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Original Article

The Inhibitory Effects of *Glycyrrhiza uralensis* on human Platelet Aggregation and Thrombus Formation

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Platelets are activated at the sites of vascular injury by a number of molecules, including adenosine diphosphate, collagen and thrombin. The full platelet aggregation is absolutely essential for the normal hemostasis. *Glycyrrhiza glabra* is a well-known medicinal herb that grows in various parts of the world and is known to have various effects such as antioxidant, anti-inflammatory, anti-atherogenic, anti-osteoporotic and skin-whitening. However, the platelet inhibitory effect of *Glycyrrhiza glabra* extract (GGE) has not been identified. In this study, we investigated if GGE inhibited platelet aggregation. We observed that GGE inhibited collagen-induced platelet aggregation, Ca^{2+} mobilization, and thromboxane A2 generation. In addition, GGE suppressed phosphorylation of phosphatidylinositol-3 kinase (PI3K), Akt and elevated phosphorylation of inositol 1,4,5-trisphosphate receptor (IP3R), vasodilator stimulated phosphoprotein (VASP). Taken together, GGE showed strong antiplatelet effects and may be used to block platelet-mediated cardiovascular diseases.

Key Words: *Glycyrrhiza glabra*, Calcium mobilization, Fibronectin adhesion, Thromboxane A2, Clot retraction

INTRODUCTION

Platelets, the commander of the hemostatic process, interact with collagen exposed in damaged blood vessels to initiate the hemostatic response (Moroi and Jung, 2004). After binding action, calcium concentration in platelet is increased (Varga-Szabo et al., 2009). Elevated intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) activates granule release (Farndale, 2006) and agonist-induced signaling cascade activates glycoprotein IIb/IIIa (integrin αIIb/β_3), which triggers full platelet spreading and aggregation (Phillips et al., 2001). Platelets are essential cells for hemostasis, but they can also cause thrombosis. Hyperactivity of platelets can form a hemostatic plug even with minor stimulation, blocking blood vessels or forming thrombosis. For people exposed to hyperlipidemia, high blood pressure, or cardiovascular disease, this effect can be fatal. Therefore, platelets are important regulators of cardiovascular diseases (Jackson, 2011). However, the mortality rate from cardiovascular disease is still high (Lee et al., 2020). Therefore, we focused on *Glycyrrhiza glabra*. *Glycyrrhiza glabra* extract has been kwon to have anti-inflammatory effect, anti-microbial effect, anti-oxidant effect and have various phytochemicals such as alkaloids, phenolic compounds, flavonoids, saponins, lipids and tannins (Kaur et al, 2013). However, the platelet inhibitory effect of *Glycyrrhiza glabra* extract (GGE) has not been identified. In this study, we conducted experiments focusing on three

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mechanisms of platelet activity. The first is the platelet aggregation reaction and calcium release, the second is the thromboxane A2 production process, and the last is the activation of integrins $\alpha IIb/\beta_3$. We evaluated the effects of GGE on three platelet mechanisms in collagen-stimulated human platelets.

MATERIALS AND METHODS

Materials

Glycyrrhiza glabra was purchased from Jecheon herb (Jecheon, Korea). The dried *Glycyrrhiza glabra* was pulverized by grinder and a powdered larvae was successively extracted with 70% ethanol (350 mL) using a soxhlet apparatus (JISICO, Seoul, Korea) at 150℃ for 1 hours. The extract was concentrated in a vacuum evaporator and lyophilized. The lyophilized extract was re-dissolved in dimethyl sulfoxide to a concentration of 100 mg/mL. Human platelets were obtained from the Korean Red Cross Blood Center (Suwon, Korea). Platelet agonists, collagen, and thrombin were bought from Chrono-Log Co. (Havertown, PA, USA). Fura 2-acetoxymethyl (Fura-2 AM) was purchased from Invitrogen (Eugene, OR, USA). Phospho-inositol 1,4,5-trisphosphate receptor (IP3R), Phospho-VASP (Ser^{157} and Ser^{239}), Phospho-PI3K, Phospho-Akt (Ser^{473} and Thr^{308}), phosphor-p38, phosphor-cytosolic phospholipase A2 (cPLA2) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Bicinchoninic acid protein assay kit was purchased form Pierce Biotechnology (IL, USA). Fibronectin-coated cell adhesion kit as procured from Cell Biolabs (San Diego, CA, USA). A serotonin detection kit was purchased from Labor Diagnostika Nord GmbH and Co. (Nordhorn, Germany).

