Food and Chemical Toxicology 131 (2019) 110553

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

# Ginsenoside Rg3, a component of ginseng, induces pro-thrombotic activity of erythrocytes via hemolysis-associated phosphatidylserine exposure

Yiying Bian[a], Gwang-Jin An[a], Keunyoung Kim[a], Thien Ngo[a], Sue Shin[b], Ok-Nam Bae[c], Kyung-Min Lim[d], Jin-Ho Chung[a,*]

[a] College of Pharmacy, Seoul National University, Seoul, 151-742, South Korea
[b] Department of Laboratory Medicine, Boramae Hospital, Seoul, 156-707, South Korea
[c] College of Pharmacy, Hanyang University, Ansan, Gyeonggi-do, 426-791, South Korea
[d] College of Pharmacy, Ewha Womans University, Seoul, 03760, South Korea

ARTICLE INFO

*Keywords:*
Ginseng
Ginsenoside Rg3
Erythrocyte debris
Hemolysis
Phosphatidylserine (PS)
Thrombosis

ABSTRACT

Ginseng and its active gradient, ginsenoside Rg3 (Rg3), are widely used for a variety of health benefits, but concerns over their misuses are increasing. Previously, it has been reported that Rg3 can cause hemolysis, but its health outcome remains unknown. Here, we demonstrated that Rg3 could promote the procoagulant activity of erythrocytes through the process of hemolysis, ultimately leading to increased thrombosis. In freshly isolated human erythrocytes, Rg3 caused pore formation and fragmentation of the erythrocyte membrane. Confocal microscopy observation and flow cytometric analysis revealed that remnant erythrocyte fragments after the exposure to Rg3 expressed phosphatidylserine (PS), which can promote blood coagulation through providing assembly sites for coagulation complexes. Rat *in vivo* experiments further confirmed that intravenous administration of Rg3 produced PS-bearing erythrocyte debris and increased thrombosis. Collectively, we demonstrated that Rg3 could induce the procoagulant activity of erythrocytes by generating PS-bearing erythrocyte debris through hemolysis, which might provoke thrombosis.

## 1. Introduction

Ginseng, the root of *Panax ginseng* C. A. Meyer, has long been consumed as a herbal remedy in Asian countries for diverse health benefits. People over Asian countries widely consumed ginseng products as dietary supplements, even believing them as a panacea for a wide spectrum of abnormal health conditions. Indeed, ginseng possesses many pharmacological effects that include vasorelaxation, anti-cancer, anti-aging, anti-inflammatory, and anti-oxidant activities (Helmes, 2004; Karmazyn et al., 2011), which are mostly attributable to ginsenosides (e.g., ginsenoside Rg3), a major saponin component of ginseng. In recent decades, however, concerns over the ginseng-associated health hazards have been escalating, which is mostly from excessive consumptions and misuses unproven for their safety (Ernst, 2002). Even an intravenous formula of ginseng has been developed as a line of pharmacotherapeutic strategy in some countries (Haijiang et al., 2003).

Many reports have illuminated various side effects of ginseng ranging from insomnia, diarrhea, vaginal bleeding, nausea, vomiting, mastalgia, hypertension and hypotension to drug interactions, which have been strongly supported by lines of evidence from clinical trials and case reports (Coon and Ernst, 2002; Siegel, 1979, 1980). Of these, the potential cardiovascular toxicity of ginsenoside Rg3 concerning hemolysis, hypertension/hypotension, vascular dysfunction and hematological toxicity (Jung et al., 2018; Lee et al., 2010; Liu et al., 2003, 2012; Siegel, 1980) alludes a profound and broad impact of ginsenoside Rg3 on cardiovascular tissue integrity and function. We came to notice that ginsenoside Rg3 has a potent hemolytic effect (Li and Liu, 2008; Liu et al., 2002, 2003) but its health outcome is relatively unknown.

Lysis of erythrocytes, i.e., hemolysis can ultimately result in hemolytic anemia, a major pathophysiological phenomenon involving erythrocytes (Rao, 2014). Hemolysis often concurs with various cardiovascular adverse effects such as increased blood pressure, heart attack or thrombotic complications. Recently, the role of erythrocytes in thrombosis is gathering increasing attention (Byrnes and Wolberg, 2017; Weisel, 2015). Erythrocytes can promote the coagulation cascade and clot formation by providing a site for the assembly of prothrombinase and tenase complexes, ultimately resulting in a hypercoagulable state (Zwaal and Schroit, 1997). Externalization of

* Corresponding author. Research Institute of Pharmaceutical Sciences & Seoul National University.
*E-mail addresses:* byy19900719@snu.ac.kr (Y. Bian), adslagn28@snu.ac.kr (G.-J. An), millio1014@naver.com (K. Kim), ngothien86@snu.ac.kr (T. Ngo), jeannie@snu.ac.kr (S. Shin), onbae@hanyang.ac.kr (O.-N. Bae), eusapien@yahoo.com (K.-M. Lim), jhc302@snu.ac.kr (J.-H. Chung).

https://doi.org/10.1016/j.fct.2019.05.061
Received 20 March 2019; Received in revised form 19 May 2019; Accepted 31 May 2019
Available online 01 June 2019
0278-6915/ 

