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Baicalin promotes migration and angiogenesis of endothelial progenitor cells but impedes thrombus formation via SIRT1/NF-κB signaling in a rat model of deep vein thrombosis

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Summary. Background. Deep vein thrombosis (DVT) is the third most prevalent vascular disease worldwide, seriously threatening human health. Baicalin, a flavonoid isolated from the roots of *Scutellaria baicalensis*, has been identified to play a crucial role in various vascular diseases. The study aimed to explore the efficacy and underlying mechanisms of baicalin in DVT.

Methods. Endothelial progenitor cells (EPCs) were differentiated from peripheral blood mononuclear cells isolated from rat bone marrow. Dil-ac-LDL/FITC-UEA-1 double staining and flow cytometry analysis were conducted for the identification of EPCs. The angiogenesis and migration of EPCs *in vitro* were tested by a tube formation assay and Transwell assay, respectively. DVT rat models were established by stenosis of the inferior vena cava (IVC). After the euthanasia of rats, thrombi in the IVC were collected and weighed, and histological alterations in IVC tissue were measured by H&E staining. The protein levels of SIRT1, p-P65, and p65 in rat IVC tissues were quantified via western blotting.

Results. EPCs used in this study displayed a spindle-like shape and were positive for endothelial cell-specific markers, suggesting the phenotypic characteristics of EPCs. Baicalin enhanced the migratory and angiogenetic abilities of EPCs *in vitro*. For *in vivo* experiments, baicalin reduced thrombus weight and mitigated DVT formation in model rats. Moreover, baicalin activated SIRT but repressed NF-κB signaling in IVC tissues of DVT rats.

Conclusion. Baicalin facilitates migration and angiogenesis of EPCs but impedes thrombus formation via regulation of SIRT1/NF-κB signaling in DVT model rats.

Key words: Deep vein thrombosis, Baicalin, Endothelial progenitor cells, SIRT/NF-κB signaling, Angiogenesis

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Introduction

Deep vein thrombosis (DVT) refers to the formation of blood clots in deep veins, mainly in leg veins but can also occur in arms, cerebral veins, and mesenteric veins (Wang et al., 2023). DVT can be life-threatening when blood clots rupture and develop into pulmonary embolism, resulting in sudden cardiovascular failure and death (Boon et al., 2018). Endothelial progenitor cells (EPCs) can be isolated from the bone marrow and cord and peripheral blood (Peters, 2018). EPCs possess proliferative and migrative capabilities and can differentiate into endothelial cells to form new blood vessels (Zhang et al., 2020b). Recent articles illustrated that EPCs can be recruited into a thrombus to facilitate its resolution and thus influence pathological and physiological neovascularization (Zhang et al., 2020a; Ling et al., 2021). However, the number of EPCs and the capacity for angiogenesis can be impaired by many complex conditions in the microenvironment (Li et al., 2018). Thus, it is essential to find approaches to improve EPC recruitment and promote angiogenesis during the thrombus formation process.

Baicalin, a flavonoid active substance derived from the roots of *Scutellaria baicalensis* Georgi (Lamiaceae), possesses many biological properties, such as anti-inflammatory, anti-tumor, antioxidant, anti-apoptosis, neuroprotective, and anti-viral activities (Huang et al., 2019a). Baicalin was reported to promote the

angiogenesis of human umbilical vein endothelial cells and MRC-5 fibroblast cells (Zhang et al., 2011; Chen et al., 2019). Moreover, baicalin plays a critical role in coagulation, inhibition of platelet aggregation, and anti-thrombosis. For example, baicalin inhibited thrombin-catalyzed fibrin polymerization and platelet functions while prolonging the *in vivo* bleeding time, which indicates the antithrombotic activity of baicalin and its potential to be a new anticoagulant agent (Lee et al., 2015). Baicalin mitigates the thrombin-induced inflammatory response in vascular smooth muscle cells and protects human umbilical vein endothelial cells and SH-SY5Y cells from thrombin-induced cell injury (Ju et al., 2015; Zhang et al., 2019; Zheng et al., 2022). Baicalin inhibits platelet aggregation and exerts antithrombotic activity in ischemia/reperfusion-induced cerebral stroke (Liu et al., 2021). However, whether baicalin affects angiogenesis in EPCs and influences thrombus formation in DVT animal models remains unclarified.