Determination of platelet aggregation

Platelets were separated and washed in washing buffer (pH 6.5) and adjusted in suspension buffer (pH 6.9) to 10^8/mL. GGE was dissolved in dimethyl sulfoxide (0.1%). Platelets (10^8/mL) were preincubated with different GGE concentrations (75, 100, 150, and 200 μM) at 37℃ while stirring, and collagen was added for full platelet aggregation using an aggregometer (Chrono-Log). This study was conducted with approval from the Public Institutional Review Board at the National Institute for Bioethics Policy (PIRB-P01-201812-31-007, Seoul, Republic of Korea).

Cytotoxicity analyses

We investigated if GGE concentrations affected lactate dehydrogenase (LDH) levels in platelets. Platelets (10^8/mL) were preincubated with different GGE concentrations for 15 min at 37℃ while stirring. After centrifugation at 12,000× *g*, supernatants were separated and LDH levels analyzed using an enzyme-linked immunosorbent assay (ELISA) kit and ELISA plate reader (TECAN, Salzburg, Austria).

Ca^{2+} mobilization analyses

To measure $[Ca^{2+}]_i$, the Grynkiewicz method (Grynkiewicz et al., 1985) was used. Platelets were incubated with Fura-2 AM for 20 min, washed, and platelet concentrations adjusted to 10^8/mL using suspension buffer. Platelets (10^8/mL) were incubated with different GGE concentrations (75, 100, 150, and 200 μM) at 37℃ for 5 min and then stimulated with collagen (2.5 μg/mL). Ca^{2+} concentrations were analyzed using a fluorescence spectrophotometer (F-2700; Hitachi, Japan).

Serotonin release analyses

Platelet aggregation was conducted for 7 min at 37℃ with GGE, then reaction cuvette place onto ice in order to terminate release action for 3 min. After termination, the reaction mixture was centrifuged and the supernatant was used. The serotonin and ATP were detected using ELISA reader.

Thromboxane A2 (TXA2) production

Activated platelets synthesize TXA2 via an "inside-out signaling cascade". TXA2 acts as a strong agonist and is quickly converted to thromboxane B2 (TXB2), which was measured. After collagen-induced platelet aggregation with GGE, indomethacin was added to stop reactions and mixtures centrifuged briefly to generate TXB2-containing supernatants, which were analyzed using an ELISA plate reader (TECAN, Salzburg, Austria).

Western blotting

To investigate phosphorylation events, platelet aggregation was performed and platelet lysates quantified. Proteins were separated by electrophoresis and then transferred to polyvinylidene fluoride membranes. Primary antibodies were incubated with membranes overnight at 4℃, and after washing (Tris-buffered saline plus 0.1% tween 20), a secondary antibody was added and incubated with membranes at room temperature for 2 h. Then, protein signals were developed in a darkroom. Western blotting results were calculated using the Quantity One program (Bio-Rad, Hercules, CA, USA).

Analyzing αIIb/β_3 adhesion to fibronectin

Fibronectin is a plasma protein and functions as an adhesive protein to bind platelet integrin αIIb/β_3. Therefore, we analyzed αIIb/β_3 activity in fibronectin-coated wells. Platelets and different GGE concentrations (75, 100, 150, and 200 μM) were added to fibronectin-coated wells and stimulated by collagen. In normal reactions, platelets adhere to fibronectin-coated wells to form thin films. After reactions, wells were washed twice in buffer, and platelet layers stained using cell staining solution. After this, extract solution was added to extract stained platelet layers and absorbances analyzed using an ELISA plate reader (TECAN, Salzburg, Austria) to determine platelet adhesion.