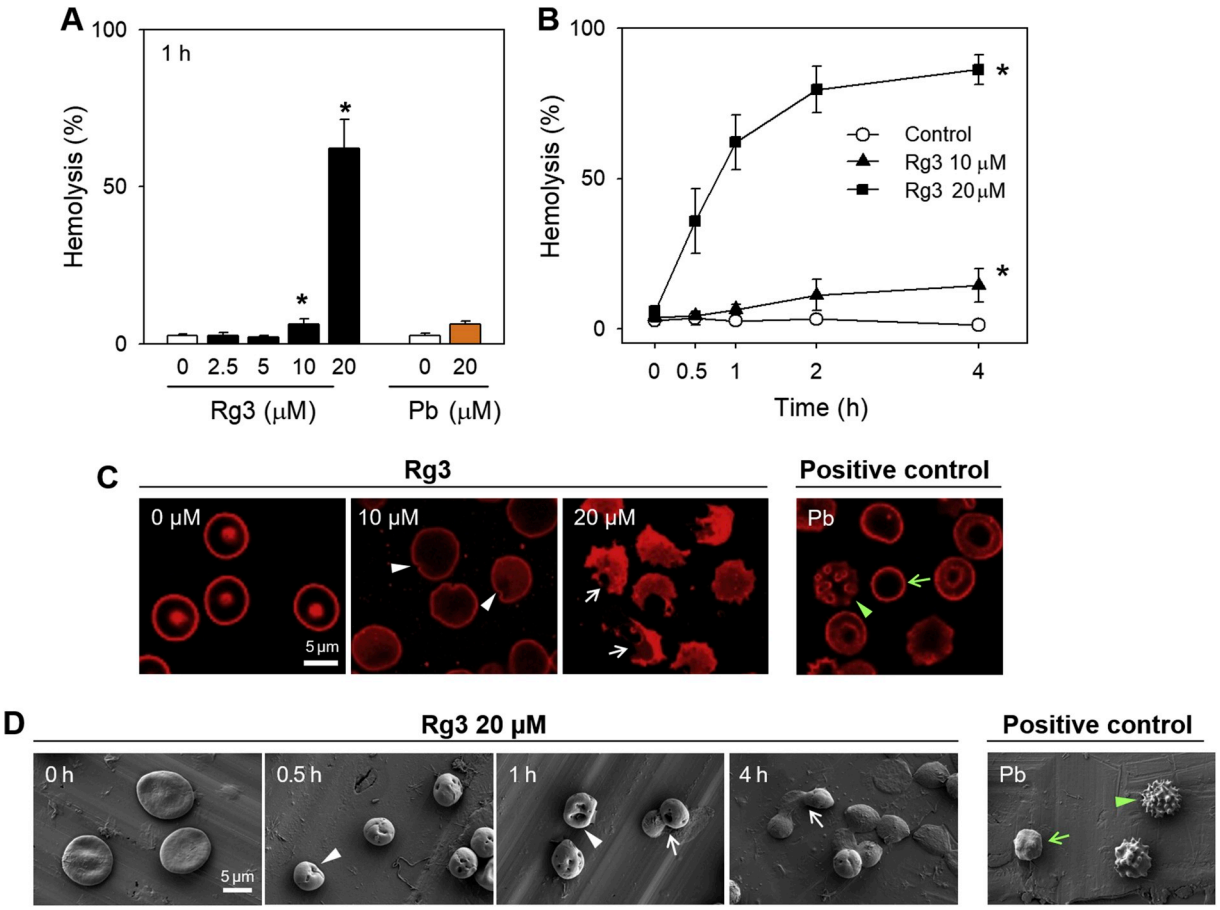



**Fig. 1.** Effects of Rg3 on hemolytic activity and morphological changes in human erythrocytes. (A) After erythrocytes were treated with 0, 2.5, 5, 10, 20 μM Rg3 and 0, 20 μM Pb for 1 h, hemolysis was measured at 540 nm. (B) Time-dependent hemolysis was determined after erythrocytes were exposed to various concentrations of Rg3 for 0, 0.5, 1, 2 and 4 h. (C) After 4 h exposure of erythrocytes to 0, 10, 20 μM of Rg3 as well as 20 μM Pb as a positive control, morphological changes were observed under confocal microscopy. (D) For further observation of morphological changes on the membrane surface, erythrocytes were treated with 20 μM Rg3 over time (0, 0.5, 1 and 4 h), and fixed to be examined using scanning electron microscope (SEM). Pore formation (white arrowheads) and burst cells to fragments or debris (white arrows) were shown in Rg3 groups. Here, 20 μM Pb at 4 h was used as a positive control. Echinocytes (green arrowheads) and spherocytes (green arrows) were shown in Pb-treated erythrocytes. Bar = 5 μm. Values are mean ± SE of more than five independent experiments, * represents significant differences from control group ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

phosphatidylserine (PS), an anionic phospholipid, from inner to the outer leaflet of the lipid bilayer is central in this process, resulting in the procoagulant activity of erythrocytes. Microvesicles (MV, ≦ 1 μm) may also be generated from surface lipids through exocytosis, which can also contribute to thrombosis (Shantsila et al., 2010). Many chemicals that include lysophosphatidic acid, lead (Pb), mercury and vitamin C promote PS externalization and MV generation, which may ultimately promote thrombosis (Daleke, 2008; Kim et al., 2015; Lim et al., 2010).

Here, we aimed to elucidate whether ginsenoside Rg3 can affect the procoagulant activity in freshly isolated human erythrocytes *in vitro* and increase thrombosis *in vivo* rat venous thrombosis model. Lead (II) acetate (Pb) was selected as a positive control, which is well established to induce thrombosis through PS-exposure in erythrocytes (Shin et al., 2007). We discovered that Rg3 could produce irregular fragmentation of erythrocytes and PS-bearing erythrocytes debris during hemolysis, which may ultimately contribute to thrombosis *in vivo*. We believe that our results will help to illuminate the potential risk of safety-unproven misuses of ginseng.

## 2. Materials and methods

### 2.1. Materials

The following chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) : Rg3, Lead(II) acetate (Pb), $CaCl_2$, glucose, ethylenediaminetetraacetic acid (EDTA), bovine serum albumin (BSA), N,N,N',N-tetrakis(2-pyridylmethyl) ethylene diamine (TPEN), N-[2Hydroxyethyl]piperazine- N'-[2-ethanesulfonic acid] (HEPES), sodium dodecyl sulfate, glutaraldehyde solution and osmium tetroxide, and purified human thrombin. Phycoerythrin-labeled monoclonal mouse anti-human CD235a (anti-glycophorin-A-PE) and fluorescein isothiocyanate (FITC)-labeled annexin V (annexin V-FITC) were from BD Pharmingen (San Diego, CA). PE anti-rat erythroid cells antibody was from Biolegend (San Diego, CA). Purified human prothrombin (factor II), factor Xa and factor Va were obtained from Hematologic Technologies, Inc. (Essex Junction, VT), and S2238 was purchased from Chromogenix (Milano, Italy). Human umbilical vein endothelial cells (HUVECs) and the endothelial cell growth media (EGM) kit were purchased from Lonza (Basel, Swiss). Calcein-green AM was from Invitrogen (Carlsbad, CA). Thromboplastin was from Instrumentation Laboratory (Lexington, Massachusetts). Prothrombin time (PT) reagent and activated partial thromboplastin time (aPTT) reagent were from Instrumentation Laboratory (Bedford, MA).

### 2.2. Preparation of erythrocytes and measurement of hemolysis

With the approval from the Ethics Committee of Health Service Center at Seoul National University, human blood was obtained from

