The combination of EGCG with warfarin reduces deep vein thrombosis in rabbits through modulating HIF-1α and VEGF via the PI3K/AKT and ERK1/2 signaling pathways

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Available online 20 Sep., 2022

[ABSTRACT] Deep venous thrombosis (DVT) poses a major challenge to public health worldwide. Endothelial cell injury evokes inflammatory and oxidative responses that contribute to thrombus formation. Tea polyphenol (TP) in the form of epigallocatechin-3-gallate (EGCG) has anti-inflammatory and oxidative effect that may ameliorate DVT. However, the precise mechanism remains incompletely understood. The current study was designed to investigate the anti-DVT mechanism of EGCG in combination with warfarin (an oral anticoagulant). Rabbits were randomly divided into five groups. A DVT model of rats was established through ligation of the inferior vena cava (IVC) and left common iliac vein, and the animals were orally administered with EGCG, warfarin, or vehicle for seven days. In vitro studies included pretreatment of human umbilical vein endothelial cells (HUVECs) with different concentrations of EGCG for 2 h before exposure to hydrogen peroxide. Thrombus weight and length were examined. Histopathological changes were observed by hematoxylin-eosin staining. Blood samples were collected for detecting coagulation function, including thrombin and prothrombin times, activated partial thromboplastin time, and fibrinogen levels. Protein expression in thrombosed IVCs and HUVECs was evaluated by Western blot, immunohistochemical analysis, and/or immunofluorescence staining. RT-qPCR was used to determine the levels of AGTR-1 and VEGF mRNA in IVCs and HUVECs. The viability of HUVECs was examined by CCK-8 assay. Flow cytometry was performed to detect cell apoptosis and ROS generation was assessed by 2′,7′-dichlorofluorescein diacetate reagent. In vitro and in vivo studies showed that EGCG combined with warfarin significantly reduced thrombus weight and length, and apoptosis in HUVECs. Our findings indicated that the combination of EGCG and warfarin protects HUVECs from oxidative stress and prevents apoptosis. However, HIF-1α silencing weakened these effects, which indicated that HIF-1α may participate in DVT. Furthermore, HIF-1α silencing significantly up-regulated cell apoptosis and ROS generation, and enhanced VEGF expression and the activation of the PI3K/AKT and ERK1/2 signaling pathways. In conclusion, our results indicate that EGCG combined with warfarin modifies HIF-1α and VEGF to prevent DVT in rabbits through anti-inflammation via the PI3K/AKT and ERK1/2 signaling pathways.

[KEY WORDS] Deep vein thrombosis; EGCG; Warfarin; HIF-1α; VEGF; PI3K/AKT; ERK1/2

[Introduction]

Deep vein thrombosis (DVT) is a common condition that may lead to debilitating conditions such as pulmonary embolism and also the cause of complications in hospitalized patients that may result in death [1-3]. Endothelial cells play a role in the development of DVT through participating in thrombus formation in response to injury [4, 5]. Normally, endothelial cells prevent platelet and thrombin activation by expressing and producing mediators such as heparan sulfate, thrombomodulin, and nitric oxide (NO), which inhibit platelet adhesion and thrombin-induced coagulation [6-9]. Furthermore, endothelial cells help reduce blood loss and clot formation by producing endothelin-1 (ET-1), angiotensin II (Ang II), and adhesion glycoproteins, which enhance vasoconstriction and platelet aggregation in response to injury [9, 10]. According to previous studies, Ang II and ET-1 induced the production of 20-HETE, which activated the ERK pathway, resulting in endothelial activation [11]. Blood coagulation can be initiated by multiple pathways,
such as ERK1/2, which are activated by damaged blood vessels or exposure to collagen [12]. These pathways can trigger the generation of thrombin, which converts fibrinogen to fibrin for scaffold formation [10]. Thrombin can also stimulate endothelial cells to release cell surface adhesion molecules, such as P-selectin and von Willebrand factor (vWF), which attract platelets and leukocytes to the injury site [14]. In the absence of injury, endothelial cell dysfunction can cause fibrin production and platelet aggregation through stimulating inflammation, contributing to DVT-associated damage in endothelial cells [15-17]. Previous studies showed that coagulation factor XII (FXII) promoted DVT via the activation of PI3K/AKT signaling by inducing an inflammatory response [18].

