Antithrombotic effect of grape seed proanthocyanidins extract in a rat model of deep vein thrombosis

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Objective: Proanthocyanidins are abundantly found in grape seeds and have been suggested to inhibit the pathogenesis of systemic diseases. We investigated the antithrombotic effects of proanthocyanidins in a rat model of deep vein thrombosis (DVT) and examined the underlying mechanisms.

Methods: DVT was induced in rat model by inferior vena cava (IVC) ligation. Grape seed proanthocyanidins extract (GSPE, 400 mg/kg/d) dissolved in saline (2 mL) was orally administered to the experimental rats. Control rats were administrated saline (2 mL) only. The thrombi were harvested and weighed. The IVC was analyzed histologically and by transmission electron microscopy. The cytokines interleukin (IL)-6, IL-8, and tumor necrosis factor-α (TNF-α) were detected by enzyme-linked immunosorbent assay. Expression of cellular adhesion molecules (CAMs) in thrombi was examined by Western blot.

Results: GSPE significantly reduced thrombus length and weight (P < .01) and protected the integrity of the endothelium. GSPE inhibited thrombogenesis-promoting factors P-selectin, von Willebrand factor, and CAMs, and promoted thrombogenesis-demoting factors CD34, vascular endothelial growth factor receptor-2, and ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type one motif, member 13). Compared with the control, GSPE significantly lowered the cytokines IL-6 (74.19 ± 13.86 vs 189.54 ± 43.76 pg/mL; P < .01), IL-8 (80.71 ± 21.42 vs 164.56 ± 39.54 pg/mL; P < .01), and TNF-α (43.11 ± 17.58 vs 231.84 ± 84.11 pg/mL; P < .01).

Conclusions: GSPE significantly inhibited the propagation of thrombus induced by IVC ligation in a rat model. The antithrombotic properties of proanthocyanidins are likely to be directly associated with endothelial protection and regeneration, platelet aggregation, and inhibition of inflammatory cell and thrombus adhesion. Thus, proanthocyanidins may have a clinical application in DVT treatment. (J Vasc Surg 2011;53:743-53.)

Clinical Relevance: The major presenting symptoms of deep vein thrombosis (DVT) are pain, swelling, and sometimes intractable venous ulceration. These are mainly caused by intravascular thrombosis occlusion and local inflammatory reaction. Two recent clinical studies reported that the levels of inflammatory markers such as interleukin-6, intercellular adhesion molecule 1 (ICAM-1), and C-reactive protein were associated with the development of postthrombotic syndrome (PTS). Our current results show that grape seed proanthocyanidins extract (GSPE) inhibited interleukin-6 release and ICAM-1 expression, which may suggest that prompt administration of GSPE is instrumental to thrombus clearance, removal of venous outflow obstruction, and attenuation of venous endothelium loss in PTS. Clinically, oral anticoagulants, heparin, and urokinase/streptokinase regimens are routinely used for treating DVT patients. These medicines are effective in correcting bloodstream stasis and hypercoagulable state; however, they are unable to cure endothelium injury caused by preceding thrombus occlusion that induces endothelial inflammation, compression, and hypoxia. This pathology may contribute to the recurrence of DVT, PTS, and other complications. Therefore, it is necessary to find a novel regimen that is effective and safe for the treatment of DVT. GSPE may be such a promising candidate.

Recent studies reveal that proanthocyanidins are one of the most important polyphenolic antioxidants present in grape seeds.1,2 Potential therapeutic values of grape seed proanthocyanidins extract (GSPE) are associated with its antioxidant and anti-inflammatory properties.3-7 Several studies have suggested that proanthocyanidins may beneficially affect endothelium injury, platelet aggregation, and inflammation involved in the pathogenesis of systemic diseases.5-9 However, the antithrombotic effects of GSPE on venous thrombosis have not been reported yet.

The classic risk factors for deep vein thrombosis (DVT) are described as Virchow’s triad—endothelium injury, blood stasis, and hypercoagulability—which are closely associated with inflammation. Inflammation is important in the formation and progress of DVT.10,11 The theory of thrombotic inflammation resulting in endothelial injury and platelet aggregation has received more attention nowadays; however, the precise mechanisms are still unclear. Previous studies have suggested that prothrombotic inflammatory mediators, endothelium injury, and platelet aggregation are important components in the pathogenesis...
of DVT.\textsuperscript{12-15} However, the endothelium protection and regeneration has not yet drawn sufficient attention in the field of DVT therapeutics.