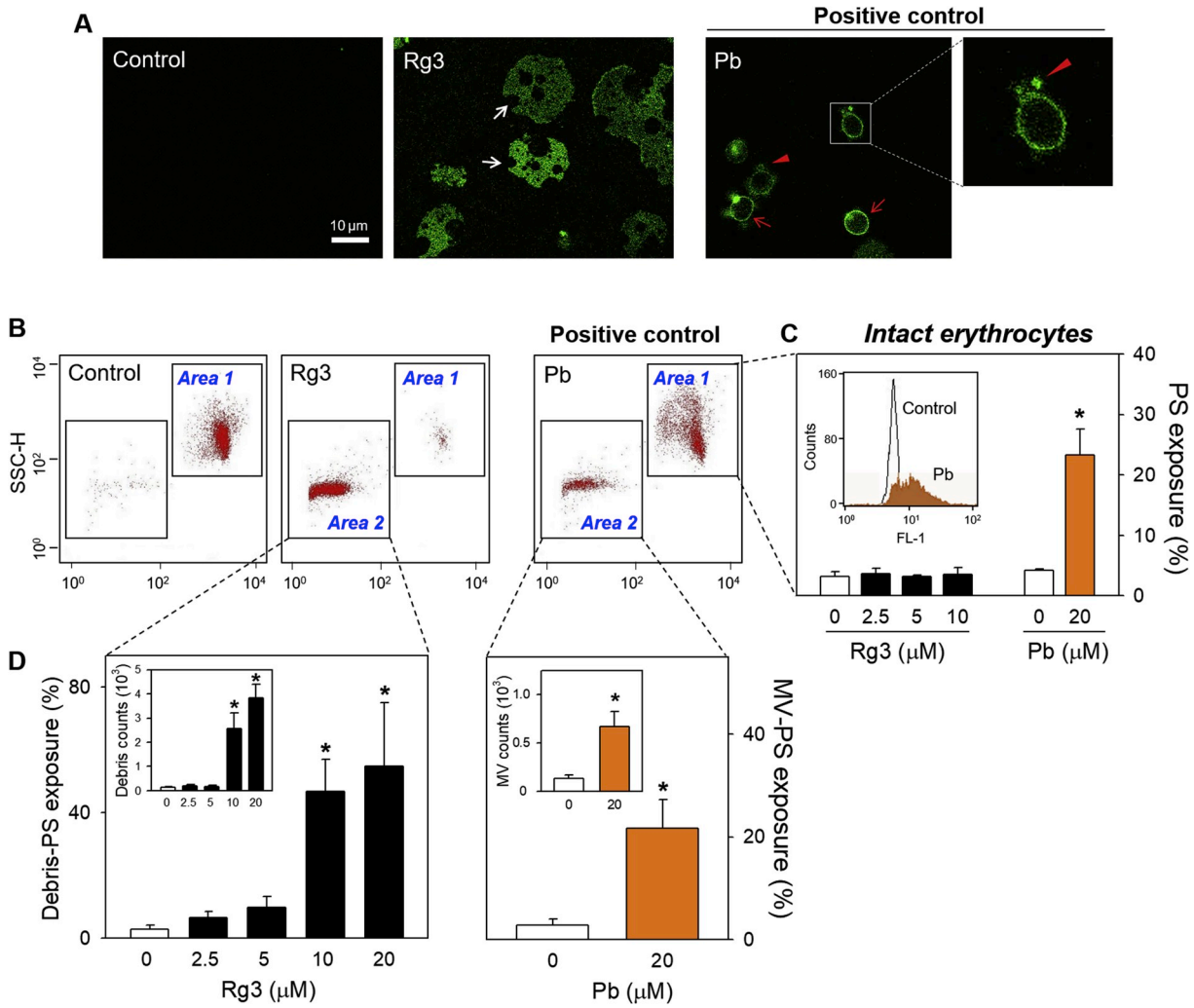



**Fig. 2.** Confocal identification and flow cytometric detection of PS exposure on erythrocytes debris induced by Rg3. (A) Erythrocytes were treated with 20 μM Rg3 and 20 μM Pb (positive control) for 1 h at 37 °C, and annexin V-FITC was treated for binding exposed phosphatidylserine (PS). Annexin-positive erythrocytes debris (white arrows) were irregular in size and shape after Rg3 treatment. As a positive control, a regular distribution of PS was shown as annexin-positive intact erythrocytes (red arrow) and annexin-positive MV (red arrowheads) releasing after Pb treatment. Bar = 10 μm. (B-D) Intact cells (area 1) and debris/MV (area 2) were measured using flow cytometry, respectively. (C) In intact cells (area 1), no PS exposure was observed in Rg3 group. (D) In area 2, erythrocytes debris induced by Rg3 and (D, left) PS exposure of such debris significantly increased in a concentration-dependent manner, and (D, right) MV released from erythrocytes and PS exposure of MV increased as well. Values are mean ± SE of more than five independent experiments, * represents significant differences from control group ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

healthy male donors (20-30 years old) using a vacutainer with acid citrate dextrose and a 21 gauge needle (Becton Dickinson, U.S.A.) on the day of each experiment. Platelet-rich plasma and buffy coat were removed by aspiration after centrifugation at 200 *g* for 15 min. Packed erythrocytes were washed 2 times with phosphate buffered saline (PBS: 1.06 mM $KH_2PO_4$, 154 mM NaCl and 2.96 mM $Na_2HPO_4$ at pH 7.4) and once more with Ringer solution (125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 32 mM HEPES, 5 mM glucose, pH 7.4). Washed erythrocytes were resuspended in TBS buffer to a cell concentration of $5 \times 10^7$ cells/mL, and the final $CaCl_2$ concentration was adjusted to 1 mM prior to use. After erythrocytes incubation with chemicals, samples were centrifuged (10,000 g for 1 min) and the extent of hemolysis was determined spectrophotometrically at 540 nm.

### 2.3. Microscopic observation

For confocal microscopy, 500 μL erythrocytes suspension was added and attached for 1 h to a four-chambered coverslip (Lab-Tek® from Thermo Fisher, NY, USA). After washing the coverslip once with Ringer's solution containing 2% BSA, erythrocytes were then incubated with vehicle (dimethyl sulfoxide, DMSO), various concentrations of Rg3 or 20 μM Pb. After incubation, the coverslips were washed once again with Ringer's solution and then, erythrocytes were stained with Ringer's solution containing anti–glycophorin-A–PE for 30 min. Finally, samples were washed once and resuspended to be observed using confocal microscopy equipped with an argon laser (TCS SP8, Leica, Germany). Excitation and emission filters were set at 488 nm and 550–600 nm, respectively. For SEM observation, after fixation with 2% glutaraldehyde solution for 1 h at 4 °C, the erythrocytes were centrifuged and washed three times with PBS, followed by post-fixation with 1% osmium tetroxide for 30 min at room temperature in the hood. After washing with PBS twice, the samples were dehydrated serially with 50%, 70%, 80%, 90%, and 100% ethanol. After drying and coating with gold, the images were observed on a field emission scanning electron microscope (Merlin Compact FE-SEM, Zeiss, Germany).

### 2.4. Flow cytometric analysis

Annexin V-FITC was used as a marker for phosphatidylserine (PS) detection, whereas anti-glycophorin A-PE was used as an identifier of