Polyphenols from tea (Camellia sinensis) are plant-based antioxidants with a wide range of health benefits [19, 20]. Tea polyphenols (TPs), also known as catechins, make up 30%–42% of the dry weight of tea leaves [19]. Their antioxidant effects were enhanced by their vicinal dihydroxy or trihydroxy structure, which allowed for electron delocalization and chelation of metal ions, resulting in free radical quenching [21]. TPs have been found to attenuate oxidative stress by regulating the Keap1/Nrf2/ARE pathway [22]. The reagent utilized in this investigation is epigallocatechin-3-gallate (EGCG), which is one of the key polyphenols known to exhibit anti-inflammatory and antioxidant properties in C. sinensis.

Compared with venous blood, newly formed thrombus is hypoxic, which results in the expression of hypoxia-inducible factor 1 (HIF-1) [23]. HIF-1α is one of two subunits that make up HIF1 and its expression increases during hypoxia. According to a previous report, HIF-1α translocated to the nucleus under hypoxia, where it dimerized with HIF-1β to activate the HIF-1 downstream genes [24]. HIF-1 may accelerate transcription of angiogenesis-related genes such as vascular endothelial growth factor (VEGF) [25, 26]. VEGF was elevated during DVT and has been found in endothelial cells in thrombus resolution and newly formed vascular tissue [27].

Through ligating the inferior vena cava (IVC) and left common iliac vein, we were able to test whether the combination of EGCG and warfarin can reduce the formation of blood clots. In human umbilical vein endothelial cells (HUVECs) exposed to hydrogen peroxide (H$_2$O$_2$), we measured hemorheological parameters, the levels of HIF-1α, VEGF, AGTR-1, and the components of the PI3K/AKT and ERK1/2 signaling pathways. Our results demonstrated that the combination of EGCG and warfarin can be a beneficial complementary medication in the treatment of DVT.

Materials and Methods

Reagents

EGCG was purchased from Hangzhou Yibeijia Tea Technology Co., Ltd. (S18152, Zhejiang, China). Warfarin was purchased from Qilu Pharmaceutical Co., Ltd. (H37021314, Jinan, China). HIF-1α (NB100-105, 1 : 1000, Novus, USA) and AGTR-1 (NB01-7708, 1 : 1000, Novus, USA) were purchased from Novus (Colorado, USA). p-ERK (bs-3016R, 1 : 1500), ERK (bs-0022R, 1 : 1000), VEGF (bs-1313R, 1 : 1500), VEGFR-1 (bs-20692R, 1 : 1500), Akt (bs-0115M, 1 : 1000), p-PI3K (bs-3332R, 1 : 1500), p-P70S6K (bs-1656R, 1 : 1000), P70S6K (bs-3617R, 1 : 1000), p-Akt (bs-0876R, 1 : 1000) and PI3K (bs-2067R, 1 : 2000) were purchased from Bioss antibodies (Beijing, China). GAPDH (ab9485, 1 : 1000) antibodies were purchased from Abcam (Cambridge, MA, USA). Primary HUVECs were purchased from ThermoFisher Scientific (C0035C, Waltham, MA, USA). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from GE Healthcare Life Sciences (SH30022, Logan, UT, USA).

Animals

Male New Zealand white rabbits weighing 2.5 kg on average were obtained from the Medical Animal Experimental Center at Nanjing Medical University. The rabbits were caged at a controlled temperature of 24 °C with free access to food and water. The animals were maintained following the ethical guidelines recommended by our institute (Ethical code: IACUC-20210207, time: 2021-07-02) and all experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, 8th edition (revised in 2011).

Animal treatment and sampling

The rabbits were randomly divided into five groups (n = 10): a control group, a model group, a model with warfarin group (2.00 mg·kg$^{-1}$·d$^{-1}$), model with warfarin and EGCG low-dose (200 mg·kg$^{-1}$·d$^{-1}$), middle-dose (600 mg·kg$^{-1}$·d$^{-1}$), and high-dose (1000 mg·kg$^{-1}$·d$^{-1}$) groups. The animals were first anesthetized with 20% urethane (5 mL·kg$^{-1}$). Then, a DVT model was established through ligation of the IVC and left common iliac vein with 6-0 silk thread via midline laparotomy. Then, animals in each group were orally administered with an appropriate dose of EGCG and/or warfarin (2.00 mg·kg$^{-1}$·d$^{-1}$) or vehicle after on day 7 after surgery.

