Journal of Ginseng Research 48 (2024) 428–434

Contents lists available at ScienceDirect

Journal of Ginseng Research

journal homepage: www.sciencedirect.com/journal/journal-of-ginseng-research

Research Article

Red ginseng extract inhibits lipopolysaccharide-induced platelet–leukocyte aggregates in mice

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ARTICLE INFO

Keywords:
Platelet activation
Platelet–leukocyte aggregates
Red ginseng extract
Inflammation
Thrombosis

ABSTRACT

Background: Platelet–leukocyte aggregates (PLAs) play important roles in cardiovascular disease and sepsis. Red ginseng extract (RGE) has been well-studied for its antiplatelet and anti-inflammatory activities. However, the potential inhibitory effects of RGE on PLA have not been investigated.

Methods: Six-week-old ICR mice were given oral gavage of RGE for 7 days, followed by an intraperitoneal injection of 15 mg/kg of lipopolysaccharide. Mice were euthanized 24 h later, and blood samples were collected for further analysis. Flow cytometry was utilized to sort populations of PLAs and platelet–neutrophil aggregates (PNAs). By using confocal microscopy, PNAs were validated. Morphological changes in platelets and leukocytes were visualized with scanning electron microscopy. Expressions of tissue factor (TF) and platelet factor 4 (PF4) were investigated using enzyme-linked immunosorbent assay.

Results: Populations of activated platelets, PLAs and PNAs, were significantly increased with LPS-induction. Treatment with 200 and 400 mg/kg of RGE decreased platelet activation. Moreover, the populations of PLAs and PNAs were reduced. PNAs were visible in the blood of septic mice, and this was attenuated by treatment with 400 mg/kg of RGE. Morphologically, sepsisinduced platelet activation and fibrin formation in the blood. This was reduced with RGE treatment. Sepsis-induced increase in the plasma levels of TF and PF4 was also reduced with RGE treatment.

Conclusion: This study shows that RGE is a potential therapeutic that reduces the activation of platelets and targets PLA and PNA formation. Detailed inhibitory mechanisms of RGE should be studied.

1. Introduction

Platelets and leukocytes form aggregates during vascular remodeling, mainly via CD62P (P-selectin) on platelets and the P-selectin glycoprotein ligand-1 on leukocytes [1]. This interaction was widely reported to be involved in atherogenesis by upregulating tissue factor (TF) and increasing levels of various inflammatory cytokines [2]. Platelets were also reported to bind monocytes [3], neutrophils [4], and lymphocytes [5]. This formation of aggregates can be detrimental and contributes to the progression of atherosclerosis. According to Schrottmaier et al. (2020), the interaction of platelet and leukocytes contribute to the (1) infiltration of leukocytes in the vascular endothelium, (2) secretion of factors that contribute to the formation of foam cells and differentiation of macrophages and T-cell activation, (3) secretion of an array of inflammatory cytokines and chemokines by macrophages and neutrophils alongside the differentiation of T cells into regulatory T cells and Th1 cells, (4) anti-inflammatory regulation of platelets via CD40L and JamA, (5) dysregulation of homeostasis whereby macrophages secrete TF, and activation of FXa of the coagulation cascade. Neutrophil extracellular traps (NETs) are also formed at this stage [6]. Based on previous studies, the formation of platelet–leukocyte aggregates (PLAs) further activate a series of mechanisms that lead to atherogenesis.

Sepsis induces cardiac dysfunction [7], and platelets were reported to be activated in sepsis as they express TLR1-7 and 9 [8]. The

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https://doi.org/10.1016/j.jgr.2024.03.009
Received 22 November 2023; Received in revised form 27 March 2024; Accepted 29 March 2024
Available online 7 April 2024

