integrin's play a vital role in communication among cells and attract other platelets to adhere for thrombus formation. The structural modification in integrin $\alpha_{\text{IIb}}\beta_3$ allows and enhances its binding affinity toward fibrinogen, which is known as an inside-out signaling event. Later, this activation further transduces the signal into the cell and causes shape changes in the cytoskeletal structure, which leads to platelet spreading, adhesion, and finally clot retraction [32]. Our results demonstrated obvious inhibitory effects on fibrinogen binding to integrin $\alpha_{\text{IIb}}\beta_3$ and platelet adhesion on the immobilized fibrinogen surface.

To rule out mechanistic aspects, specific signaling events were analyzed, and we observed that molecules of the MAPK pathway (p38 and ERK), which are commonly expressed in agonist-stimulated platelets and contribute to granule secretion and platelet activation [22], were inhibited. The glycoprotein VI pathway is activated upon collagen stimulation and first encountering protein which get activated is Src, then comes PLCγ2 or PI3K/Akt. Here, PI3K can also be the upstream molecule of PLCγ2 and their inhibition significantly reduces platelet shape change, granule secretion, and aggregation [19]. Our results demonstrated that these signaling events were attenuated in the RGE- and FRG-treated groups. Fig. 6 summarizes the inhibitory effects of RGE and FRG on platelet functions.

Cyclic-AMP-enhancing agents or integrin $\alpha_{\text{IIb}}\beta_3$ antagonists have been reported as antiplatelet drugs. It is known that increased levels of cAMP or enhanced expression of VASP may result in the inactivation of platelets [25]. It has also been reported that several ginseng preparations and ginsenoside-Rg3(s) may enhance cAMP levels and VASP expressions, including the inhibition of fibrinogen binding to integrin $\alpha_{\text{IIb}}\beta_3$ [12,33–35]. Moreover, the linkage between calcium mobilization, inositol 1,4,5-triphosphate receptor (IP$_3$R) and cAMP has been established [18], while recent studies have proved that some ginsenosides and ginseng preparations modulate platelet functions through upregulation of IP$_3$R and cAMP/VASP$^{\text{Ser}}$-157 [36–38]. They have also proved the linkage between calcium mobilization, IP$_3$R and cAMP/VASP$^{\text{Ser}}$-157 phosphorylation (Fig. 6), while inhibiting calcium mobilization (Fig. 2A). Considering available literature, it is quite possible that RGE and FRG may also act through IP$_3$R but needs further evidences. It must be noted that RGE contains a higher amount of ginsenoside-Rg3(s) than FRG (Table S1), which could contribute to its specific
antiplatelet functions. Moreover, previously [12], we have summarized that Rg1 and Rg2 are weak antiplatelet agents which could contribute an additive effect in RGE. Our HPLC results reveal that RGE contains higher amount of Rg1, Rg2 and Rg3 in RGE than FRG (Table S1), which could be the possible reason behind stronger antiplatelet effects of RGE compared to FRG.

Intravenous administration of thrombus-initiating factors such as thrombin, ADP, and collagen with epinephrine may cause fatal and acute platelet-rich thrombosis in the vasculature of lungs within a short period of time, and such models have been extensively used to evaluate antithrombotic drugs [26]. It is necessary to consider possible drug-associated complications while evaluating any antiplatelet or antithrombotic agent, and one of the common complications is impaired hemostasis or thrombocytopenia as reported in the case of aspirin or clopidogrel, and a significant proportion of population is also resistant to such drugs [6–8]. Therefore, we evaluated the antithrombotic properties of RGE and FRG along with possible effects on hemostasis and found that RGE-treated mice significantly survived the thrombosis (comparatively with better percentage of survival rate than FRG) without affecting hemostasis, whereas only a higher dosage of FRG moderately increased the bleeding time in mice.

It is evidenced from the available literature that several ginseng and ginsenosides preparations are effective in modulating platelet functions [11,12,14,33–35]. The present study was limited to a rodent model and can be correlated but of course a separate study should be conducted to verify and explore more antiplatelet parameters on human platelets. Our future prospects includes the evaluation of mechanistic aspects of RGE and FRG on a human

Fig. 4. RGE enhances cAMP levels and VASP$^{\text{Ser-157}}$ expressions. Washed platelets obtained from various treatment groups were incubated at 37°C for 1 min in an aggregometer and then stimulated with agonist for 5 min. (A-B) After terminating the aggregation, the cAMP and cGMP immunoassays were performed using the cAMP and cGMP EIA kits, respectively, according to the manufacturers’ protocol. (C-D) Proteins were extracted from the platelets and analyzed for the phosphorylation of total VASP and p-VASP$^{\text{Ser-157}}$ by immunoblotting. Shown are the representative immunoblot images and graphs of the mean ± SD and the data of at least four independent experiments. *p < 0.05 vs. control.
Fig. 5. Effects of RGE and FRG on pulmonary thromboembolism and hemostasis. (A-B) PPP obtained from rats orally administered with various treatments was incubated at 37°C for 7 min. Then, 100 μL of the incubated PPP was mixed with 50 mL of cephalin in the process plate, and coagulation was initiated by adding 1 mM CaCl₂ and 100 mL thromboplastin to the PPP for the aPTT and PT assays, respectively. (C) Mice were orally administered various treatments once a day for 7 days (n = 5 in each group), and tail bleeding assay was performed. (D) Mice were orally administered various treatments once a day for 7 days (n = 10 in each group). One hour after the final administration, mice were challenged with a collagen + epinephrine mixture and checked for survival at 10 min. (E) Arrows show the microvasculature occluded due to collagen-epinephrine-induced thrombosis. The graph shows the mean ± SD of at least five independent experiments. *p < 0.05 and ***p < 0.01 vs. control.

Fig. 6. Summary of inhibitory effects of RGE and FRG on platelet intracellular signaling pathways.
model, and further exploring underlying antiplatelet and antithrombotic mechanism.

5. Conclusion

Both RGE and FRG exhibited potent antiplatelet and antithrombotic properties, with better differentiating antiplatelet effects of RGE, which could be attributed to its higher content of ginsenoside-Rg3(s). These extracts are potential candidates to treat and prevent platelet-related cardiovascular disorders and could be recommended for dietary intake to obtain their cardioprotective effects.

Acknowledgment

The project was funded by the Korean Society of Ginseng (2020).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2021.05.010.

Abbreviations

| Term       | Definition                                      |
|------------|-------------------------------------------------|
| ADP        | Adenosine diphosphate                           |
| Akt        | Protein kinase B                                |
| ASA        | Acetylsalicylic acid                            |
| ATP        | Adenosine triphosphate                          |
| cAMP       | Cyclic adenosine monophosphate                  |
| COX-I      | Cyclooxygenase I                                |
| ERK        | Extracellular signal-regulated kinase           |
| GPVI       | Glycoprotein VI                                 |
| MAPK       | Mitogen-activated protein kinase                |
| PLC        | Phospholipase C                                 |
| SFPK       | Src family kinase                               |
| VASP       | Vasodilator-stimulated phosphoprotein           |

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