Hemorheology and coagulation function detection

The whole blood (5 mL) was collected from the ear artery of rabbits 1, 3, and 7 days after DVT procedure. Plasma viscosity, whole blood viscosity at high and low shear, and red cell assembly, and other hemorheology indexes were measured using a hemorheology detector (Beijing Succeeder Science and Technology Development, Beijing, China). Coagulation function indexes, such as fibrinogen content, prothrombin time (PT), thrombin time (TT), and activated partial thromboplastin time (APTT), were determined using a coagulation reaction detection method (Nanjing Perlong Image Documentation Equipment, Nanjing, China).

Cell treatment

HUVECs were cultured in DMEM supplemented with 10% fetal bovine serum and 100 U·mL$^{-1}$ penicillin/streptomycin (15140122, Gibco, Gaithersburg, MD, USA) in a humidified atmosphere at 37 °C with 5% CO$_2$. After 24 h, the
cells (1 × 10^4 cells/mL) were resuspended in DMEM containing 0, 5, 10, and 20 μmol L^−1 EGCG. Then, the cells were incubated for 2 h before exposure to 200 μmol L^−1 H_2O_2 for 24 h. The cells were harvested at 150 × g for 5 min for analysis.

**Hematoxylin and eosin (H&E) staining**

To assess the severity of injury, the femoral veins were fixed with 4% paraformaldehyde before dehydrated in a series of ethanol and finally embedded in paraffin. The paraffin-embedded tissue blocks were cut into 4 μm sections and dewaxed. The resultant sections were stained with H&E and images were captured at × 400 magnification using a light microscope (IX-81, Olympus, Tokyo, Japan).

**TUNEL staining**

Femoral vein tissues were fixed, sliced into 4 μm sections, dehydrated and rehydrated. A TUNEL apoptosis detection kit (40306ES20, Yeasen Biotechnology, Shanghai, China) was used according to the manufacturer’s instructions. The sections were counterstained with DAPI (C1002, Beyotime, Nanjing, China).

**Immunofluorescence (IF) assay**

The sections of femoral vein tissue were incubated with antibodies against HLA-DR (1 : 100, bs-0646R, Bioss, Beijing, China). Then, they were incubated in HRP-conjugated with secondary antibodies (1 : 50) at room temperature for 1 h. After washing, the sections were counterstained with DAPI and visualized by a Dako REAL EnVision Detection System (K5007, Agilent, Santa Clara, CA, USA).

**MTT assay**

The viability of HUVECs was measured by MTT assay. After treatment, the cells were washed and incubated with MTT solution (5 mg·mL^−1, M5655, Sigma-Aldrich, St Louis, MO, USA) at 37 °C for 4 h. The cells were exposed to dimethyl sulfoxide (67-68-5, 100 μL, Carl Roth GmbH, Karlsruhe, Germany) before cultivation at 37 °C for another 4 h. Optical density was determined at 570 nm in a microplate reader (Model 680, Bio-Rad, Hercules, CA, USA).

**Cell viability and ROS content**

The number of viable HUVECs was determined by CCK-8 assay. Briefly, HUVECs (2 × 10^5 cells/well) were seeded into 96-well plates containing 10 μL CCK-8 solution (M4839, ApexBio, Shanghai, China) and incubated for 1 h. The cellular apoptosis was measured by a CytoFLEX flow cytometer (Beckman Coulter, Miami, FL, USA). The cells were stained with 2',7'-dichlorofluorescein (DCF) and ROS content was calculated according to the percentage of the stained cells.

**Immunohistochemical (IHC) analysis**

Slides were dewaxed with xylene, rehydrated with ethanol in gradient concentrations, recovered with EDTA solution (pH 9.0), and heated at 95–100 °C for 15 min. Next, the sections were immersed in 3% H_2O_2 for 10 min, blocked with 10% goat serum, and incubated with antibodies against VEGF and HIF-1α (1 : 200) at 4 °C overnight. Then, the sections were incubated with an HRP-conjugated secondary antibody (KGAA35, 1 : 125, KeyGEN BioTECH, Nanjing, China) at room temperature for 1 h, before stained with DAB for 40 s. Meanwhile, normal goat IgG was used as a negative control. The pathological changes were observed under an optical microscope (BX53; Olympus, Tokyo, Japan).