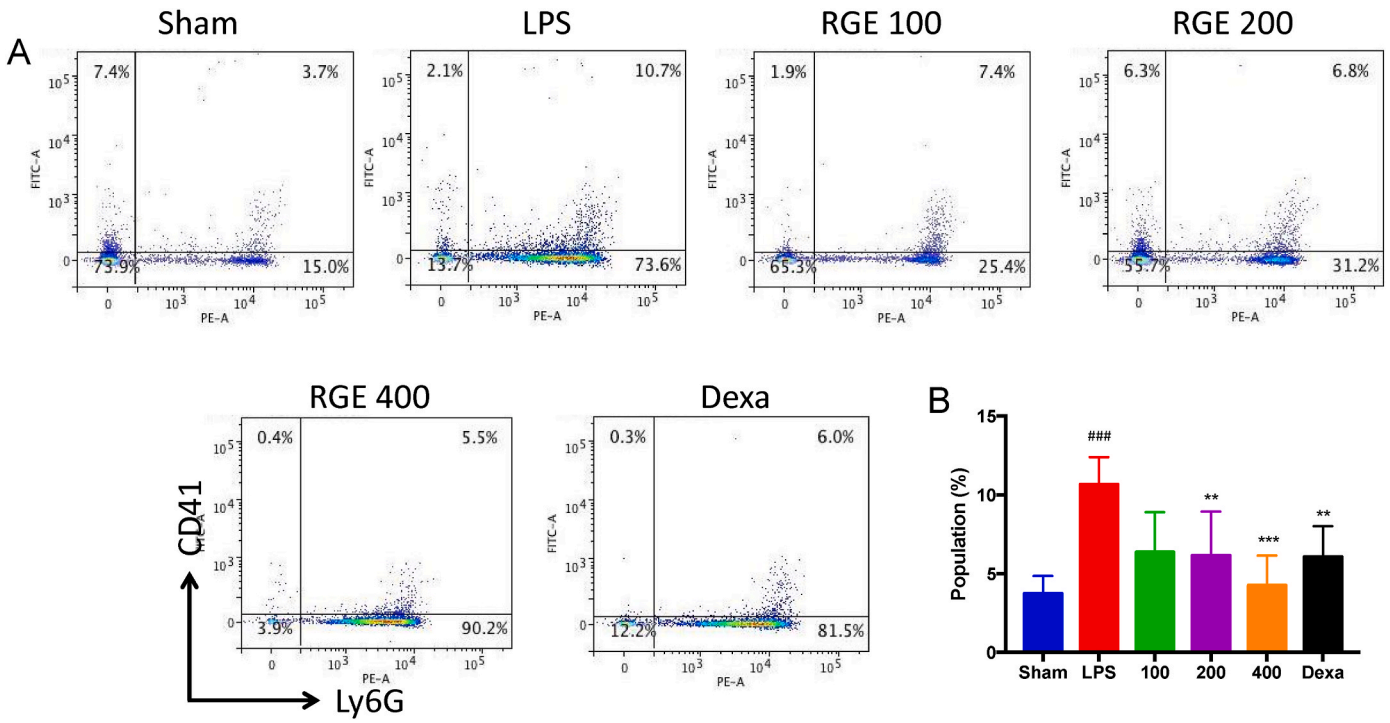

Fig. 1. The treatment with red ginseng extract (RGE) inhibits platelet–neutrophil aggregates (PNAs). An intraperitoneal injection of lipopolysaccharide (LPS) induced an increase in $CD41^{+}Ly6G^{+}$ population (PNA) in the blood. RGE was given orally for seven consecutive days. Representative flow cytometry data are shown in (A), with an average of three replicates (B). Data are presented as average ± SEM, and $^{**}p < 0.01$ and $^{***}p < 0.001$ were considered significant. Dexa, dexamethasone.

pathophysiology of sepsis mirrors that of cardiovascular disease, as patients with sepsis also exhibited increased platelet activation and were hyperadhesive to neutrophils, which may cause microcirculatory arrest [9]. A study reported that acetylsalicylic acid, an anticoagulant, has preventive effects against coagulation in *Staphylococcus aureus*-induced sepsis in a mouse model by preventing the extent of inflammation and tissue damage [10]. This further demonstrates the role of platelet aggregation in sepsis progression. However, immunothrombus formation includes a cascade of mechanisms. Activated platelets bind to neutrophils, forming platelet-neutrophil aggregates (PNAs) that are upregulated in sepsis as a response to inflammation [11]. In a mice study of early sepsis-induced hepatic dysfunction, extravasated platelets and NET formation was observed after sepsis-induction, which eventually leads to hepatic dysfunction [12]. Other than neutrophils, activated platelets bind to monocytes, another type of leukocyte, forming platelet-monocyte aggregates (PMAs). In older septic patients, PMA population is correlated to risk of mortality [13]. Moreover, a clinical investigation has shown that patients with sepsis have higher occurrence of PLAs [14]. Therefore, we are in search of a therapeutic approach that serves as an anti-inflammatory agent while having anticoagulatory properties, targeting PLAs to potentially suppress sepsis from the early stages of its initiation.

Red ginseng extract (RGE) from *Panax ginseng*, a medicinal herb, is widely studied for its anti-inflammatory effects whereby it was reported to target inflammasome in macrophages [15] and target the nuclear factor kappa B (NFκB) and activator protein 1 pathway to prevent inflammation in macrophages [16]. Moreover, RGE exhibits antithrombotic and antiplatelet activities and is proposed to prevent atherosclerosis [17–19]. However, no studies have been conducted on the effect of RGE against PLA. Thus, in this study, we utilized lipopolysaccharide (LPS) induced sepsis in mice as a model, and results showed that RGE effectively prevented PLA formation. This study is limited by the lack of mechanistic studies of which receptor RGE targets to prevent PLA that should be elucidated in future studies.

2. Materials and methods

2.1. Animal treatment regimen

Six-week-old ICR male mice were acquired from Orient-Bio (Gyeonggi-do, Republic of Korea) and were acclimatized for 1 week in a pathogen-free facility (humidity of 50 % ± 10 %, temperature of 23 °C ± 2 °C) with a 12 h light–dark cycle. Experiments were conducted according to the IACUC guidelines, and this study was approved by the Ethics Committee of the College of Veterinary Medicine of Kyungpook National University (2022-0088). Animals were separated into groups: sham, LPS, RGE 100 mg/kg, RGE 200 mg/kg, RGE 400 mg/kg, and dexamethasone 10 mg/kg (n = 6 per group). Dexamethasone was used as a positive control. Oral gavage of RGE was given to mice for seven consecutive days, followed by an intraperitoneal injection of 15 mg/kg of LPS to induce sepsis. The mice were euthanized for blood collection 24h later.