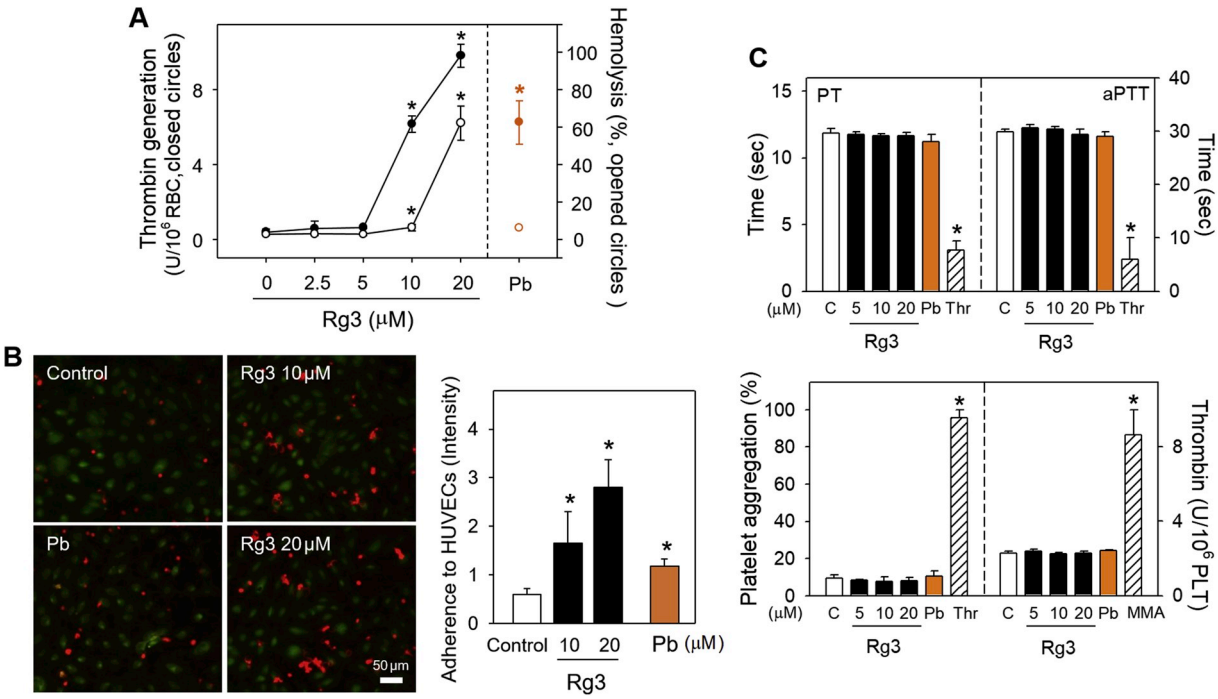



**Fig. 3.** Biological effects of erythrocytes by Rg3 treatment. (A) After erythrocytes were treated with various concentrations of Rg3 and 20 μM Pb (as a positive control) for 1 h at 37 °C, enhancement of thrombin generation (closed circles) was measured by pro-thrombinase assay at 405 nm, and hemolytic activity (opened circles) was measured at 540 nm. (B) Rg3 and Pb-treated erythrocytes were further incubated with pre-stained HUVECs (green fluorescence) for 1 h at 37 °C in 5% $CO_2$ incubator and then enhanced adherent erythrocytes (red fluorescence) to HUVECs were examined using fluorescence microscope. (C) Platelet activation by Rg3 was evaluated and no significance was detected in partial thromboplastin time (aPTT) and prothrombin time (PT), as well as platelet aggregation and thrombin generation of platelets (PLT) by both chemicals. Thrombin (Thr) 1 unit/mL and monomethylarsonic acid (MMA) 50 μM were used as positive control initiating plasma coagulation and platelets activation. Bar = 50 μm. Values are mean ± SE of more than five independent experiments, * represents significant differences from control group ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

erythrocytes. Negative controls for annexin V binding were stained with annexin V-FITC in the presence of 2.5 mM EDTA instead of 2.5 mM $CaCl_2$. Samples were analyzed on the flow cytometer FACS Calibur (Becton Dickinson, U.S.A.) equipped with an argon-ion laser emitting at 488 nm. The light scatters, and fluorescence channels were set on a log scale. Data from 5,000 events were collected and analyzed using Cell Quest Pro software. Microvesicles (MV) were identified by forward scatter characteristics after calibration by 1% standard beads.

### 2.5. Prothrombinase assay

After treatment with chemicals, samples were incubated with 5 nM factor Xa and 10 nM factor Va in Tyrode buffer (134 mM NaCl, 10 mM HEPES, 5 mM glucose, 2.9 mM KCl, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 0.34 mM $Na_2HPO_4$, 0.3% BSA, and 2 mM $CaCl_2$ at pH 7.4) for 3 min at 37 °C. Thrombin formation was initiated by the addition of 2 μM prothrombin. Exactly 3 min after the addition of prothrombin, an aliquot of the suspension was transferred to a tube containing stop buffer (50 mM Tris-HCl, 120 mM NaCl, and 2 mM EDTA at pH 7.9). Thrombin activity was determined using the chromogenic substrate S2238 (chromogenic substrate for thrombin; Chromogenix, Milano, Italy). We calculated the rate of thrombin formation from the change in absorbance at 405 nm using a calibration curve generated with active-site–titrated thrombin.

### 2.6. Fluorescence observation of adhered erythrocytes to human umbilical vein endothelial cells (HUVECs)

Endothelial cells ($2 \times 10^4$ cells) were seeded into 4-well-chamber for 2 days and stained with calcein green for 20 min. Chemical-treated erythrocytes were washed once and resuspended in EBM-2 to a cell concentration of $5 \times 10^7$ cells/mL. After HUVECs were washed twice with EBM-2, chemical-exposed erythrocytes were layered onto confluent HUVEC monolayer and incubated for 60 min at 37 °C. After the incubation, the chambers were rinsed once with EBM-2 to remove nonadherent erythrocytes, and glycophorin A-PE was added for staining erythrocytes. Adhered erythrocytes to HUVECs were observed using fluorescent microscopy.

### 2.7. Experiments with platelets

Briefly, platelet-rich plasma (PRP) was prepared by centrifugation for 15 min at 150 g, and platelet-poor plasma (PPP) was obtained from the precipitated fraction of PRP by centrifugation for 20 min at 2,000g. The platelet count in PRP was adjusted to $3 \times 10^8$ platelets/mL by using PPP. In PPP, PT and aPTT were measured in BBL Fibrometer (Becton Dickinson, Cockeysville, Maryland), according to the procedures in PT and aPTT reagent kit, respectively. In PRP, platelet aggregation was measured by light transmission in an aggregometer (Chrono-log Corp., USA), with 100% calibrated as the absorbance of PPP and 0% calibrated as the absorbance of PRP. Thrombin generation by platelet was determined using prothrombinase assay as described above.

### 2.8. In vivo experiments

All the protocols of *in vivo* experiments were approved by the Ethics Committee of Animal Service Center at Seoul National University. Male Sprague-Dawley rats (SamTako, Osan, Korea), weighing 300-400 g were anesthetized with urethane (1.25 g/kg, i.p.). Here, 0, 25 and 50 mg/kg of Rg3 were evaluated with 25 mg/kg Pb as a positive control. After intravascular injection of chemicals, whole blood (3.8% sodium citrate) was collected from the abdominal aorta. The extent of hemolysis was determined spectrophotometrically at 540 nm. The fragility of chemical-exposed erythrocytes was evaluated in 0.45% NaCl