**Treatment with small interfering RNA (siRNA)**

In Table S1, the sequences of HIF-1 siRNAs are presented. A random siRNA sequence (5′-GGCGCGCUUGAUAGGAUUCG dTdT-3′) was used as a control for each siRNA. The Lipofectamine™ 3000 reagent (Invitrogen, Grand Island, NY, USA) was used to transfect cells with corresponding siRNA according to the manufacturer’s instructions.

**RNA extraction and quantitative real-time PCR**

RNA was extracted with Trizol reagent (15596018, Invitrogen, Carlsbad, CA, USA). An RT reagent kit (RR037, Takara, Shiga, Japan) was used to synthesize cDNA. Genes were amplified with SYBR Green Supermix (1725121, Bio-Rad, Hercules, USA). The PCR condition was listed as follows: 95 °C for 5 min, 95 °C for 3 s and 60 °C for 20 s for 40 cycles using a LightCycler480 II (Roche, Basel, Switzerland). Relative gene expression was determined against GAPDH using the 2^−ΔΔCt method. The primer sequences are listed in Table 2.

**Western blot analysis**

Cells were first lysed with RIPA buffer, and protein levels were measured using a protein quantification kit (P0012S, Beyotime, Shanghai, China). The proteins were then separated by SDS-PAGE, before transferred to PVDF membrane (1620177, Bio-Rad, Hercules, CA). The membrane was blocked in non-fat milk and then incubated with antibodies against HIF-1α (1 : 1000), VEGF (1 : 1500), VRGFR-1 (1 : 500), AGTR-1 (1 : 1000), p-PI3K (1 : 1500), PI3K (1 : 2000), p-Akt (1 : 1000), Akt (1 : 1000), p-ERK (1 : 1500), ERK (1 : 1000), p-70S6K (1 : 1000), P70S6K (1 : 1000) and GAPDH (1 : 1000) at 4 °C overnight. Then, the membrane was probed with secondary antibody at room temperature for 1 h and the immunoreactive bands were detected with an ECL luminescence kit (PE0010, Solarbio, Beijing, China). Images were processed by ImageJ2x software (v2.1.5.0, Rawak Software, Stuttgart, Germany).

**Statistical analysis**

Data are presented as the mean ± standard error of the mean (SEM). Differences among groups were compared with one-way ANOVA and the Tukey post-hoc test. Student’s t-test was used to compare the differences between two groups. P < 0.05 was considered statistically different. All statistical analyses were conducted using SPSS 20.0 (SPSS, Chicago, IL USA) and GraphPad Prism v8.0 (GraphPad Software, San Diego, CA, USA) software.

**Results**

EGCG combined with warfarin ameliorated thrombosis in vivo

To determine whether the combination of EGCG and warfarin can ameliorate the formation of blood clots, a rabbit model of DVT was established through ligation of the IVC and left common iliac vein. The numerical data of hemorheological parameters and coagulation function measured seven days after modeling is shown in Table S3. There was a
significant difference between the model group and the control group in terms of PT, TT, APTT, and fibrinogen levels, indicating enhanced blood clotting in the model group. PT, TT, and APTT values were significantly improved as EGCG doses increased in animals receiving EGCG combined with warfarin for seven days, where the fibrinogen levels were lower than those in model rabbits or after treatment with warfarin alone. Similarly, when EGCG was administered in combination with warfarin, thrombus formation, measured by length and weight, (Figs. 1A−1C) and vein wall thickness

![Fig. 1](https://example.com/fig1.jpg)

**Fig. 1** EGCG combined with warfarin improved thrombosis *in vivo*. (A) Images of thrombus from different groups. (B and C) Thrombus length and weight in each group. (D) H&E-stained sections of vein wall thickness indicated by arrows (bar = 20 μm). (E) Vein wall thickness in different treatment groups. Data are expressed as the mean ± SEM (n = 10). *P* < 0.05, the model group vs the control group; *P* < 0.05, the model + warfarin group vs the model group; and *P* < 0.05, the model + warfarin + EGCG groups vs the model + warfarin group.
Combined use of warfarin and EGCG regulated the levels of HIF-1 and VEGF protein in vivo