2.1.1. Flow cytometry analysis on blood samples of mice

Blood was harvested through cardiac puncture and stored in tubes with sodium citrate as an anticoagulant (BD Biosciences, Franklin Lakes, NJ, USA). Blood samples were immediately fixed with 1 % paraformaldehyde, and the red blood cells (RBCs) were lysed using an ammonium-chloride-potassium lysing buffer to obtain the blood cells. Blood samples were stained with Alexa Fluor 488-conjugated CD41, PE/Cy7-conjugated CD62P (BioLegend, San Diego, CA, USA), PE-conjugated Ly6G, and PerCP-conjugated CD11b (BD Biosciences). FACS analysis was conducted using the BD FACSAria^{TM}III (BD Biosciences). Data were post-analyzed using Flowlogic version 7 (Miltenyi Biotec, Bergisch Gladbach, Germany).

2.1.2. Peripheral hemogram of mice

Blood was harvested and stored in a microvette with ethylenediaminetetraacetic acid as an anticoagulant (BD Biosciences) for

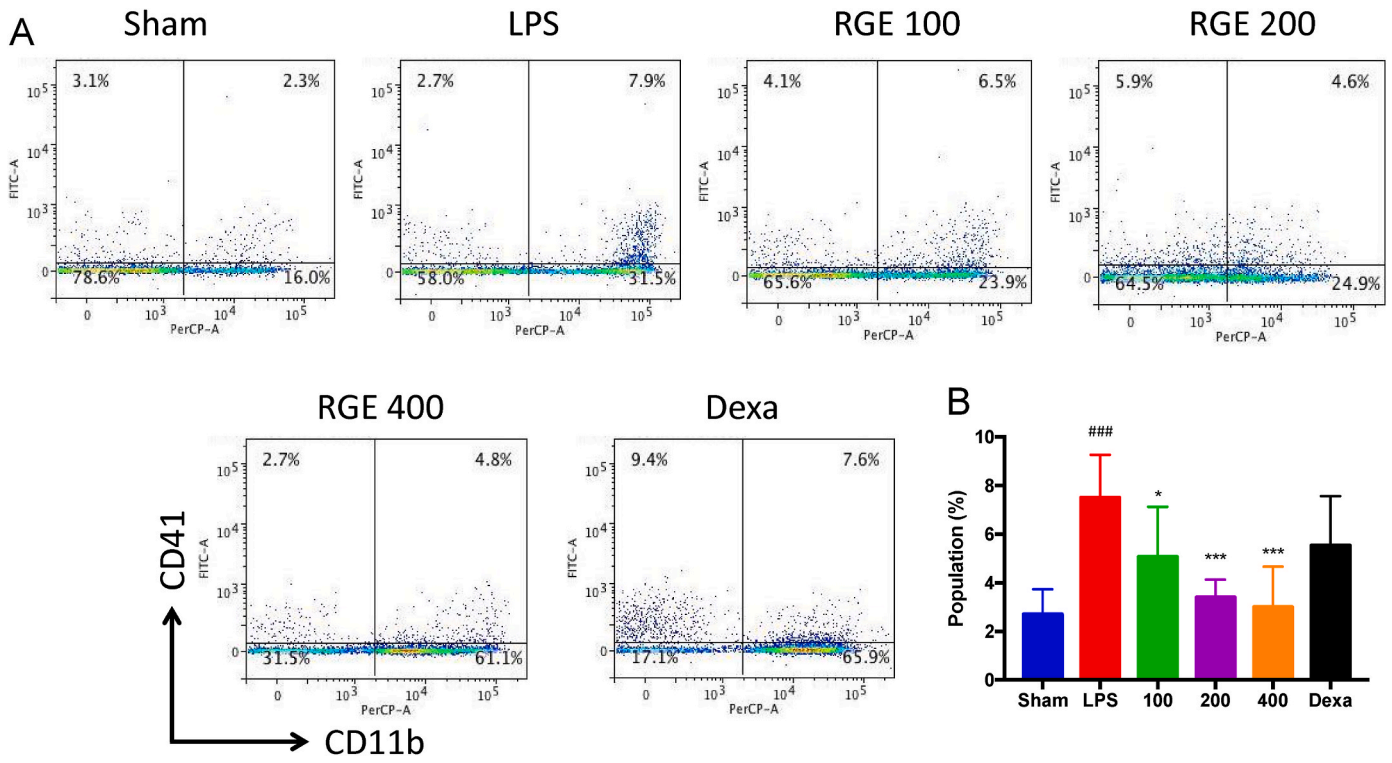

Fig. 2. Red ginseng extract inhibited platelet–leukocyte aggregate (PLA) formation. The population of $CD11b^{+}CD41^{+}$ represents the PLA population in the blood. RGE was given orally for seven consecutive days, followed by intraperitoneal injection of lipopolysaccharide to induce sepsis. Mice were euthanized 24 h later. Representative flow cytometry data are presented in (A). Data are presented as average ± SEM, where n = 3 (B), and $^{**}p < 0.01$ and $^{***}p < 0.001$ were considered significant in comparison to LPS-treated group. Significant difference was indicated as $^{###}p < 0.001$ of the LPS-group in comparison to the Sham group. Dexa, dexamethasone.