Platelet-mediated fibrin clot retraction

Human platelet-rich plasma (300 μL) was incubated with glabridin for 30 min at 37℃, and clot retraction was triggered by adding thrombin (0.05 U/mL). After reacting for 15 min, pictures of fibrin clot were taken using a digital camera.

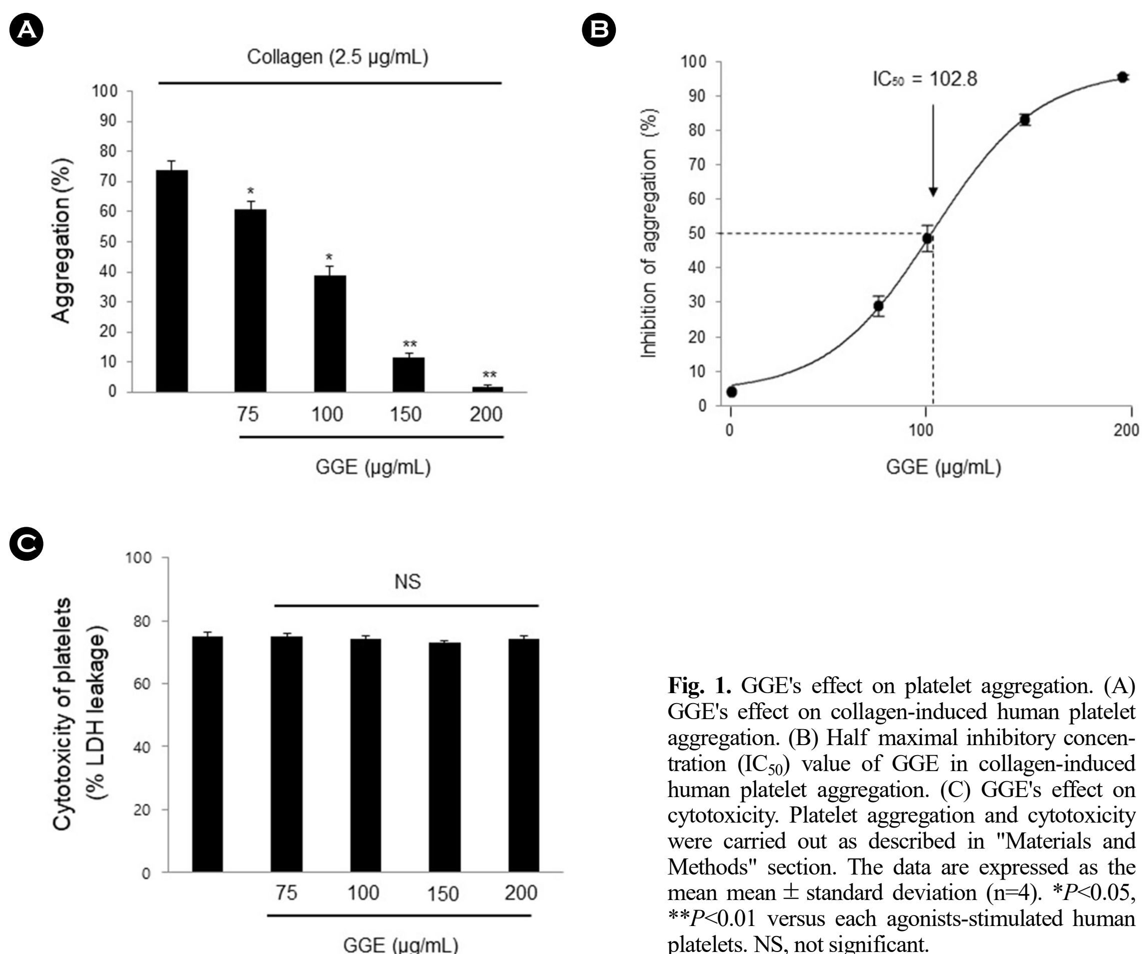

Fig. 1. GGE's effect on platelet aggregation. (A) GGE's effect on collagen-induced human platelet aggregation. (B) Half maximal inhibitory concentration (IC_{50}) value of GGE in collagen-induced human platelet aggregation. (C) GGE's effect on cytotoxicity. Platelet aggregation and cytotoxicity were carried out as described in "Materials and Methods" section. The data are expressed as the mean mean ± standard deviation (n=4). *P<0.05, **P<0.01 versus each agonists-stimulated human platelets. NS, not significant.

Data analysis

All data are presented as the mean ± standard deviation with various numbers of observations. To determine major differences among groups, analysis of variance was performed, followed by the Tukey-Kramer method. SPSS 21.0.0.0 software (SPSS, Chicago, IL, USA) was used for statistical analysis and $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Human platelet activity and GGE-induced cytotoxicity

To check the activity of platelets, we used human platelets. The platelet suspension was reacted with collagen in an aggregator, and the aggregation rate was 74.0%. However, the aggregation rate was suppressed by GGE in a concentration-dependent manner (Fig. 1A), the half maximal inhibitory concentration (IC_{50}) was 102.8 μg/mL (Fig. 1B). We confirmed that GGE can damage platelets and confirmed that it does not increase the secretion of LDH (Fig. 1C). Therefore, we confirmed that GGE does not kill platelets, but rather inhibits aggregation by a different mechanism.

Ca^{2+} mobilization, serotonin release and inositol 1,4,5-trisphosphate receptor phosphorylation

To investigate the platelet inhibitory activity of GGE, its effect on calcium release was evaluated. As shown (Fig. 2A), collagen-increased Ca^{2+} mobilization but was suppressed by GGE. Increased calcium in platelet activates kinase that triggers granule release, thus we examined serotonin release in δ-granules. As shown in Fig. 2B, GGE inhibited the serotonin secretion. Next, we investigated if GGE could control inositol 1,4,5-trisphosphate receptor (IP3R) phospho-