Hypoxia is associated with DVT. In the current study, the levels of HIF-1α, VEGF, and components of the PI3K/AKT and ERK1/2 signaling pathways in each group were determined. Immunofluorescence analysis indicated that the levels of CD34, a cell surface glycoprotein that functions as a cell-cell adhesion factor in the endothelial cells of blood vessels, were higher in the model group than those in the model group. CD34 expression was considerably reduced in the model group, but dramatically increased after treatment with warfarin alone. The combined use of different doses of EGCG and warfarin increased CD34 expression in a dose-dependent manner (Fig. 3A). Furthermore, immunohistochemistry analysis revealed higher levels of HIF-1 and VEGF in the periphery of blood arteries and thrombus in the model group than those in the control group. EGCG decreased the levels of HIF-1 and VEGF in the DVT model, and high-dose EGCG had a more significant effect (Figs. 3B–3F). The protein and mRNA levels of HIF-1α, VEGF, VEGFR1 and ATGR-1 were measured, which showed the similar trend (Figs. 3D–3F). The model group presented the highest levels of HIF-1, ATGR-1, VEGF, and VEGFR-1, while the control group and animals that received warfarin and high-dose EGCG showed the lowest levels. We also measured the levels of total PI3K, AKT, and ERK protein and their phosphorylated protein in each group (Fig. 3D). The levels of phosphorylated PI3K, AKT, and ERK were elevated in the model group, but dramatically increased after treatment with EGCG. These results suggest that the combination of EGCG
with warfarin reduces the levels of HIF-1α and VEGF, which may be associated with the PI3K/AKT and ERK1/2 signaling pathways.

**Endothelial cell damage was prevented in vitro by the combination of EGCG with warfarin**

To verify the protective effect of EGCG combined with warfarin on endothelial cell injury, we measured the effect of the combination of EGCG with warfarin on cell proliferation in HUVECs treated with 0, 5, 10, and 20 μmol·L⁻¹ EGCG for 2 h before exposure to 200 μmol·L⁻¹ H₂O₂ for 24 h. Cell proliferation and apoptosis in HUVECs are shown in Figs. 4A−4D. Cell proliferation was greatly reduced in cells exposed to H₂O₂ without the presence of warfarin (Fig. 4A). Furthermore, cell proliferation was improved by the addition
of warfarin, and enhanced by EGCG treatment in a dose-dependent manner. Similar results were obtained in terms of apoptosis (Figs. 4B and 4C). The number of cells survived after \(H_2O_2\) treatment without warfarin or EGCG was low. Flow cytometry and DCF staining confirmed that EGCG combined with warfarin protected endothelial cells from oxidative stress injury in vitro.

**EGCG combined with warfarin reduced the expression of HIF-1\(\alpha\) and VEGF in vitro**

To verify that combination of EGCG with warfarin influences the expression of HIF-1\(\alpha\) and VEGF in vitro, we assessed the levels of HIF-1\(\alpha\) in HUVECs using immunofluorescence (Fig. 5A). The levels of HIF-1\(\alpha\) increased in response to oxidative stress in vitro but decreased after treatment with the combination of EGCG and warfarin. The levels of VEGF, VEGFR-1, AGTR-1, and ratios of p-PI3K/PI3K, p-AKT/AKT, p-P70S6K/P70S6K, and p-Erk/Erk in vitro showed similar changes with those in vivo (Fig. 5B). HIF-1\(\alpha\), VEGF, VEGFR-1 and ATGR-1 were all up-regulated in response to oxidative stress. However, the addition of warfarin combined with EGCG protected the cells from the damage caused by oxidative stress. In addition, the levels of phosphorylated PI3K, AKT, P70S6K and ERK1/2 increased under oxidative stress but decreased in cells pretreated with warfarin and EGCG. The relative expression of HIF-1\(\alpha\) and ATGR-1 mRNA was also elevated under oxidative stress but not reach to the same level in cells pretreated with warfarin and EGCG. Overall, our results suggested that EGCG combined with warfarin protect the cells from oxidative stress and reduce the expression of HIF-1\(\alpha\) and VEGF, which may be associated with the PI3K/AKT and ERK1/2 signaling pathways.