The goal of present study was to determine whether GSPE inhibits thrombogenesis in an inferior vena cava (IVC) ligation-induced rat DVT model and to examine the underlying mechanisms. We hypothesized that GSPE would inhibit the propagation of thrombus induced by IVC ligation, and a few lines of experimental evidence support that the antithrombotic properties of GSPE are directly associated with inhibition of inflammatory cell and thrombus adhesion, endothelial cell proliferation signal transduction, and endothelial progenitor cell-induced endothelium regeneration. Our results may suggest that GSPE is a promising candidate for clinical use in treatment of DVT.

**MATERIALS AND METHODS**

This project was approved by the Animals Ethics Committees of Sun Yat-sen University.

**Animal care.** The study used 50 male Sprague-Dawley rats (weight, 280-330 g). The animals were kept in conditions of 23 ± 2°C temperature at 12-hour dark-light cycle in the specified pathogen free animal house of the Central Animal Laboratory, Sun Yat-sen University, Guangzhou, China. The rats had free access to standard rat chow and water.

**Animal model operating procedures.** Rats were randomly divided into three groups: group A (control, n = 15), group B (GSPE-treated, n = 25), and group C (sham-operated, n = 10). Each rat in these groups received an operative procedure. DVT was induced in the rats of groups A and B according to the previously described method.\textsuperscript{10,16} Briefly, anesthesia was initiated and maintained by the combination of oxygen (0.5 L/min) and isoflurane (1% to 4%), and all rats underwent laparotomy. Rats in groups A and B underwent ligation of the IVC below the renal veins and all visible side branches involved. Consequent obstruction and stasis of the bloodstream resulted in rapid IVC dilatation and thrombosis, and thrombi were detected ≤24 hours. The remaining rats received a sham operation, undergoing laparotomy and suturing, but without infrarenal IVC ligation. The sham group was set to obviate the operation-related traumatic effects.

Whole blood was collected from the caudal vein before the operation and every 12 hours after. Thrombi harvested from IVC were gently washed by phosphate-buffered saline (PBS) and weighed (with no vein walls and drops) at 1, 3, 5, 7, and 14 days in groups A and B. IVC tissues were carefully dissected and removed from the area 1 to 2 mm from the figure for histologic analysis at 1, 3, and 5 days. Serum was obtained by centrifugation and stored at −80°C until use for biochemical and Western blot analyses. All rats were euthanized at various times up to 2 weeks.

**GSPE administration.** The rats in group A were intragastrically administrated saline (2 mL) every 24 hours. After IVC ligation, the group B rats received daily intragastric administration of GSPE (400 mg/kg/d) dissolved in saline (2 mL). The GSPE (sources: grape seed; ethanol extraction oligomeric proanthocyanidins B2 and B3; purity: 95%) was obtained from BAOXING Bio-technologies Co., Ltd, Guangzhou, China.

**Morphologic examination.** Vascular tissues were fixed with cold 2% paraformaldehyde and 2% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4; Electron Microscopy Sciences, Hatfield, Pa) at 4°C overnight. Tissues were washed in sodium cacodylate buffer, rinsed with distilled water, and trimmed into smaller pieces. IVC tissues were postfixed in 1% osmium tetroxide and potassium ferrocyanide (Electron Microscopy Sciences, San Diego, Calif) to enhance membrane contrast.

After extensive rinsing with distilled water, tissues were dehydrated in a graded series of ethanol and embedded in Araldite (Electron Microscopy Sciences). All specimens were cut into semithin sections of 0.5 to 1 μm in thickness, counter-stained with toluidine blue/basic fuchsin stain, and examined using a Zeiss Light Microscope (Carl Zeiss, Jena, Germany). All ultrathin sections of 60 to 80 nm were collected on copper grids, double-stained with uranyl acetate and lead citrate for 8 minutes each, and then viewed and photographed on a CM-10 transmission electron microscope (TEM; Philips Electronics, Mahwah, NJ).