SIRT1 is the most important member of Sirtuins and participates in the angiogenic process in many vascular diseases (Huang et al., 2019b; Dou et al., 2020; Liu et al., 2020). SIRT1 can repress the acetylation of nuclear factor-κB (NF-κB) p65 to reduce the release of proinflammatory factors (Kauppinen et al., 2013). Mounting evidence has demonstrated the involvement of SIRT1/NF-κB signaling in the pathogenesis of DVT (Tang et al., 2020). Importantly, baicalin was previously reported to upregulate SIRT1 expression and inhibit NF-κB signaling in a collagen-induced rat model of arthritis and rats with depression-like symptoms (Wang et al., 2014; Yu et al., 2019). Nonetheless, whether baicalin can modulate the SIRT1/NF-κB pathway in DVT remains unknown.

In this study, the role of baicalin in regulating EPC migration and angiogenesis was explored. Additionally, rat models of DVT induced by inferior vena cava (IVC) stenosis were established to investigate the effect of baicalin on thrombosis formation and its histo-pathological analysis. The current work might provide a novel antithrombotic component for DVT prevention and treatment.

Materials and methods

Isolation and culture of EPCs

Six Sprague Dawley rats (three weeks old, 80-100 g) were purchased from Vital River Technology (Beijing, China). Animal experiments were approved by the animal ethics committee of The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology. The rats were housed in an environment with a 12-hour (h) day/night cycle at 22-24°C and with free access to standard diet and tap water. Best efforts were made to minimize the suffering of rats. The rats were anesthetized and euthanized using an intraperitoneal injection of 3% pentobarbital sodium (3 g pentobarbital sodium dissolved in 100 ml of 0.9% saline) at a dose of 30 mg/kg. Then, rat bone marrow was collected from femurs and tibias. Mononuclear cells were harvested after density-gradient centrifugation with Ficoll-Paque (GE Healthcare, Buckinghamshire, UK). EPCs (1×10^6 cells/cm^2) were seeded in a culture flask and incubated with microvascular endothelial cell growth medium-2 (Lonza, Greenwood, USA) with penicillin-streptomycin and 5% CO_2 at 37°C. The culture medium was changed every two days. After cell culture for four days, the non-adherent cells were washed away. EPCs in passage 3 were utilized for the following experiments. The process was conducted as previously described (Du et al., 2019; Li et al., 2019; Yang et al., 2020).

Identification of EPCs

EPCs were identified by performing Dil-ac-LDL/FITC-UEA-1 double staining and detecting expression levels of cell surface markers, including cluster of differentiation (CD)31, CD133, von Willebrand factor (vWF), and VEGF receptor 2 (VEGFR2), using flow cytometry. For the double staining, EPCs (on day 7) were washed with phosphate-buffered saline (PBS; 0.1 M, pH=7.4; Bioswamp, Wuhan, China) in triplicate, followed by incubation with 20 μg/ml Dil-ac-LDL (ANGYUBIO, Shanghai, China) at 37°C for 4h without light exposure. Then, EPCs were treated with 4% paraformaldehyde for fixation and incubated with 10 μg/ml FITC-UEA-1 (Maokangbio, Shanghai, China) for 1h at room temperature in the dark. The binding of FITC-UEA-1 and incorporation of Dil-ac-LDL were evaluated, and the stained cells were observed under laser scanning confocal microscopy (Keyence, Osaka, Japan). For FITC-UEA-1, the excitation (Ex)/emission (Em) wavelength was 494 nm/520 nm. For Dil-ac-LDL, the Ex/Em wavelength was 549 nm/565nm.

For flow cytometry, EPCs (on day 7) were blocked with 2% fetal bovine serum for 10 minutes at 4°C. Then, cells were washed with PBS and incubated with primary antibodies (Biorbyt, UK) against fluorescein isothiocyanate (FITC)-conjugated CD31 (#orb15292), CD133 (#orb526485-CF594), vWF (#orb158717-CF594), VEGFR2 (#orb461490) for 30 minutes at 4°C. After that, flow cytometry (BD LSR Fortessa, Franklin Lakes, USA) and FlowJo software (Version 10.6.2; TreeStar, Ashland, USA) were used to analyze the expression of these surface antigens in EPCs. EPCs in passage 4 were used in the following experiments.