**HIF-1\(\alpha\) silencing impeded the beneficial effect of EGCG and warfarin on HUVECs**

Next, we determined whether silencing of HIF-1\(\alpha\) influenced the effects of EGCG combined with warfarin on

**Fig. 4** Endothelial cell damage was prevented in vitro by combining EGCG with warfarin. (A) Proliferation of HUVECs by CCK8 assay. (B and C) Effects of EGCG on cell apoptosis by flow cytometry in three independent experiments. (D) Determination of apoptosis in HUVECs stained with DCF. (E) Statistical analysis of flow cytometry. Data are presented as the mean ± SEM (n = 3). *P < 0.05, the model groups vs the control group; †P < 0.05, the model + warfarin group vs the model group; and ‡P < 0.05, the model + warfarin + EGCG groups vs the model + warfarin group.
HUVECs. HIF-1α was silenced in HUVECs by small interfering RNA (Fig. 6A). Then, the proliferation and apoptosis of HUVECs were measured (Figs. 6B−6D). We found that warfarin and EGCG (20 μmol·L−1) promoted proliferation and reduced apoptosis, while silencing of HIF-1α in combination with warfarin and EGCG (20 μmol·L−1) decreased proliferation and increased apoptosis (Figs. 6B−6D). Cell migration and invasion were also enhanced when the cells were treated with both warfarin and EGCG, but silencing of HIF-1α suppressed migration and invasion (Figs. 6E−6G). Similar results were obtained for cell viability by flow cytometry (Fig. 6H). DCF staining indicated that the levels of ROS were higher in the model group (Fig. 6I). Due to their oxidative properties, EGCG and warfarin lowered the levels of ROS. HIF-1α silencing appeared to increase the levels of ROS and indicated that HIF-1α may be over-expressed in the hypoxic thrombus environment. These results suggested that EGCG may be involved in the modulation of HIF-1α to protect HUVECs against oxidative stress.

EGCG and warfarin combination regulated HIF-1α and VEGF expression through the PI3K/AKT and ERK1/2 signaling pathways and attenuated cellular injury

To further explore the role of EGCG and warfarin in protecting cells against H2O2-induced damage, we detected the levels of HIF-1α protein and mRNA under the experimental conditions. According to immunofluorescence analysis, HIF-1α was up-regulated in cells exposed to oxidative stress (Fig. 7A). The addition of warfarin and EGCG (20 μmol·L−1) greatly reduced the levels of HIF-1α. Likewise, the addition of EGCG and warfarin reduced the levels of proteins associated with HIF-1α (VEGF, VEGFR-1 and ATGR-1) and phosphorylated proteins in related singaling pathways (P13K, Akt, P70S6K, and Erk1/2) in cells exposed to oxidative stress (Fig. 7B). Furthermore, the levels of VEGF and ATGR-1 mRNA were significantly higher in the model group and in the model + warfarin group. The levels of VEGF and ATGR-1 mRNA were significantly reduced in the model + warfarin + EGCG group, but began to increase when HIF-1α was inhibi-
These results indicated that the antioxidative effects of EGCG and warfarin combination on HUVECs are associated with the expression of HIF-1α and the activity of the PI3K/AKT and ERK1/2 signaling pathways.

**Discussion**

DVT is a major cause of mortality and morbidity worldwide and causes complications in hospitalized patients [28, 29].
Anticoagulants like heparin and warfarin are frequently used to treat DVT but may cause spontaneous bleeding. Therefore, it is essential to search for treatments with little adverse reactions. According to previous reports, EGCG interacted with Trp-213 at the drug binding site I via π-π stacking. Moreover, warfarin was found to bind to drug binding sites I, II, and III. In the current study, we investigated the role of EGCG as a complementary medication to improve anticoagulant properties in a rabbit model of DVT and in HUVECs exposed to H$_2$O$_2$.