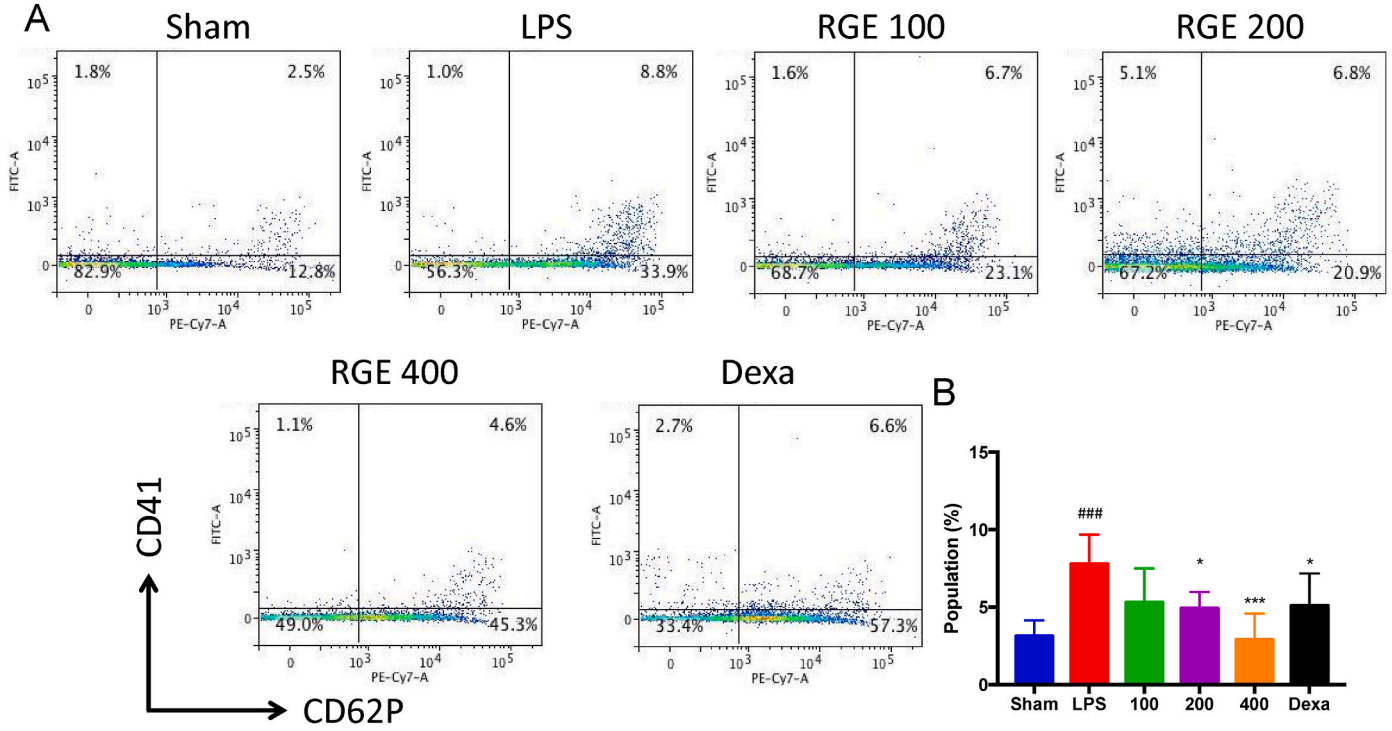

Fig. 3. Red ginseng extract reduced sepsis-induced platelet activation in the blood of mice. The $CD41^{+}CD62P^{+}$ population indicates activated platelets. Red ginseng extract was given orally to mice for seven consecutive days, followed by sepsis induction. Flow cytometry was conducted with the blood of mice (A). Data are presented as mean ± SEM (B), n = 3 and $^{*}p < 0.05$ and $^{***}p < 0.001$ were considered significant in comparison to LPS-treated group. Significant difference was indicated as $^{###}p < 0.001$ of the LPS-group in comparison to the Sham group. Dexa, dexamethasone.