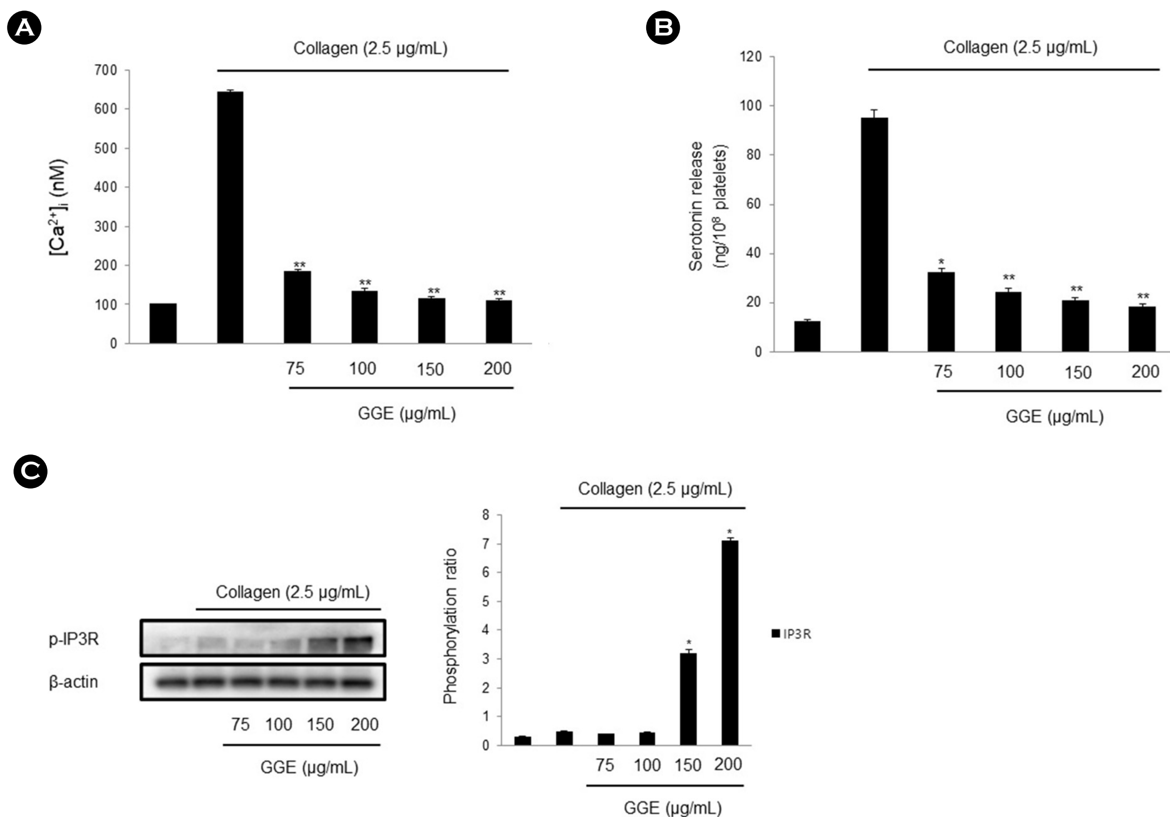

Fig. 2. GGE's effect on $[Ca^{2+}]_i$ mobilization, serotonin release, IP3R phosphorylation (A) Effect of GGE's effect on collagen-induced $[Ca^{2+}]_i$ mobilization. (B) GGE's effect on collagen-induced serotonin release. (C) GGE's effect on collagen-induced IP3R phosphorylation. All experiments were performed as described in "Materials and Methods" section. The data are expressed as the mean ± standard deviation (n=4). *P<0.05, **P<0.01 versus the collagen-stimulated human platelets.

rylation. IP3R is located on the surface of the endoplasmic reticulum and IP3R is phosphorylated by cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP)-dependent kinases. We observed that glabridin increased IP3R phosphorylation when induced by collagen (Fig. 2C).

Thromboxane A2, cPLA2-, and p38 MAPK-phosphorylation

TXA2 acts as an agonist which stimulates platelet activation (Needleman et al., 1976; FitzGerald, 1991). TXA2 production is regulated by two signaling molecules, cytosolic phospholipase A2 (cPLA2) and mitogen-activated protein kinase p38 (p38) (Kramer et al., 1996). As shown (Fig. 3A), TXA2 was dose-dependently inhibited by GGE, and collagen-elevated cPLA2 and p38 phosphorylation was dose-dependently inhibited by GGE (Fig. 3B).

Fibronectin adhesion, clot retraction and phosphorylation of αIIb/β_3-related signaling molecules

Next, we examined αIIb/β_3 function. αIIb/β_3-mediated signaling actually starts as soon as a binding molecule binds to the integrin, allowing various signaling pathways and it makes platelet aggregation more powerful. Therefore, we confirmed whether GGE affects the binding between platelet integrins and fibronectin. As shown (Fig. 4A), GGE suppressed collagen-elevated binding forces. Next, we examined whether GGE affects the binding effect as a clot retraction test. Fig. 4B shows the retraction was suppressed by GGE dose-dependently. Finally, with regard to the inactivation of αIIb/β_3 by GGE, we analyzed the phosphorylation molecules (PI3K/Akt/VASP) (Sudo et al., 2003; Guidetti et al., 2015) and we confirmed that GGE significantly reduced PI3K, Akt (Ser^{473}, Thr^{308}) phosphorylation and elevated VASP (Ser^{157}, Ser^{239}) phosphorylation (Fig. 4C).