Cell treatment

EPCs were treated with baicalin (20 μM) for 12h, and cells in the control group were incubated with the same concentration of medium. The concentration of baicalin and treatment time was confirmed based on previous studies (Duan et al., 2021; Zhang et al., 2021).

Transwell assay

EPC migration was estimated using 8 μm Transwell inserts (Corning Inc., Corning, USA). EPCs (2×10^4) maintained in 200 μL of serum-free EBM-2 medium were seeded in the upper chamber. Then, 900 μL of EGM-2MV medium containing 10% fetal bovine saline was placed into the lower chamber. After 24h of incubation, cells on the top surface of the membrane were wiped off using a cotton swab, and cells on the lower surface were dyed with crystal violet (Solarb, China) for 15 min. Next, cells were washed with PBS three times and observed under a microscope (Olympus, Japan).

Tube formation assay

To determine the capacity of angiogenesis *in vitro*, EPCs (5×10^4 cells/well) were seeded in 24-well plates coated with Matrigel and cultured for 12h. The capillary network formation was detected using an inverted microscope (Logos Biosystem, Villeneuve d'Ascq, France), and ImageJ software was utilized to assess the extent of tube formation by measuring tube number and length. The quantity of the nodes in five fields (randomly selected) of every plate was examined.

Establishment of DVT rat models

A total of 40 age-matched Sprague-Dawley rats (eight weeks old, 250-300 g) were purchased from Vital River Animal Technology. All rats were kept in standard pathogen-free conditions (12h/12h light/dark cycle, 24±2°C) with free access to water and food. The experiments were performed in compliance with the Animal Ethics Committee of The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology.

The establishment of a DVT rat model induced by IVC stenosis was implemented as previously described (Sahu et al., 2017). Briefly, rats were anesthetized with an intraperitoneal injection of 3% pentobarbital sodium (36 mg/kg; Merck, Germany) and placed in the supine position. Afterward, a 2-cm incision was made along the abdominal midline and the intestines were exteriorized. The IVC just caudal to the left renal vein was partially ligated, along with ligation of side branches. To prevent bacterial infection after surgery, the wound was treated with ceftriaxone sodium (Lijian, China). The rats in the control group received the same surgical procedure without IVC stenosis.

At 24h post ligation, rats were euthanized and the IVCs were carefully resected. Thrombi were obtained and the weight measured.

Animal grouping and baicalin administration

To assess the impact of baicalin on thrombus formation in DVT *in vivo*, rats were divided into four groups (n=6 in each group): control, thrombotic, thrombotic + dimethyl sulfoxide (DMSO), and thrombotic + baicalin. Rats in the control group were fed with standard diet and received the same surgical procedure without IVC ligation. Rats in the thrombotic group received IVC stenosis surgery. Rats in the thrombotic + Baicalin group received 2 ml baicalin (80 mg/kg) (CAS NO. 21967-41-9, HPLC Purity ≥98%; Aladdin, Shanghai, China) via intragastric administration 1h after IVC stenosis surgery (Zhang et al., 2021). Rats in the thrombotic + DMSO group received an equal amount of DMSO (8%) in the same way. After modeling for 24h, rats were anesthetized via intraperitoneal injection of pentobarbital sodium. The IVCs were resected from rats in each group, and thrombi were extracted for weighing.

Hematoxylin and eosin (H&E) staining

After the rats were sacrificed, the IVCs of different groups were resected and fixed in 4% paraformaldehyde for 24h at 26°C. The fixed tissues were embedded in paraffin and then sectioned (4 μm thick). The sections were then dewaxed in xylene twice for 15 min, rehydrated in pure ethanol for 10 min, and then treated with a series of ethanol (95, 90, 80, and 70%) for 5 min each. The sections were then incubated with 3% hydrogen peroxide (diluted in methanol) for 10 min at 26°C in the dark to suppress endogenous peroxidase activity, followed by incubation with 3% bovine serum albumin for 30 min at 26°C to avoid any non-specific binding. Tissue sections were then stained with 0.1% hematoxylin for 10 minutes and with 0.5% eosin for 1 minute, dehydrated with ethanol, cleared with xylene, and mounted in neutral balsam. The pathological changes in the samples were observed employing an inverted microscope (Olympus, Japan).