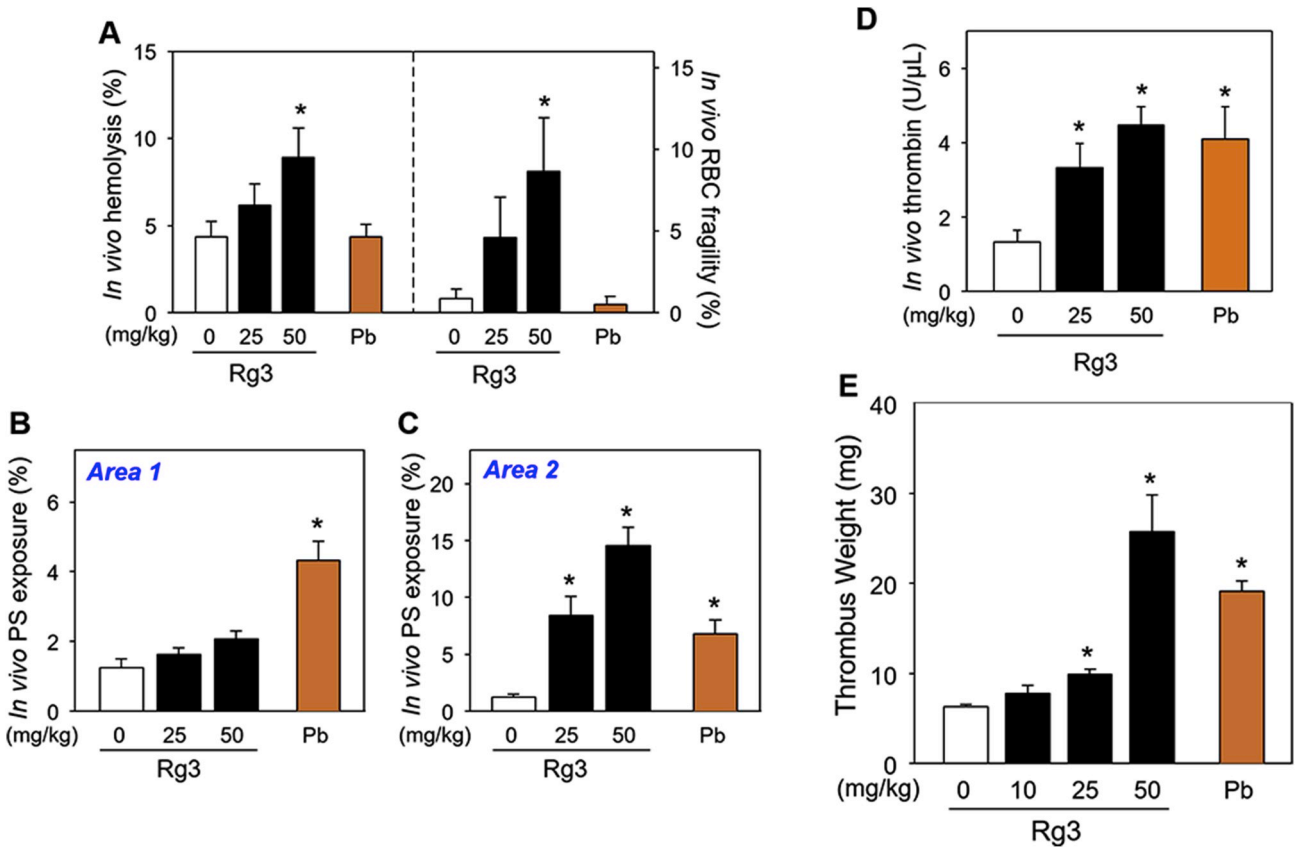



**Fig. 4.** *In vivo* effects of Rg3 in rats. 10 min after intravenous injection of 5% DMSO in saline (as control for Rg3), Rg3 25 mg/kg or 50 mg/kg, blood was collected to determine (A) *in vivo* hemolysis and *in vivo* fragility of erythrocytes in 0.45% NaCl, (B) *in vivo* PS exposure of intact erythrocytes, (C) *in vivo* PS exposure of MVs and debris in whole blood as well as (D) *in vivo* thrombin generation. (E) Thrombus formation was induced by thromboplastin in a rat venous thrombosis model. As a positive control, 25 mg/kg Pb exposure to rats for 1 h was employed. Values are mean ± SE of more than five independent experiments, * represents significant differences from control group ($p < 0.05$).

spectrophotometrically at 540 nm.

To measure PS exposure on erythrocytes (including residual erythrocytes and MV/debris), an aliquot of the blood sample was diluted 200-fold with the following buffer (10 mM HEPES, 136 mM NaCl, 2.7 mM KCl, 2.0 mM $MgCl_2$, 1.0 mM $NaH_2PO_4$, 5.0 mM glucose, 5 mg/ml BSA, 2.5 mM $CaCl_2$, pH 7.4) and then was stained with annexin V-FITC and PE anti-rat erythroid cells antibody for 15 min in the dark. Samples were analyzed using flow cytometry.

Thrombus formation was induced by stasis combined with hypercoagulability. The abdomen was surgically opened, and the vena cava was exposed after careful dissection. Two loose cotton threads were prepared 16 mm apart around the vena cava. All side branches were ligated tightly with cotton threads. After the intravenous injection of chemicals was injected for platelet inhibition into a left femoral vein, 500-fold diluted thromboplastin was infused for 1 min to induce thrombus formation. Stasis was initiated by tightening the two threads, first the proximal and the distal after that. The abdominal cavity was provisionally closed, and blood stasis was maintained for 15 min. After the abdomen was reopened, the ligated venous segment was excised and opened longitudinally to remove the thrombus. The isolated thrombus was blotted of excess blood and immediately weighed.

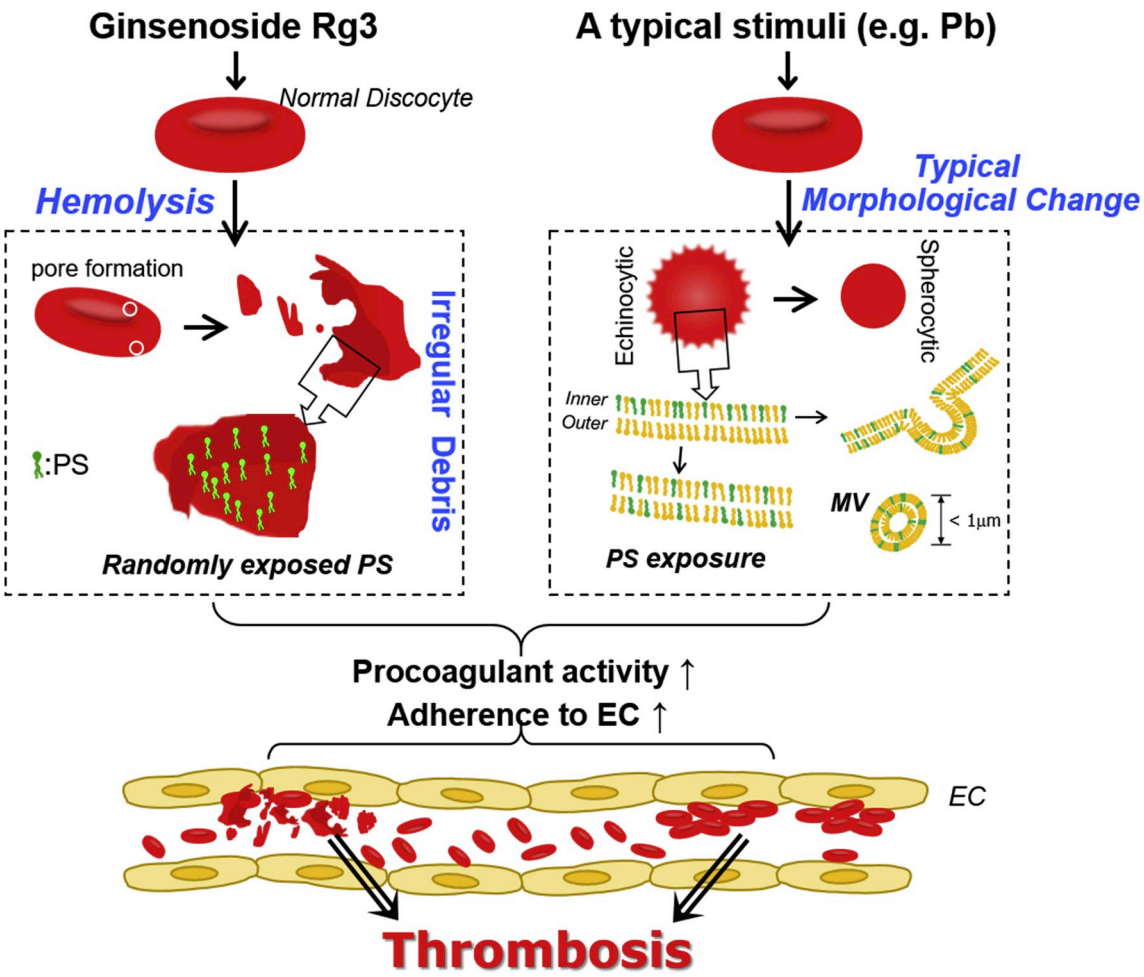
### 2.9. Statistical analysis

The means and standard errors of means were calculated for all treatment groups. The data were subjected to 1-way analysis of variance followed by Duncan's multiple range test or student t-test to determine which means were significantly different from the control. In all cases, a P value of $< .05$ was used to determine significant differences.