The most representative extract for improving blood circulation is *Ginkgo biloba*. *Ginkgo biloba* extract is actually used as a health supplement to improve blood circulation. According to a paper on the antiplatelet effect of *Ginkgo biloba*, the maximum inhibitory activity was shown at a concentration of 1 to 2 mg/mL (Dutta-Roy et al., 1999; Shiyong et al., 2015). Compared to *Ginkgo biloba* extract, GGE shows inhibitory activity at lower concentrations and thus has the potential as a health functional food. However, our study had some limitations, in that it was conducted *in vitro* and did not considered other factors *in vivo*. Therefore, it is difficult to prove its effect in the human body. In order to resolve these questions, animal tests (*in vivo*, *ex vivo*), and clinical trials in humans should be accompanied. We would like to clarify this point through future research.

In order to investigate which components of GGE exhibit antiplatelet effects, we searched papers related to component analysis (Li et al., 2016). 14 ingredients were identified in the paper, and antiplatelet effects were reported for 2 substances.

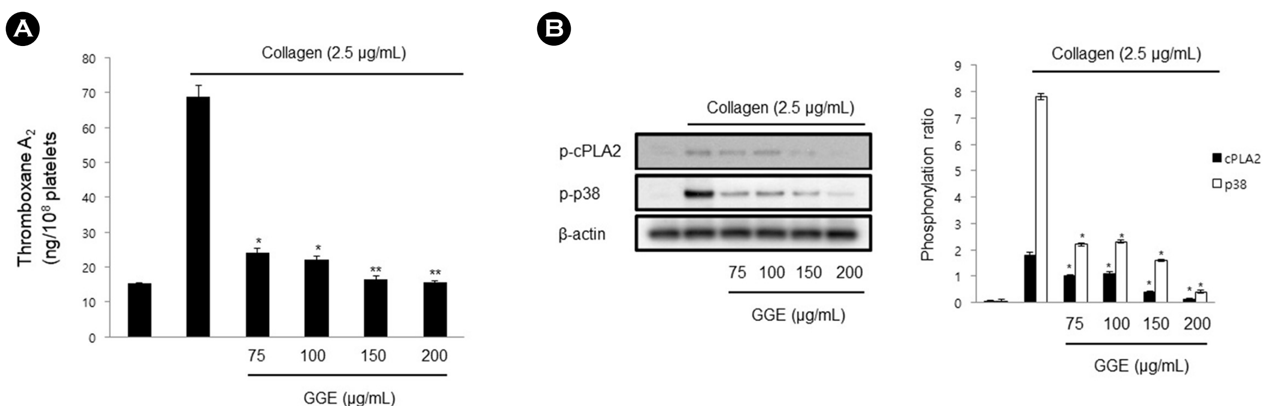

Fig. 3. GGE's effect on TXA2 production, cPLA2, p38 phosphorylation. (A) GGE's effect on collagen-induced TXA2 generation. (B) GGE's effect on collagen-induced cPLA2 and p38 phosphorylation. All experiments were performed as described in "Materials and Methods" section. The data are expressed as the mean mean ± standard deviation (n=4). *$P<0.05$, **$P<0.01$ versus the agonists-stimulated human platelets.