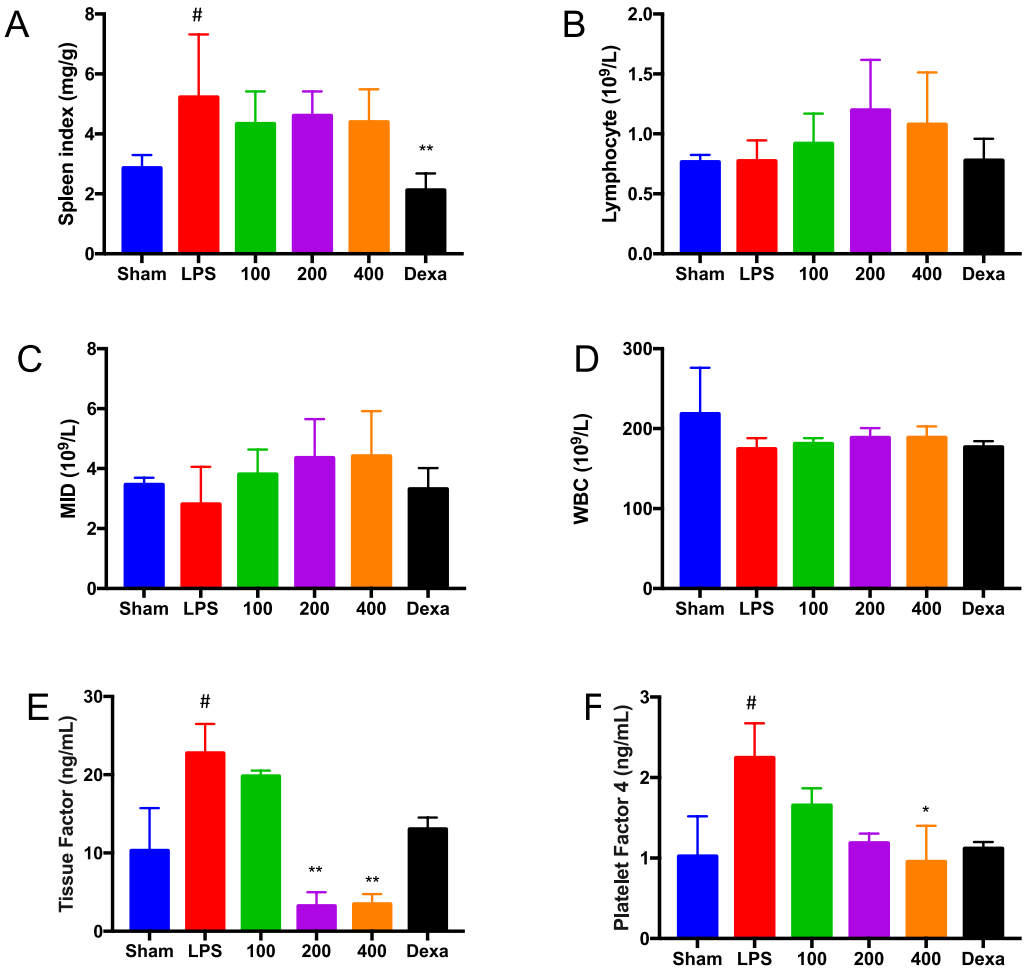

Fig. 4. Red ginseng extract therapy in sepsis-induced mice reduced platelet activation-related factors. The spleen was weighed to obtain the spleen index (A) where n = 6, and the lymphocyte (B), monocyte (C) and white blood cell (D) counts were quantified using a blood analyzer (n = 6). Plasma was collected from mice, and the levels of tissue factor (E) and platelet factor 4 (F) were investigated using ELISA assay. Plasma was collected from 6 mice, whereby 3 samples were pooled to form two replicates. Data are presented as mean ± SEM, and $^{*}p < 0.05$ and $^{**}p < 0.01$ were considered significant in comparison to LPS-treated group. Statistical significance was indicated ($^{\#}p < 0.05$) between the LPS-treated group and Sham group. Dexa, dexamethasone.

blood analysis. White blood cells (WBCs), lymphocytes, and monocytes (MID) were evaluated using a URIT-3000 Vet Plus hematology analyzer (Diamond Diagnostics Inc., Holliston, MA, USA).

2.1.3. Immunofluorescence of blood samples

Blood was collected via cardiac puncture and fixed as mentioned above. Then, samples were stained with PE-conjugated Ly6G (neutrophil marker) and Alexa Fluor 488-conjugated CD41 (platelet marker). To allow attachment of blood cells, stained samples were incubated on Poly-L-lysine-coated coverslips. Then, coverslips were sealed with ProLong® Gold Antifade Reagent with DAPI (Cell Signaling Technology, Danvers, MA, USA) that was used as a nuclear stain. Slides were analyzed under a super-resolution confocal laser scanning microscope (LSM800, Carl Zeiss, Jena, Germany).

2.1.4. Scanning electron microscopy of blood samples

Whole blood was collected as mentioned above and directly fixed with 1 % paraformaldehyde. Then, platelets were fixed with osmium tetraoxide and lyophilized before visualization with a field emission scanning electron microscope (SU8220; Hitachi, Tokyo, Japan).

2.2. Enzyme-linked immunoassay (ELISA)

Plasma levels of TF (Invitrogen, Waltham, MA, USA) and platelet factor 4 (PF4, MyBioSource Inc., San Diego, CA, USA) were investigated using ELISA kits according to the manufacturer's instructions.

2.3. Statistical analysis

The statistical significance in this study was investigated using Graphpad Prism 7.00 (San Diego, CA, USA) analyzed using one-way ANOVA. Data were presented as means ± SDs, and $p < 0.05$ were considered significant.