**Immunohistochemical staining.** Vessel specimens were collected from experimental rats. Immunohistochemical staining was performed on the paraffin-embedded tissue sections (10 μm) as described.\textsuperscript{10} Briefly, samples were fixed in formalin or snap-frozen and stored at −70°C. Formalin-fixed tissues were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. For CD34, vascular endothelial growth factor receptor-2 (VEGFR-2) and P-selectin localization, serial sections fixed in paraformaldehyde-lysine-periodate were incubated at 4°C overnight with anti-CD34 (1:100; mouse monoclonal anti-CD34, Santa Cruz Biotechnology, Santa Cruz, Calif), anti-VEGFR-2 (1:100; mouse monoclonal anti-VEGFR-2, Abcam, Cambridge, Mass), and anti-P-selectin polyclonal antibody (1:100; goat anti-rat P-selectin, Santa Cruz Biotechnology). Sections were washed, and bound primary antibodies were detected by successive incubations with bridging antibodies, peroxidase-antiperoxidase complexes (DAKO, Glostrup, Denmark), the substrate, diaminobenzidine, and finally, counterstained with hematoxylin.

**Immunofluorescence.** Cryostat sections obtained from snap-frozen tissues were used for the localization of von Willebrand factor (vWF) and ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type one motif, member 13). Specimens were stored at −80°C before immunofluorescence detection. Serial sections were fixed in 4% paraformaldehyde in PBS at 4°C. After three washes with PBS, the specimens were incubated with a blocking buffer (PBS, 0.5% Triton X-100, and 5% goat serum) for 4 to 16 hours at 4°C. After removal of the blocking buffer, they were subsequently incubated with anti-vWF (H-300) polyclonal antibody (1:100; rabbit anti-rat vWF, Santa Cruz Biotechnology) or anti-ADAMTS13 (H-300) polyclonal antibody (1:100; rabbit anti-rat vWF).
ADAMTS13, Santa Cruz Biotechnology) overnight in PB lectin-staining buffer (PBS, 1% Triton X-100, 0.1 mM CaCl₂, and 0.1 mM MnCl₂) on a rotator. After three washes with PBS, specimens were detected by successive incubation with cyanine3/fluorescein isothiocyanate-conjugated antirabbit immunoglobulin G (IgG).

**Enzyme-linked immunosorbent assay.** IL-6, IL-8, and tumor necrosis factor (TNF)-α in rat serum were prepared by centrifugation at 800 g for 15 minutes and deproteinated. Concentrations of these inflammatory factors were determined by enzyme-linked immunosorbent assay (ELISA) kits (Sigma-Aldrich, San Diego, Calif) using matched antibody pairs, according to the manufacturer’s instructions. Briefly, samples were added into 96-well plate, incubated at room temperature for 2 hours, and then washed three times with PBS.

After washing to remove the unbound molecules, samples were incubated with 3% bovine serum albumin to block nonspecific binding for 30 minutes. These inflammatory markers were detected by incubation with rabbit anti-rat monoclonal antibody for 1 hour, followed by an additional 30 minutes of incubation with horseradish peroxidase-conjugated goat antirabbit IgG. Positive color was developed with o-phenylenediamine and detected in a spectrophotometer (Bio-Rad Laboratories, Hercules, Calif) at 450 nm.

**Western immunoblotting.** Protein was isolated from thrombus lysed in a lysis buffer (50 mM Tri-HCl, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1% Nonidet P-40, 1 mM Na₃VO₄, 1 mM phenylmethyl sulfonyl fluoride, 1 mg/mL aprotinin, and leupeptin, at pH 7.4) for 20 minutes on ice. The lysates were centrifuged at 14,500 rpm for 20 minutes at 4°C. The concentration of protein in lysate was measured with a bicinchoninic acid kit (Pierce Co). A 15-µg protein lysate was subjected to electrophoresis on 8% sodium dodecyl sulfate polyacrylamide gels to detect vascular cell adhesion molecule (VCAM)-1 and intercellular cell adhesion molecule (ICAM)-1.

The samples were then electroblotted onto nitrocellulose paper. After blocking, blots were incubated with anti-VCAM-1 (1:500; rabbit anti-rat VCAM-1, Santa Cruz Biotechnology) and anti-ICAM-1 (1:500; mouse anti-rat ICAM-1, Santa Cruz Biotechnology) in PBS/Tween 20 for 2 hours at 37°C and then incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (KPL, Gaithersburg, Md) for 30 minutes. The expression of VCAM-1 and ICAM-1 was determined using the enhanced chemiluminescence kit (Amersham Pharmacia Biotech, Piscataway, NJ), and chemiluminescence exposure was detected by the filters of Kodak X-Omat films (Carestream Health Inc, Rochester, NY).