A large number of studies have reported that the application of Chinese medicinal materials in alleviating DVT. For instance, the aqueous extract of *Whitmania pigra* was found to reduce the burden of DVT by alleviating inflammatory responses through the SIRT1/nuclear factor-kappa B (NF-κB) signaling pathway and antioxidation. Similarly, *Spatholobi Caulis*, a traditional Chinese herbal medicine used to treat blood-related illnesses, was found to ameliorate DVT by suppressing platelet aggregation and reducing inflammation. Furthermore, Danhong Huayu koufuye (DHK) prevented DVT in rats through inhibiting inflammation. Administration of DHK to rats with DVT significantly reduced the levels of neutrophils, lymphocytes, and matrix metalloproteinases-9, resulting in a significant reduction in the size of thrombi and vein wall thickness. EGCG has been found to have antioxidant and anti-inflammatory properties. EGCG was discovered to regulate the expression of HIF-1α and VEGF in age-related macular degeneration, inhibiting the progression of choroidal neovascularization. EGCG has also been successfully used as a complementary medication due to its antioxidant and apoptotic effects on cancer.

Fig. 7  EGCG and warfarin combination regulated HIF-1α and VEGF expression through the PI3K/AKT and ERK1/2 signaling pathways and attenuated cellular injury. (A) Immunofluorescence analysis of HIF-1α in different groups (bar = 20 μm). (B) Expression of HIF-1α, VEGF, VEGFR-1, AGTR-1, PI3K/AKT, and ERK1/2 proteins *in vitro* by Western blot, where GAPDH was used as the loading control. (C) Expression of AGTR-1 and VEGF mRNA in different groups by reverse transcription-quantitative polymerase chain reaction. Data are presented as the mean ± SEM (n = 5). *P < 0.05, the model group vs the control group; $^b$P < 0.05, the model + warfarin group vs the model group; $^c$P < 0.05, the model + warfarin + EGCG-H (20 μmol·L$^{-1}$) group vs the model + warfarin group; and $^d$P < 0.05, the model + warfarin + EGCG-H (20 μmol·L$^{-1}$) + si-HIF-1α group vs the model + warfarin + EGCG-H (20 μmol·L$^{-1}$) + si-NC group.
a complementary medication to prevent and resolve thrombus formation.

The antioxidant properties of EGCG and warfarin combination during DVT were also investigated by measuring the levels of HIF-1, VEGF, VEGFR1, and ATGR-1 in rabbits and HUVECs. DCF reagent was also used to measure ROS production. There was a dose-dependent decrease in the levels of HIF-1, VEGF, VEGFR1, and ATGR-1 in the model group in response to H2O2. Although HIF-1 and VEGF are known to be up-regulated in DVT, they may function independently in IVC. VEGF is an angiogenic growth factor that promotes epithelial cells growth and infiltrates the thrombus during resolution.[23] However, the levels of HIF-1α in the thrombus were lower than those in the IVC group, which suggested that HIF-1α may induce the expression of VEGF through interaction with VEGFR-1. Another study demonstrated that suppressing HIF-1 by rapamycin inhibited the expression of VEGF and reduced the levels of metastatic and angiogenic activities in cancer.[43] Similar results were obtained in our study, where HIF-1 suppression impeded the migration and invasion of hampered HUVECs while increasing ROS levels. When HIF-1 was inhibited, the levels of VEGF and AGTR-1 mRNA in HUVECs increased.

The PI3K/AKT and ERK1/2 signaling pathways are associated with DVT.[18, 42] Recent research suggests that thrombin activates the ERK1/2 pathway to initiate a VEGF-induced proangiogenic response.[43] Rumex acetosa’s antiplatelet action was reported to reduce thrombus formation in rats through modulating PI3K/AKT and ERK1/2.[44] Elevated ROS levels are known to activate platelets by disrupting the PI3K/AKT signaling pathway.[45] Our results demonstrate that the combination of EGCG and warfarin reduce the levels of HIF-1α and phosphorylated HIF-1α in multiple pathways, such as PI3K, Akt, P706SK, and Erk1/2, in HUVECs under oxidative stress.

**Conclusion**

In the current study, the combined use of EGCG and warfarin is explored using a rabbit DVT model through ligation of the IVC and using HUVECs exposed to oxidative stress. Together with warfarin, EGCG significantly reduces thrombus formation and protects HUVECs from oxidative stress and apoptosis. However, HIF-1α silencing reduces the advantages of combining EGCG and warfarin, up-regulates VEGF expression and activates the PI3K/AKT and ERK1/2 signaling pathways. Overall, EGCG combined with warfarin alleviates DVT by modulating the levels of HIF-1α and VEGF via the PI3K/AKT and ERK1/2 signaling pathways.

**References**