## 3. Results

### 3.1. Effects of Rg3 on hemolytic activity and morphological changes in human erythrocytes

Firstly, we investigated the hemolytic responses of human erythrocytes following exposure to Rg3 or Pb. Treatment with Rg3 for 1 h induced hemolysis in a concentration- and time-dependent manner as compared to the absence of hemolysis in case of Pb (Fig. 1A). Rg3 caused time-dependent hemolysis and at 20 μM Rg3, hemolysis up to 50% was observed within 1 h (Fig. 1B). Next, the morphological changes were observed on confocal microscopy and scanning electron microscope (SEM). In confocal microscopy, treatment with 10 μM Rg3 for 4 h induced pore formation (white arrowheads) on the cell surface of which extent gradually increased in a time-dependent fashion. At 20 μM, almost all cells were burst with the releases of intracellular contents, leaving debris (Fig. 1C white arrows). In contrast, as a positive control used in this study, 20 μM Pb induced echinocytes and spherocytes (green arrowheads and green arrows). Changes of the cell surface and the shape over time (0, 0.5, 1, 4 h) could be also observed in SEM, where Rg3 induced pore formation and cell burst could be evidently observed, which was distinct from Pb-induced echinocytes and spherocytes (Fig. 1D).




**Scheme. 1.** Suggested scheme of hemolysis-dependent thrombosis by Rg3.

### 3.2. Confocal identification and flow cytometric detection of PS exposure on erythrocytes debris induced by Rg3

Phosphatidylserine (PS) exposure has been suggested as the main event conferring erythrocytes with hypercoagulability (Atichartakarn et al., 2002; Gao et al., 2012), which frequently concurs with morphological alteration from discocytes into echinocytes and spherocytes (Iglič et al., 1998). As expected, annexin-positive cells induced by Pb (the positive control) could be observed with PS-bearing micro-sized vesicles (MV) shedding from outer cell membranes (Fig. 2A red arrowheads). Notably, the annexin-positive cells resulting from Pb exposure always maintained a normal shape (Fig. 2A red arrow), indicating that Pb-induced PS exposure and MV generation are the regulated events occurring on the outer membrane of cells. In contrast, although annexin-positive cells were observed following Rg3 treatment, they occurred as irregular debris with annexin-positive, that is, annexin-positive debris (Fig. 2A white arrows), indicating that PS exposure induced by Rg3 may result from the destruction of erythrocytes rather than regulated events as shown with Pb.

With flow cytometry, intact erythrocytes (area 1) and debris or MV (area 2) could be analyzed, respectively (Fig. 2B). Compared to the positive control (Pb), Rg3 depleted intact erythrocytes significantly, and furthermore, the intact erythrocytes remaining did not express any PS exposure (Fig. 2C), which was in clear contrast with Pb. On the other hand, numerous counts of erythrocytes debris were generated by Rg3, and showed significant PS exposure which is comparable to MV induced by Pb (Fig. 2D).

### 3.3. Biological effects of erythrocytes by Rg3 treatment

PS-exposing erythrocytes exhibit procoagulant activity, which is manifested by thrombin generation and adhesion to the endothelial membrane (Closse et al., 1999; Wautier et al., 2011). Rg3-treated erythrocytes also promoted thrombin generation even during minimal hemolysis at 10 μM (Fig. 3A). The procoagulant activation of erythrocytes by 10 and 20 μM Rg3 was further confirmed by increased adherence of Rg3-treated erythrocytes to endothelial cells (Fig. 3B). On the contrary, Pb treatment induced both thrombin generation and adherence of erythrocytes to endothelial cells in the absence of hemolysis (Fig. 3A and B), which matched well with the results above. In addition to erythrocytes, plasma coagulation system and platelets are well known to contribute to thrombosis (Wagner and Burger, 2003). However, no significant effects of Rg3 or Pb were shown in activated partial thromboplastin time (aPTT) and prothrombin time (PT), platelet aggregation as well as thrombin generation (Fig. 3C), demonstrating that the prothrombotic effects of Rg3 were erythrocyte-mediated.

### 3.4. Effects of Rg3 in rats in vivo

In good accordance with *in vitro* results, significant hemolysis was observed after the intravenous administration of Rg3 in rats *ex vivo* while positive control (Pb) did not (Fig. 4A left). Rg3-induced erythrocyte damage was further confirmed by the erythrocyte fragility assay in hypotonic 0.45% NaCl solution (Fig. 4A right). In area 1 (the intact cell region) of flow cytometric analysis, Rg3 did not induce any PS exposure while Pb did (Fig. 4B). Meanwhile, Rg3, as well as Pb,

induced PS exposure in area 2 (debris or MV region) significantly (Fig. 4C). Furthermore, the thrombogenic activity of Rg3 or Pb was confirmed *ex vivo* as determined by increased thrombin generation (Fig. 4D). Most importantly, thrombus formation in the venous thrombosis rat model was significantly augmented by Rg3 or Pb treatment (Fig. 4E).

## 4. Discussion

Here, we demonstrated that ginsenoside Rg3 could induce distinctive PS-bearing erythrocytes debris, procoagulant activity and ultimately promote thrombosis. This phenomenon was closely linked with the hemolytic activity of Rg3, during which PS-bearing erythrocyte debris was generated. PS–bearing erythrocyte debris derived from Rg3-induced hemolysis could be clearly identified in confocal microscope observation using annexin V-FITC, which have contributed to the procoagulant activity of erythrocytes and thrombosis.

We observed that Rg3 induced the procoagulant activity of erythrocytes in a distinctive way, as shown in Scheme 1. Generally, PS externalization is commonly the consequence of tipping of the balance of lipid translocation from inner towards outer membrane of erythrocytes and accompanies with morphological changes of normal discocytes to echinocytes or spherocytes with microvesicles shedding from outer membrane (Iglič et al., 1998). On the other hand, Rg3 induced PS exposure through the rupture of erythrocytes and the externalization of inner membrane leaflet, which is clearly different from the typical ways as observed with Pb. Nevertheless, PS-bearing debris behaved similarly to PS-exposed erythrocytes and MV, which ultimately promoted procoagulant activity of erythrocytes and thrombosis.

The pharmacologically effective levels of Rg3 given by previous studies was rather high, and the toxicity may occur with its efficacy. A study in healthy volunteers showed the maximum plasma concentration ($C_{max}$) of Rg3 was about 770.2 ± 275.4 ng/mL (approximately 1.0 ± 0.4 μM) (Zhao et al., 2016). However, *in vitro* studies observed the efficacy of Rg3 in various cells at around 50 μg/mL (approximately 60 μM) (Chen et al., 2008; Jiang et al., 2011; Joo et al., 2004; Lee et al., 2009). We showed that Rg3 induces the procoagulant activity of erythrocytes at the much lower concentration of 10 μM within 1 h, which may provide a strong clinical relevance to our findings.