**Statistical analysis.** Data were reported as means ± SE and were analyzed statistically by analysis of variance, independent-samples t test, and linear correlation analysis at certain time points and with appropriate controls using SPSS 15.0 software (SPSS Inc, Chicago, Ill). A value of P < .05 was considered significant. All experiments were repeated at least three times to confirm reproducibility.

**RESULTS**

GSPE reduced thrombus formation after IVC ligation. Thrombi were formed by IVC ligation and collected at 1, 3, 5, 7, and 14 days, and their sizes and weights were measured. In group A (control), the lengths of thrombi increased from 0.17 ± 0.04 cm (day 1) to 0.89 ± 0.13 cm (day 14; Fig 1, a), and the weights increased from 0.0078 ± 0.0014 g (day 1) to 0.0531 ± 0.0043 g (day 14; Fig 1, b). Thrombus size peaked at days 5 to 7 and thereafter remained at a plateau. Stasis and trauma are two main causes of DVT. In control rats, these factors triggered the release of prothrombotic molecules and cytokines, activated platelets, and induced endothelium injury, thus accelerating the progress of thrombosis.

In the GSPE-treated group, lengths of the thrombi averaged at 0.16 ± 0.02 cm, significantly smaller than the 0.51 ± 0.08 cm for the control rats (P < .01), and weights
of the thrombi averaged 0.0027 ± 0.0006 g, significantly lighter than 0.0314 ± 0.0046 g for the control rats (P < .01). The index of weight (g)/length (cm) in the GSPE group was significantly lower than in the control rats (P < .01, Fig 1, c).

In addition, linear regression analysis revealed linear correlations between thrombus weight and cytokine production: IL-6 and thrombus weight (r = 0.874, P < .001), IL-8 and thrombus weight (r = 0.878, P < .001), and TNF-α and thrombus weight (r = 0.919, P < .001; Fig 2). However, the linear correlations were not shown in the GSPE group for IL-6 (r = 0.005), IL-8 (r = 0.029), and TNF-α (r = 0.353).

Histologic analysis showed that IVC endothelium was subjected to thrombi compression and adhesion, and fibrin strands and platelet aggregates were attached to a grossly intact vein wall, whereas these were not found in the GSPE and sham group (Fig 3). Collectively, these results indicate that the process of thrombogenesis is associated with inflammatory cytokine release and endothelial injury.

**GSPE protected the integrity of the endothelium.** To further investigate the protective effects of GSPE on the vein wall endothelium, we used a TEM to analyze endothelial morphologic changes. Fig 4, a shows that prethrombotic vein wall endothelium had integrity and was smooth, without leukocytes adhesion or infiltration. In contrast, Fig 4, b shows that activated macrophages and monocytes adhered to the endothelium after IVC ligation. Furthermore, the thrombotic compression and inflammation resulted in endothelial cell necrosis and apoptosis (Fig 4, c) and subendothelial extracellular matrix exposure and contact with blood cells (Fig 4, d), thus destroying the integrity of endothelium (Fig 4, e). Leukocyte recruitment exacerbated the development of thrombosis, and inflammation-activated leukocytes released molecules to induce cell adhesion (Fig 4 g). In the GSPE-treated group, however, the cell-cell junction was closed, without leukocyte adhesion or infiltration (Fig 4, b), suggesting that the integrity of the endothelium was protected by the GSPE treatment. These results indicate that thrombosis induces endothelial loss by way of inflammation but that GSPE protected the integrity of the endothelium by protecting endothelial cells from inflammatory attack.

**GSPE increased CD34 and VEGFR-2 expression in the endothelium.** To assess the role of GSPE in endothelial protection and regeneration, expression of CD34 and VEGFR-2 was determined by immunohistochemical staining. The expression of CD34 and VEGFR-2 in the endothelium in the GSPE-treated group was significantly higher than in the control group at day 3, when thrombosis-induced endothelium loss was substantial (P < .05; Fig 5). The findings of histologic staining are in accord with the TEM microscopic endothelial observation (Fig 4). The results indicate that GSPE may induce endothelial cell proliferation and endothelial progenitor cell repair by increasing the CD34 and VEGFR-2 expression in the endothelium.