3. Results

3.1. RGE inhibits the formation of platelet–neutrophil aggregates in sepsis-induced mice

The PNA ($CD41^{+}Ly6G^{+}$) population was identified in the blood of sepsis-induced mice through flow cytometry. Our findings show that PNAs increased in septic mice. This shows that sepsis increases PNA formation, which was significantly reduced by treatment with 200 and 400 mg/kg of RGE for seven consecutive days (Fig. 1A and B).

3.1.1. RGE inhibits platelet-leukocyte aggregate formation in the blood of sepsis-induced mice

The PLA ($CD41^{+}CD11b^{+}$) population increased consistently with

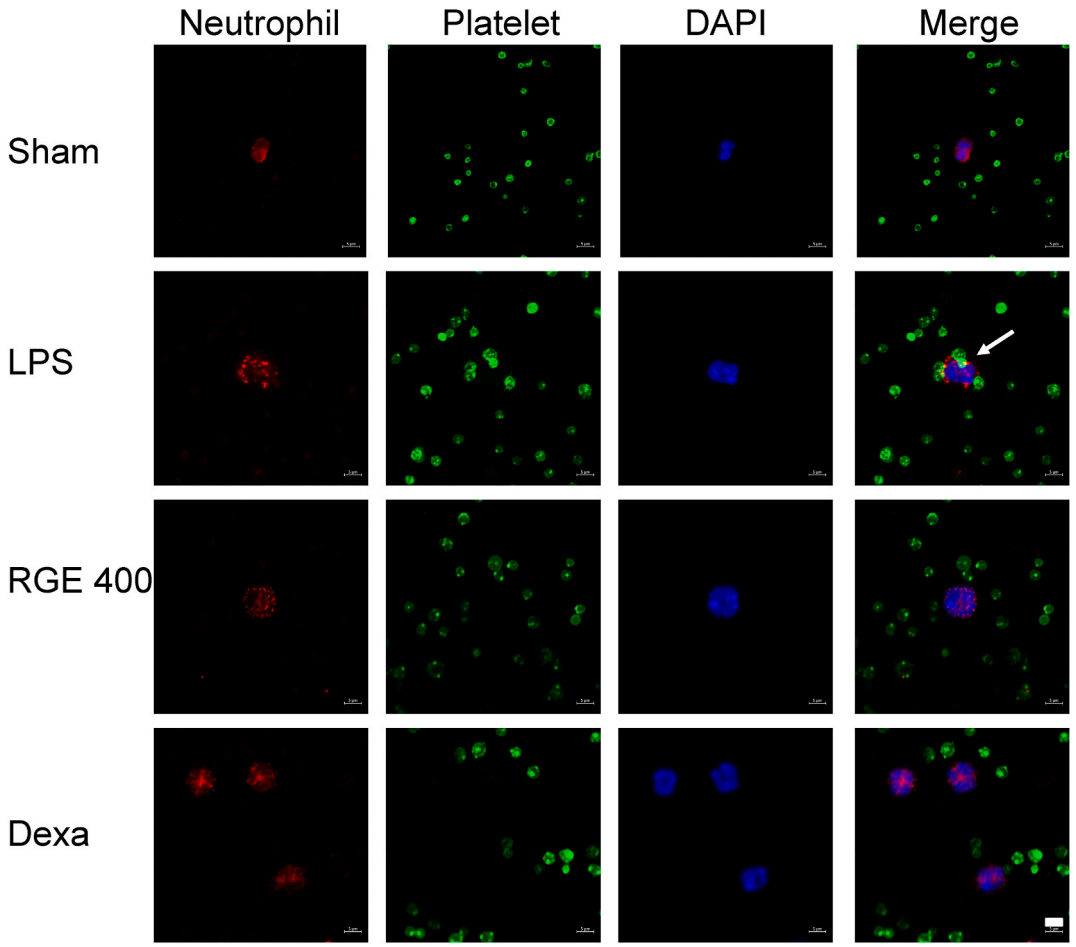

Fig. 5. Red ginseng extract prevented the formation of platelet–neutrophil aggregates. Blood was collected from mice and fixed immediately before staining with a neutrophil marker, Ly6G (red), platelet marker, CD41 (green) and DAPI. Dexa, dexamethasone. Scale bar indicates 5 μm.

PNAs by inducing sepsis in mice. This increase has been significantly attenuated with 200 and 400 mg/kg treatments in mice, showing a significant decrease in PLAs and proposing that RGE effectively inhibits platelet aggregation to form aggregates with leukocytes (Fig. 2A and B).

3.1.2. RGE prevents platelet aggregate formation in sepsis-induced mice

Populations of activated platelets ($CD41^{+}CD62P^{+}$) were identified in the blood of sepsis-induced mice whereby activated platelets have been increased in septic mice. This shows a correlation of inflammation that activates platelets. The treatment with 200 and 400 mg/kg for seven consecutive days shows a significant decrease in activated platelet population (Fig. 3A and B). Our results suggest that RGE prevents platelet activation and further reduces PLA and PNA formation.