Ginsenoside Rg3 has been used in combination therapy with a chemotherapeutic agent to achieve a higher therapeutic efficacy in cancer patients (Sun et al., 2017; Zhao et al., 2016). Many studies have documented the improved anti-cancer effect of a combination therapy against diverse cancers *in vitro* (Kim et al., 2010) and *in vivo* (Liu et al., 2009; Zhang et al., 2006). A clinical trial was also conducted for the Rg3 combination therapy against non-small cell lung carcinoma (Zhao et al., 2016). However, these studies are focusing only on its therapeutic efficacy without paying serious attention to its potential risk. It should be noted that cancer, itself, can provoke thrombotic risks by more than 4 folds and thrombotic events are prevalent in cancer patients (Heit et al., 2000; Lee and Levine, 2003). Also, chemotherapeutic agents could trigger procoagulant activity (Egler and Lang, 2017) and ultimately promote thrombosis in cancer patients (Haddad and Greeno, 2006; Zangari et al., 2001). In this context, it is highly probable that the use of Rg3 with chemotherapeutic agents in cancer patients may increase thrombotic risks as well as the anti-cancer effects, which needs full attention in the future.

In addition to ginseng, many kinds of plants contain saponins, such as *Sapindaceae*, *Gynostemma pentaphyllum* and *Manilkara zapota*, and they are frequently used as biomedical reagents for health benefits. Of note, they also carry the potential of hemolytic actions. Earlier studies demonstrated that saponin could affect membrane structure and cause hemolysis (Baumann et al., 2000; Glauert et al., 1962). Another study tested hemolytic activities of saponins extracted from various plants (Voutquenne et al., 2002). Also, a recent study described that saponin could trigger apoptosis (Bissinger et al., 2014). Unfortunately, these findings were not followed up by studies examining their health outcome. Even for ginseng, saponins other than Rg3 exist such as Rb1, Rg1 and Rh2, of which effects on hemolysis have not be illustrated. We found that Rh2 also induced hemolysis and PS exposure (data not shown). In this regard, we propose that the potential risk of their procoagulant and prothrombotic effects of saponins need to be studied in the future.

In conclusion, we demonstrated that Rg3 could generate PS-bearing erythrocyte debris through pore formation and cell burst, which could increase the procoagulant activity of erythrocytes and ultimately thrombosis. Rg3-induced procoagulant activity of erythrocytes was distinct from those observed with typical procoagulant agents with terms of mechanism and pattern of PS exposure. Nevertheless, Rg3 exposure led to a similar outcome of procoagulant activity of erythrocytes and thrombosis. Collectively, we believed that more attention shall be paid to the potential thrombotic risks of Rg3 and ginseng products.

## Ethics

With the approval from the Ethics Committee of Health Service Center at Seoul National University, human blood was obtained from healthy male donors. All the animal protocols used *in vivo* experiments were approved by the Ethics Committee of Animal Service Center at Seoul National University.

## Consent for publication

Not applicable.

## Availability of data and materials

Yes.

## Conflicts of interest

None declared.

## Funding

This research was supported by the National Research Foundation of Korea grant funded by the Korea Government (MSIP) (2015R1A2A2A01011705).

## Authors' contribution

Y. B. and K.M.L. designed and performed the experiments; G. A., K. K. and T. N. analyzed the data and performed platelet experiments; S. S. designed the RBC experiments isolated from healthy volunteers; J.H.C. supervised the study.

## Declaration of interests

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.

## Acknowledgments

Not applicable.

## Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2019.05.061.

## References


Atichartakarn, V., Angchaisuksiri, P., Aryurachai, K., Onpun, S., Chuncharunee, S., Thakkinstian, A., Atamasirikul, K., 2002. Relationship between hypercoagulable state and erythrocyte phosphatidylserine exposure in splenectomized haemoglobin E/β-thalassaemic patients. Br. J. Haematol. 118, 893–898.

Baumann, E., Stoya, G., VoÈlkner, A., Richter, W., Lemke, C., Linss, W., 2000. Hemolysis of human erythrocytes with saponin affects the membrane structure. Acta Histochem. 102, 21–35.

Bissinger, R., Modicano, P., Alzoubi, K., Honisch, S., Faggio, C., Abed, M., Lang, F., 2014. Effect of saponin on erythrocytes. Int. J. Hematol. 100, 51–59.

Byrnes, J.R., Wolberg, A.S., 2017. Red blood cells in thrombosis. Blood 130, 1795–1799.

Chen, J., Peng, H., Ou-Yang, X., He, X., 2008. Research on the antitumor effect of ginsenoside Rg3 in B16 melanoma cells. Melanoma Res. 18, 322–329.

Closse, C., Dachary-Prigent, J., Boisseau, M.R., 1999. Phosphatidylserine-related adhesion of human erythrocytes to vascular endothelium. Br. J. Haematol. 107, 300–302.

Coon, J.T., Ernst, E., 2002. Panax ginseng. Drug Saf. 25, 323–344.

Daleke, D.L., 2008. Regulation of phospholipid asymmetry in the erythrocyte membrane. Curr. Opin. Hematol. 15, 191–195.

Egler, J., Lang, F., 2017. Triggering of eryptosis, the suicidal erythrocyte death, by perifosine. Cell. Physiol. Biochem. 41, 2534–2544.

Ernst, E., 2002. The risk–benefit profile of commonly used herbal therapies: ginkgo, St. John's wort, ginseng, Echinacea, Saw Palmetto, and Kava. Ann. Intern. Med. 136, 42–53.

Gao, C., Xie, R., Yu, C., Wang, Q., Shi, F., Yao, C., Zhou, J., Gilbert, G., Shi, J., 2012. Procoagulant activity of erythrocytes and platelets through phosphatidylserine exposure and microparticles release in patients with nephrotic syndrome. Thromb. Haemost. 107, 681–689.

Glauert, A.M., Dingle, J., Lucy, J., 1962. Action of saponin on biological cell membranes. Nature 196, 953.

Haddad, T.C., Greeno, E.W., 2006. Chemotherapy-induced thrombosis. Thromb. Res. 118, 555–568.

Haijiang, Z., Yongjiang, W., Yiyu, C., 2003. Analysis of 'shenmai'injection by Hplc/ms/ms. J. Pharm. Biomed. Anal. 31, 175–183.

Heit, J.A., Silverstein, M.D., Mohr, D.N., Petterson, T.M., O'fallon, W.M., Melton, L.J., 2000. Risk factors for deep vein thrombosis and pulmonary embolism: a population-based case-control study. Arch. Intern. Med. 160, 809–815.

Helmes, S., 2004. Cancer prevention and therapeutics: Panax ginseng. Altern. Med. Rev. 9, 259–275.

Iglič, A., Kralj-Iglič, V., Hägerstrand, H., 1998. Amphiphile induced echinocyte-spheroechinocyte transformation of red blood cell shape. Eur. Biophys. J. 27, 335–339.