**GSPE inhibited P-selectin expression.** P-selectin expression in the control group reached a peak at day 3, and the upregulation of P-selectin localized prothrombotic microparticles to the area of stasis and promoted thrombogenesis (Fig 6). In addition, we observed that leukocyte recruitment was associated with high-P-selectin–stained area. In contrast, the expression of P-selectin was reduced significantly (P < .05) in the GSPE-treated group compared with the control group. These data demonstrated that GSPE reduced P-selectin expression to exert inhibitory effects on leukocyte recruitment and thrombosis.

**GSPE downregulated vWF and upregulated ADAMTS13.** We first tested whether GSPE affects the expression of vWF and ADAMTS13 by determining the extent to which vWF and ADAMTS13 localize after IVC ligation. Fluorescence staining displayed that vWF expression at day 1 was highly expressed in both the endothelium and thrombus in the control group (Fig 7, e) but was much downregulated in the GSPE-treated group (Fig 7, b). In contrast, the expression of ADAMTS13 in the GSPE-treated group (Fig 7, i) was higher than in the control group (Fig 7, f). These results suggest that GSPE may inhibit thrombogenesis by downregulating vWF expression and upregulating ADAMTS13 at the early stage of thrombosis.

**GSPE reduced IL-6, IL-8, and TNF-α production.** Previous studies suggest that IL-6, IL-8, and TNF-α contribute to the formation of thrombosis, especially in the acute phase. We performed ELISA to determine whether GSPE affects the release of these cytokines and found that the levels of IL-6, IL-8, and TNF-α were all markedly elevated after the laparotomies, with and without IVC ligation. The increased cytokine production correlated with the weight of thrombosis (Fig 2). However, the cytokine levels in the GSPE group were lower than the control: IL-6, 74.19 ± 13.86 vs 189.54 ± 43.76 pg/mL (P < .01); IL-8, 80.71 ± 21.42 vs 164.56 ± 39.54 pg/mL (P < .01); and TNF-α, 43.11 ± 17.58 vs 231.84 ± 84.11 pg/mL (P < .01). The levels of IL-6, IL-8, and TNF-α all started to increase at the early stage of thrombosis; however, GSPE treatment markedly reduced these cytokines’ release.

**GSPE reduced the VCAM-1 and ICAM-1 expression in thrombus.** VCAM-1 and ICAM-1 contribute to the congregation of inflammatory cells, platelet, and endothelial cells, so we examined whether GSPE affects the expression of these CAMs. Western blot analysis displayed that VCAM-1 and ICAM-1 expressions in the thrombus of the GSPE group were significantly lower than in the control group (P < .01, Fig 8). These results indicate that GSPE inhibited the adhesion of thrombus to vessel wall by decreasing the VCAM-1 and ICAM-1 expression.

**DISCUSSION**

In the present study, we demonstrated that thrombogenesis occurred after IVC ligation in a rat model, which involves endothelium injury, upregulation of vWF, CAMs, and inflammatory cytokines, as well as platelet aggregation and fibrin strand formation. We also found that GSPE
Fig 2. The thrombus weight has a linear correlation with the cytokine production of (a) interleukin (IL-6) \(r = 0.874\), (b) IL-8 \(r = 0.878\); and (c) tumor necrosis factor (TNF-\(\alpha\)) \(r = 0.919\); but this was not shown in the grape seed proanthocyanidins extract (GSPE) group (IL-6, \(r = 0.005\); IL-8, \(r = 0.029\); TNF-\(\alpha\), \(r = 0.353\)).

Fig 3. Mature thrombi (TH) were detected by hematoxylin and eosin staining in paraffin sections of inferior vena cava (IVC) biopsy specimens at day 3 after IVC ligation. Thrombi compression and adhesion, fibrin strands, and platelet aggregates were observed to attach to a grossly intact vein wall (a, b). However, these were not found in the grape seed proanthocyanidins extract (GSPE) (c) and sham group (d) (original magnification \(\times200\)).
reduced thrombus formation by maintaining the endothelial integrity, increasing the expression of CD34, VEGFR-2, and ADAMTS13, decreasing the expression of P-selectin, VCAM-1, ICAM-1, and vWF expression, and reducing the release of IL-6, IL-8, and TNF-α. These new data highlight the antithrombotic effects of GSPE through protecting the endothelium, inhibiting platelet aggregation, and reducing the release of prothrombotic inflammatory mediators.