Western blotting

The proteins from IVC tissues were lysed using RIPA lysis buffer (Beyotime, Shanghai, China) with protease inhibitor (Roche, Switzerland). Protein concentration was measured with a bicinchoninic acid assay kit (CWBIO, China). The total protein (35 μg) was separated on 10% SDS-PAGE and transferred onto a PVDF membrane (Millipore). The membrane was blocked with 5% bovine serum albumin for 1h and then incubated primary antibodies (Cell signaling, Danvers, USA) against SIRT1 (#9475, 1:1000), phospho-p65 (#3031, 1:1000), p65 (#8242, 1:1000), and GAPDH (#5174, 1:1000) overnight at 4°C. The membranes were washed in triplicate with Tris-buffered saline with Tween-20 for 5 minutes each and then incubated with corresponding HRP-labeled secondary antibodies for 1h at room temperature. After the reaction, band density was measured using Quantity One software (Bio-Rad Laboratories, Hercules, USA) after visualization with an enhanced chemiluminescence kit (Bio-Rad Laboratories).

Statistical analysis

SPSS 18.0 software (Statistical Package for the Social Sciences Inc., Armonk, USA) was used for the analysis of the statistics retrieved from three independent experiments. Data are displayed as mean ± standard deviation. Differences between two groups were compared using an independent sample *t*-test, and multigroup comparisons were analyzed using a one-way analysis of variance followed by Tukey's *post hoc* test. A *p*-value less than 0.05 was set as the threshold for statistical significance.

Results

Identification of EPCs

After the culture of isolated peripheral blood mononuclear cells (PBMCs) for seven days, the cells displayed a spindle-like shape and formed a central cluster (Fig. 1A). Subsequently, Dil-ac-LDL/FITC-UEA-1 double staining revealed that the isolated PBMCs uptake fluorescently labeled FITC-UEA-1 (green) and Dil-ac-LDL (red), and the double-positive cells represent EPCs (Fig. 1B). To further identify EPCs, flow cytometry was utilized to test the expression of surface antigens. As manifested in Figure 1C, endothelial cell surface marker CD31 (92.7%), vWF (96.0%), and VEGFR2 (96.1%) were overexpressed and progenitor cell surface marker CD133 (1.5%) was downregulated in EPCs. Since CD31, vWF, and VEGFR2 were positively expressed while CD133 was negatively expressed, the EPC phenotype was further identified.

Baicalin promotes the migratory and angiogenic capabilities of EPCs

EPCs can be recruited to the site of thrombosis to positively advance the vasculogenic process and thrombosis resolution (Kong et al., 2016). The chemical structure of baicalin is presented in Figure 2A. To test whether baicalin affects the migration and angiogenesis of EPCs, Transwell and tube formation assays were conducted, respectively. As denoted by Figure 2B,C, the migratory ability of EPCs was accelerated after baicalin treatment. Additionally, the administration of baicalin augmented the angiogenic ability of EPCs, as manifested by increased numbers of tube-like structures per field and relative total length per field in the baicalin-treated EPCs relative to angiogenesis in EPCs without baicalin treatment (Fig. 2D-F). Collectively, baicalin intensified EPC migration and angiogenesis *in vitro*.