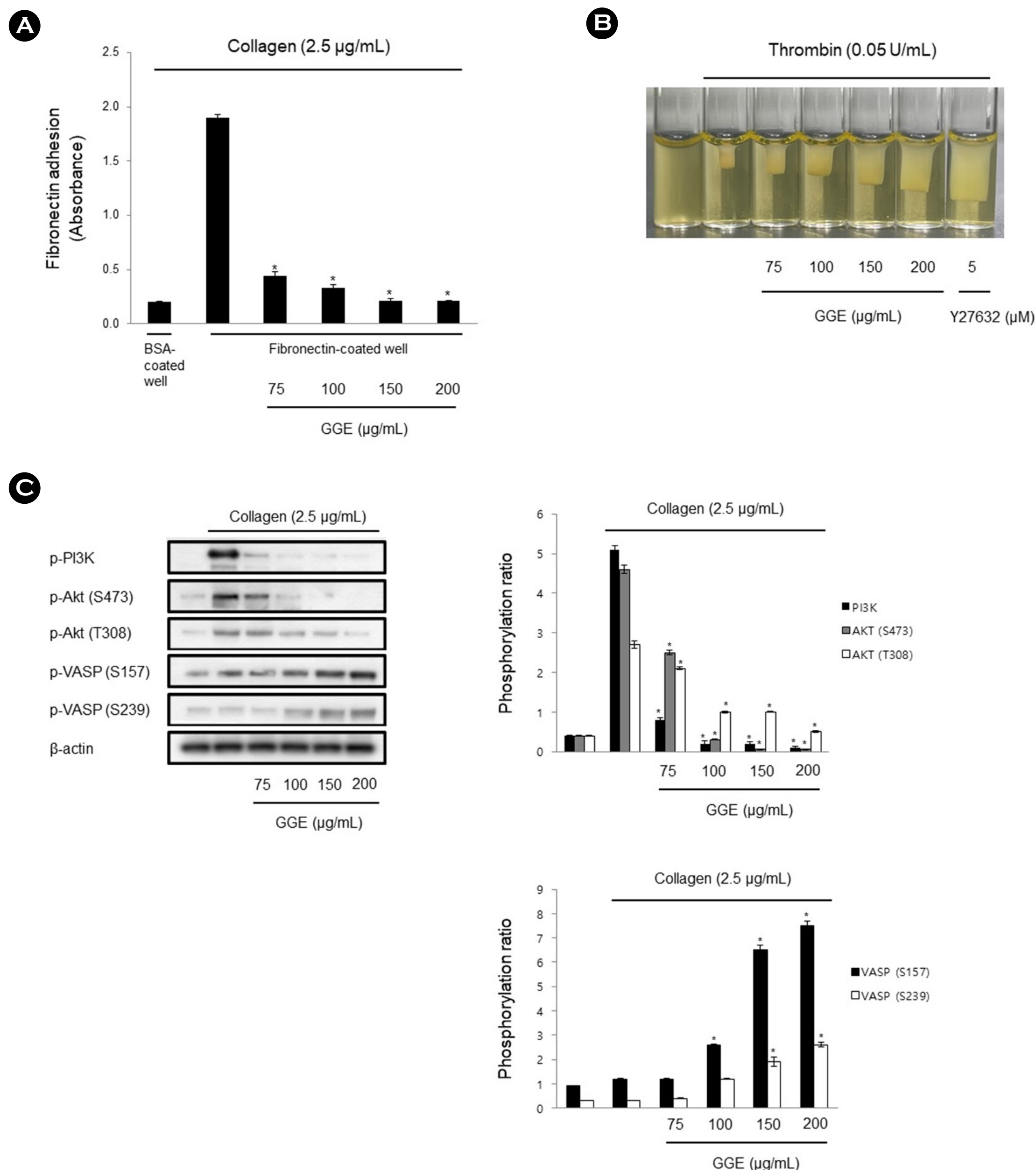

Fig. 4. GGE's effect on fibronectin adhesion, clot retraction and PI3K/Akt/VASP phosphorylation. (A) GGE's effect on collagen-induced fibronectin adhesion. (B) GGE's effect on thrombin-induced clot retraction. (C) GGE's effect on collagen-induced PI3K/Akt/VASP phosphorylation. All experiments were performed as described in "Materials and Methods" section. The data are expressed as the mean mean ± standard deviation (n=4). **P*<0.05 versus the collagen-stimulated human platelets.

Glabridin and licochalcone A were reported to be substances with representative antiplatelet effects (Chung et al., 2022; Lien et al., 2017), and we could predict that the antiplatelet effect of GGE was caused by two single substances.

This study found that GGE inhibited platelet activity without cell damage. GGE inactivated calcium release and integrin activation in collagen-stimulated platelets and ultimately delayed the contraction of blood clots. Therefore, GGE would be a useful in antithrombosis applications.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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