Jiang, J.-W., Chen, X.-M., Chen, X.-H., Zheng, S.-S., 2011. Ginsenoside Rg3 inhibit hepatocellular carcinoma growth via intrinsic apoptotic pathway. World J. Gastroenterol. 17, 3605.

Joo, S., Won, T., Kim, M., Lee, D., 2004. Hematopoietic effect of ginsenoside Rg3 in ICR mouse primary cultures and its application to a biological response modifier. Fitoterapia 75, 337–341.

Jung, Y., Kim, K., Bian, Y., Ngo, T., Bae, O.-N., Lim, K.-M., Chung, J.-H., 2018. Ginsenoside Rg3 disrupts actin-cytoskeletal integrity leading to contractile dysfunction and apoptotic cell death in vascular smooth muscle. Food Chem. Toxicol. 118, 645–652.

Karmazyn, M., Moey, M., Gan, X.T., 2011. Therapeutic potential of ginseng in the management of cardiovascular disorders. Drugs 71, 1989–2008.

Kim, K., Bae, O.-N., Koh, S.-H., Kang, S., Lim, K.-M., Noh, J.-Y., Shin, S., Kim, I., Chung, J.-H., 2015. High-dose vitamin C injection to cancer patients may promote thrombosis through procoagulant activation of erythrocytes. Toxicol. Sci. 147, 350–359.

Kim, S.M., Lee, S.Y., Cho, J.S., Son, S.M., Choi, S.S., Yun, Y.P., Yoo, H.S., Oh, K.-W., Han, S.B., Hong, J.T., 2010. Combination of ginsenoside Rg3 with docetaxel enhances the susceptibility of prostate cancer cells via inhibition of NF-κB. Eur. J. Pharmacol. 631, 1–9.

Lee, A.Y., Levine, M.N., 2003. Venous thromboembolism and cancer: risks and outcomes. Circulation 107 I-17-I-21.

Lee, J.-Y., Lim, K.-M., Kim, S.-Y., Bae, O.-N., Noh, J.-Y., Chung, S.-M., Kim, K., Shin, Y.-S., Lee, M.-Y., Chung, J.-H., 2010. Vascular smooth muscle dysfunction and remodeling induced by ginsenoside Rg3, a bioactive component of ginseng. Toxicol. Sci. 117, 505–514.

Lee, S.Y., Kim, G.T., Roh, S.H., Song, J.-S., Kim, H.-J., Hong, S.-S., Kwon, S.W., Park, J.H., 2009. Proteomic analysis of the anti-cancer effect of 20S-ginsenoside Rg3 in human colon cancer cell lines. Biosci. Biotechnol. Biochem 0903051360-0903051360.

Li, G.-X., Liu, Z.-Q., 2008. The protective effects of ginsenosides on human erythrocytes against hemin-induced hemolysis. Food Chem. Toxicol. 46, 886–892.

Lim, K.-M., Kim, S., Noh, J.-Y., Kim, K., Jang, W.-H., Bae, O.-N., Chung, S.-M., Chung, J.-H., 2010. Low-level mercury can enhance procoagulant activity of erythrocytes: a new contributing factor for mercury-related thrombotic disease. Environ. Health Perspect. 118, 928.

Liu, J.-P., Lu, D., Nicholson, R.C., Zhao, W.-J., Li, P.-Y., Wang, F., 2012. Toxicity of a novel anti-tumor agent 20 (S)-ginsenoside Rg3: a 26-week intramuscular repeated administration study in rats. Food Chem. Toxicol. 50, 3388–3396.

Liu, T.-G., Huang, Y., Cui, D.-D., Huang, X.-B., Mao, S.-H., Ji, L.-L., Song, H.-B., Yi, C., 2009. Inhibitory effect of ginsenoside Rg3 combined with gemcitabine on angiogenesis and growth of lung cancer in mice. BMC Canc. 9, 250.

Liu, Z.-Q., Luo, X.-Y., Liu, G.-Z., Chen, Y.-P., Wang, Z.-C., Sun, Y.-X., 2003. In vitro study of the relationship between the structure of ginsenoside and its antioxidative or prooxidative activity in free radical induced hemolysis of human erythrocytes. J. Agric. Food Chem. 51, 2555–2558.

Liu, Z.-Q., Luo, X.-Y., Sun, Y.-X., Chen, Y.-P., Wang, Z.-C., 2002. Can ginsenosides protect human erythrocytes against free-radical-induced hemolysis? Biochim. Biophys. Acta Gen. Subj. 1572, 58–66.

Rao, K.V., 2014. Drug-induced Hematologic Disorders. Pharmacotherapy: A Pathophysiologic Approach, ninth ed. pp. 931–944.

Shantsila, E., Kamphuisen, P., Lip, G., 2010. Circulating microparticles in cardiovascular disease: implications for atherogenesis and atherothrombosis. J. Thromb. Haemost. 8, 2358–2368.

Shin, J.-H., Lim, K.-M., Noh, J.-Y., Bae, O.-N., Chung, S.-M., Lee, M.-Y., Chung, J.-H., 2007. Lead-induced procoagulant activation of erythrocytes through phosphatidylserine exposure may lead to thrombotic diseases. Chem. Res. Toxicol. 20, 38–43.

Siegel, R.K., 1979. Ginseng abuse syndrome: problems with the panacea. Jama 241, 1614–1615.

Siegel, R.K., 1980. Ginseng and high blood pressure. Jama 243 32-32.

Sun, M., Ye, Y., Xiao, L., Duan, X., Zhang, Y., Zhang, H., 2017. Anticancer effects of ginsenoside Rg3. Int. J. Mol. Med. 39, 507–518.

Voutquenne, L., Lavaud, C., Massiot, G., Men-Olivier, L.L., 2002. Structure-activity relationships of haemolytic saponins. Pharm. Biol. 40, 253–262.

Wagner, D.D., Burger, P.C., 2003. Platelets in inflammation and thrombosis. Arterioscler. Thromb. Vasc. Biol. 23, 2131–2137.

Wautier, M.P., Heron, E., Picot, J., Colin, Y., Hermine, O., Wautier, J.L., 2011. Red blood cell phosphatidylserine exposure is responsible for increased erythrocyte adhesion to endothelium in central retinal vein occlusion. J. Thromb. Haemost. 9, 1049–1055.

Weisel, J.W., 2015. Role of red cells in thrombosis and hemostasis. Blood 126.

Zangari, M., Anaissie, E., Barlogie, B., Badros, A., Desikan, R., Gopal, A.V., Morris, C., Toor, A., Siegel, E., Fink, L., 2001. Increased risk of deep-vein thrombosis in patients with multiple myeloma receiving thalidomide and chemotherapy. Blood 98, 1614–1615.

Zhang, Q., Kang, X., Zhao, W., 2006. Antiangiogenic effect of low-dose cyclophosphamide combined with ginsenoside Rg3 on Lewis lung carcinoma. Biochem. Biophys. Res. Commun. 342, 824–828.

Zhao, Q., Li, P., Jiang, J., Hu, P., 2016. Pharmacokinetics of single ascending doses and multiple doses of 20 (S)-Ginsenoside Rg3 in Chinese Healthy Volunteers. Eur. J. Drug Metab. Pharmacokinet. 41, 845–853.

Zwaal, R.F., Schroit, A.J., 1997. Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. Blood 89, 1121–1132.