3.1.3. RGE inhibits platelet-leukocyte aggregate formation in sepsis-induced mice by targeting TF and PF4

To further elucidate the role of RGE in preventing aggregate formation, no change in spleen index was observed among the groups, other than the dexamethasone group, indicating that this inhibition of aggregate formation may not be related to lymphocyte function, as the spleen is an important organ of residence organ of T lymphocytes (Fig. 4A). The blood analyzer did not detect significant change in the lymphocyte, monocyte, and WBC counts among the groups (Fig. 4B–D). However, TF and PF4 levels were reduced in the plasma of mice treated with 400 mg/kg of RGE (Fig. 4E and F). This indicates that RGE contributed to the activation of platelets that causes reduction in PNA and PLA formation.

3.1.4. RGE inhibits platelet-leukocyte aggregate formation in sepsis-induced mice

The blood sample was stained with a neutrophil marker and a platelet marker to visualize aggregate formation in the blood of mice induced with sepsis (Fig. 5). The results revealed that the LPS-treated mice had platelets (green) attached to neutrophils (red). This has not been observed in mice treated with 400 mg/kg of RGE and dexamethasone, despite having platelets located around the neutrophils. Moreover, blood samples were visualized using scanning electron microscopy in the presence of RBCs. From our observation, platelets adhered to leukocytes in LPS-induced mice. Aggregate formation (fibrin-like mesh) and platelet activation were observable in LPS-induced mice at lower magnification (Fig. 6A). However, this was not visible in the RGE and dexamethasone groups. At higher magnification, it is observed that platelets are attached to leukocytes in mice induced with sepsis, where clusters of activated platelets were also observed. This has been attenuated with treatment of RGE at 400 mg/kg (Fig. 6B). This suggests that RGE attenuated PLA and PNA formation.

4. Discussion

In this study, a model of LPS-induced mice was utilized to simulate a septic condition to induce PLA formation. Patients who had sepsis and were admitted in the intensive care unit have significantly higher levels of PMAs and PNAs upon admission, whereby 59 % of the patients are thombocytopenic [20]. In sepsis, the activated vascular endothelium releases agonists of coagulation. Moreover, pathogens and cytokines are platelet activators. Activated platelets tether to monocytes and