In the preliminary study, bleeding time was significantly prolonged when GSPE (400 mg/kg) was used (data not shown). For the sake of preventing bleeding in the preoperative period, we used the lower dose of GSPE (250-300 mg/kg) for the preadministration and maintenance dosage, and this had the similar antithrombotic effect as the larger dose of GSPE (400 mg/kg) administration sooner after IVC ligation. No bleeding complications or death from bleeding occurred in the rats in the present study. This suggests that GSPE could be possibly used before (prevention) and after (therapeutically) thrombosis: the lower dosage could be used for prevention and higher dosage for therapeutic effect.

To our knowledge, this is the first report investigating the potential for GSPE administration to inhibit thrombus propagation in a rat model. Actually, the thrombi did not come out...
as a regular cylinder. It could be argued that the handmade measurement we used does not reflect the true size of the clot. Nevertheless, we found the clots in the GSPE group were two to three times smaller than in the control group after day 3, so we just compared the size of thrombus between the control and GSPE group by weight, longitudinal axis length, and weight/length index. We observed that the progress of venous thrombosis was accompanied with endothelial cell denudation and leukocyte recruitment, consistent with previous reports.\textsuperscript{18,19}

During the acute phase of thrombogenesis, vWF is released from endothelial cells, and platelets are thus activated. We also showed that P-selectin expression, leukocyte recruitment, and platelet aggregation significantly increased in the control group. P-selectin has been found upregulated in the vein wall as early as 6 hours after thrombus induction.\textsuperscript{20} Interactions of P-selectin and its receptor P-selectin glycoprotein ligand-1 can stimulate the production of thrombogenic microparticles from leukocytes, particularly monocytes, along with platelets and endothelial cells.\textsuperscript{15,21} Previous reports\textsuperscript{12-15} and the present study both showed that an increase in the blood level of inflammation mediators, such as CAMs and cytokines, may recruit leukocytes, trigger platelet aggregation, induce fibrin strand formation, and exacerbate endothelium injury. The initial capture of flowing platelets is mediated by the interaction of vWF and its receptor,\textsuperscript{13} and then CAMs allow leukocyte transmigration. P-selectin is also integrally involved in thrombosis.\textsuperscript{15} On the other hand, thrombus sticks to the endothelium, causing compression, hypoxia, and inflammation, which induces endothelial cell damage. At the site of endothelium injury, activated platelets contact with the subendothelial extracellular matrix, triggering the formation of a hemostatic plug. However, prothrombotic factors, such as vWF, P-selectin, ICAM-1, and VCAM-1, were significantly reduced when GSPE was administrated, accompanied with a reduction of thrombus formation.

Fig 5. CD34 and vascular endothelial growth factor (VEGFR)-2 expression in the endothelium. CD34 (a, b) and VEGFR2 (g, h) staining in the endothelium of sham group are shown as above. Representative photomicrographs of immunohistochemistry-stained paraffin sections of vein biopsy specimens are shown. c, d, The CD34 expression of endothelium in control group at day 3 shows negative or weak staining in the endothelium in the control, representing an endothelium loss (original magnification $\times 200$). e, f, Endothelial cells were positively stained in the grape seed proanthocyanidins extract (GSPE) group at day 3, indicating the maintenance of the endothelium (original magnification $\times 200$). i, j, VEGFR-2 expression in the control specimens was negatively stained on the major part of the endothelium but weakly positive at the site of the endothelium without thrombus or leukocyte adhesion on day 3 (original magnification $\times 200$). k, l, The positively-stained areas were detected at the endothelium in GSPE group specimens at day 3 (original magnification $\times 200$). m, The bar chart representing the percentage of CD34 stained at the endothelium shows that GSPE significantly increased CD34 expression compared with control ($P < .05$). n, The two bars representing the expression of VEGFR-2 in control and GSPE treatment show that the VEGFR2 expression in the GSPE treatment group is significantly higher than that of control, calculated by the staining area/total area of the inferior vena cava endothelium ($P < .05$). The solid line arrows point to the positive staining area; the dotted line arrows point to the negative staining area. TH, thrombus; vW, vein wall. *$P < .05$. 100$: a, c, e, g, i, k/$\times 200$: b, d, f, h, j, l.
A strong link has been reported between venous thromboembolism and arterial thrombosis that is represented by endothelial dysfunction and endothelium loss. Hence, maintaining the normal endothelial functions and integrity of the endothelium is crucial in the prevention and treatment of thrombosis. Here, we show that the adhesion of inflammatory cells and thrombus to the endothelial cells causes endothelium loss (Figs 3 and 4, b, c, d, and e). In contrast, GSPE restored a healthy endothelium by inhibiting inflammatory cytokines and CAMs release (Fig 6, c, d, and Fig 8) at the early stage of thrombogenesis.