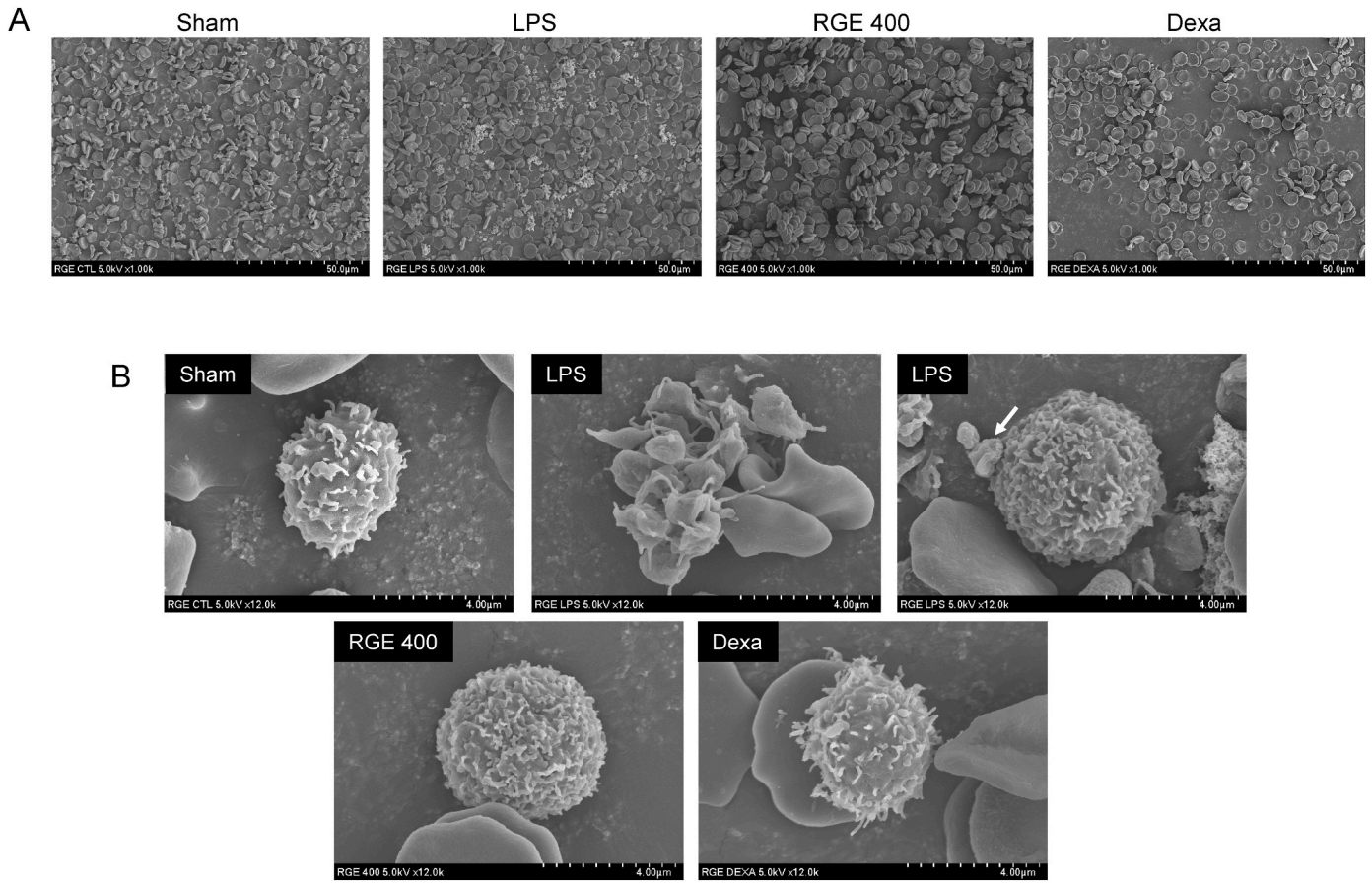

Fig. 6. Treatment with red ginseng extract prevented the aggregation of leukocyte and platelets. Blood was collected from mice and immediately fixed and processed for scanning electron microscopy. Images on the top and bottom panels were observed at 1.0k (A) and 12.0k (B), respectively. Platelets were indicated by arrow, depicting the attachment of platelets to leukocytes. Dexa, dexamethasone.

neutrophils that regulate host defense such as phagocytosis, NET formation, and cytokine release [21]. However, uncontrolled platelet activation may cause undesirable effects such as organ dysfunction because of tissue hypoxia caused by impaired vascular endothelial function in sepsis [22]. PLA formation is not only found in patients with sepsis but also in those with cardiovascular diseases, cerebral and renal inflammation, and acute lung injury (diseases that involve thrombo-inflammation) [23]. Activated platelets and leukocytes were also upregulated in patients with venous thromboembolism [24]. To date, treatments of thromboembolism include glycoprotein IIb/IIIa inhibitors such as eptifibatide, abciximab, and tirofiban. Although eptifibatide increased the recanalization rate in a metadata analysis, tirofiban and abciximab were less effective comparatively [25]. Therefore, the discovery of therapeutics from natural sources that can be consumed as a supplement to existing therapeutics is important.

Leukocytes and platelets tether via the following interactions: (1) PSGL-1 and P-selectin, (2) scCD40L, and (3) Mac-1 binding to GPIIb/IIIa on platelets via fibrinogen. Cells that can interact with platelets includes monocytes, lymphocytes, natural killer cells, neutrophils, and eosinophils [26]. Platelet activation allows platelet binding with platelets or leukocytes via fibrinogen after the conformational change in GPIIb/IIIa through the inside-out signaling [27]. Following this, P-selectin helps in stabilizing GPIIb/IIIa–fibrinogen interaction allowing the formation of larger aggregates [28]. Thus, the prevention of platelet aggregation is a strategic approach to prevent uncontrollable PLA and PNA formation.

A previous study showed that RGE was a promising candidate that exhibited antiplatelet and antithrombotic activity effectively inhibiting collagen, arachidonic acid, U46619 and thrombin-induced platelet aggregation [29]. This shows that RGE inhibits platelet activation by targeting various mechanisms including the α2β1, GPVI receptors, and thromboxane pathway. Moreover, fractions of RGE have demonstrated effective antiplatelet activity [30,31]. Not only is RGE a potent anti-inflammatory agent, the polysaccharide fractions of RGE also enhance host defense against infection by increasing phagocytic leukocytes [32]. We hypothesize that increased bacterial clearance by increasing phagocytosis could dampen bacterial-induced platelet activation via TLRs, GPIIb/IIIa, and FcγRIIa to prevent further PLA formation [33]. Our results showed that RGE inhibited PF4 and TF in the plasma of mice. Bacteria-induced platelet aggregation was reported to be induced by PF4 [33]. Moreover, platelet–monocyte interactions were related to monocyte TF in SARS-Cov-2-induced hypercoagulability [34]. Our previous study has shown that Rg3-enriched RGE has rescued mice sepsis-induced mice, potentially targeting the NFκB and mitogen-activated protein kinase (MAPK) pathway [35]. A recent review has also summarized the potential molecular actions of various ginsenosides that are studied against sepsis, showing that ginsenosides mainly target the NFκB, MAPK pathway and the NOD-like receptor protein 3 to target sepsis [36]. Taken altogether, RGE is potentially effective against sepsis.

Based on the aforementioned studies, platelet–leukocyte interactions are not only complicated and induced by various factors. In a nutshell, this study shows evidence that RGE can further target PLAs and PNAs as a pathway of mechanism against sepsis. RGE not only inhibit platelet aggregation but can also potentially act through multiple mechanisms to target PLA formation, and further prevent inflammation by targeting the NFκB and MAPK pathway in macrophages. However, we have yet to identify the actual mechanism whereby RGE inhibits PLA formation. Further studies are needed to elucidate the route of the mechanism of

RGE that potentially prevents PLA formation.

Data availability

All data were presented in the main figures in this manuscript.

Declaration of competing interest

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or nonfinancial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Ministry of Science and ICT, Republic of Korea (No. 2022R1A2C1012963).

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