CD34 is a glycoprotein expressed on the surface of endothelial progenitor cells and small-vessel endothelial cells. VEGFR-2 is a member of a receptor tyrosine kinase family whose activation plays an essential role in a large number of biologic processes such as embryonic development, wound healing, cell proliferation, migration, and differentiation. It has been suggested that endothelial progenitor cells (CD34+/VEGFR-2+) bind platelets by way of CD62P and inhibit platelet activation, aggregation, adhesion to collagen, and thrombus formation. Furthermore, VEGFR-2 mediates the endothelial cellular signal pathways, which is important in regulating vascular endothelial function and promoting vascular endothelial cell proliferation and antithrombosis. This is consistent with our data showing that GSPE mediates endothelial protection and regeneration by increasing the expression of P-selectin, a critical molecule in inflammation and thrombosis.
CD34 and VEGFR-2. Although, where these endothelial cells originated from remains to be ascertained.

The antithrombotic properties of GSPE should be ascribed to platelet antiaggregation induced by an increase of ADAMTS13 expression, a decrease of vWF and CAMs expression, and a reduced release of inflammatory cytokines at the early stage of thrombosis. It is known that vWF promotes adhesion of endothelial cells to the vessel wall and thrombogenesis. ADAMTS13 can make a precise cut in the specific peptide-bond site of vWF. We found that vWF releases at the acute phase of thrombosis and activates platelet aggregation to the site of endothelium injury, similar to the previous studies. Our data suggested that GSPE upregulated the ADAMTS13-mediated proteolytic cleavage that is essential to reducing the size of vWF polymers so that they remain functional enough to stop bleeding but not so sticky that they cause unwanted thrombosis (Fig 7).

Further, inflammatory cytokines are generated in this process and play an additional role in this pathology. IL-8 and TNF-α stimulate ultralarge vWF released from endothelial cells, and IL-6 significantly inhibits the rate of the cleavage of ultralarge vWF. The levels of IL-6, IL-8, TNF-α were all markedly reduced after the administration of GSPE, suggesting that GSPE may contribute to platelet antiaggregation by reducing the production of cytokines.

**Fig 7.** Expression is shown of von Willebrand factor (vWF) and ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) in cryostat sections of (a, b, c) prethrombosis, (d, e, f) control group, and (g, h, i) grape seed proanthocyanidins extract (GSPE) group. b, vWF expression of prethrombotic vein endothelium was weakly stained. After inferior vena cava ligation, vWF expression in the control group was upregulated at day 1. c, Endothelium and thrombus were both strongly positive stained. h, vWF expression of in the GSPE group was weaker than the control. On the contrary, the expression of ADAMTS13 in the (f) control group was weaker than in the (i) GSPE group. Black arrows point to the endothelium shown in the hematoxylin and cosin (HE)-stained slice. White arrows point to the prethrombotic endothelium. Blue arrows point to the postthrombotic endothelium in control group. Pink arrows point to the postthrombotic endothelium in the GSPE group. Th, Thrombus; vw, vein wall. 100×: a, b, c, e, g, h, i; 200×: d, f.
prothrombotic effects of P-selectin, VCAM-1, and ICAM-1 have been implicated in leukocytes transmigrating to the site of endothelial injury and in platelet aggregation.\textsuperscript{17,20,34} Our results demonstrate that GSPE may inhibit platelet aggregation by decreasing CAMs (Figs 6 and 8).

CONCLUSIONS

We conclude that GSPE significantly inhibited the formation of thrombus induced by IVC ligation in a rat model. It is reasonably speculated that the antithrombotic properties of GSPE are directly associated with inhibition of inflammatory cell and thrombus adhesion and platelet aggregation, endothelial cell proliferation signal transduction, and endothelial progenitor cell-induced endothelium regeneration. Thus, GSPE may be a promising candidate for effective and safe treatment of DVT.

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REFERENCES