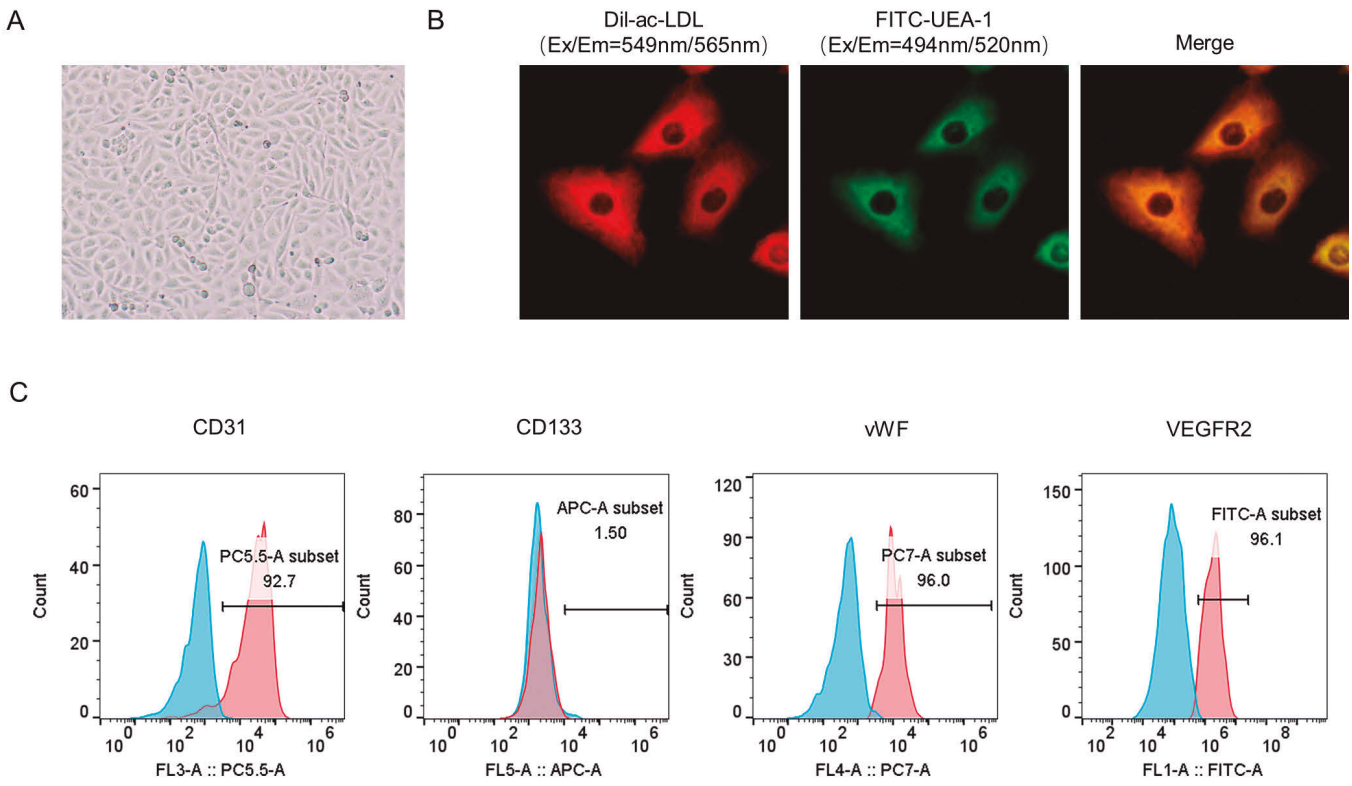

Fig. 1. Identification of EPCs. **A.** The morphology of EPCs was observed under a light microscope (200 μm, 100×). **B.** Dil-ac-LDL/FITC-UEA-1 double staining of EPCs was performed for cell identification. n=3. **C.** Flow cytometry was utilized to detect the expression of surface antigens (CD31, CD133, vWF, and VEGFR2) on EPCs. n=3.

Baicalin prevents thrombus formation in DVT rats

Next, the DVT rat models were constructed through IVC ligation, and the effect of baicalin on thrombus formation was explored. As Figure 3A illustrates, thrombi were formed in model rats, and the thrombus weight in DVT rats was markedly reduced by baicalin administration. Moreover, H&E staining was performed to analyze histopathological changes in deep veins after DVT. In the control group, the IVC lumen was normal without significant dilation and there was no substantial thrombosis in the lumen. Additionally, the intima and venous structures in the control group were intact without endothelial cell damage or necrosis. Around the vein wall of the control group, there was no inflammatory cell infiltration (Fig. 3B). There were many thrombi in the thrombotic group, staining dark red. The sites of venous thrombi were loose vascular wall tissues, red blood cell aggregation, thickening, and edema. Moreover, in the thrombotic group, inflammatory cell infiltration and irregular vascular intima were discovered around the vein wall (Fig. 3B). There were no significant changes between the thrombotic and thrombotic + DMSO groups. All the above-mentioned histopathological alterations induced by DVT were greatly improved upon baicalin administration. Thrombi in the thrombotic + baicalin group were far fewer than those in the thrombotic and thrombotic + DMSO groups (Fig. 3B). Immunohistochemistry was performed to measure the expression of endothelial cell marker CD34 in thrombi. As shown in Figure 3C, CD34 expression was upregulated in response to baicalin administration compared with that in the thrombotic and thrombotic + DMSO groups. The results suggested the abundant existence of EPCs in response to baicalin treatment, which is consistent with findings of *in vitro* experiments. Overall, baicalin exerts an antithrombotic activity in DVT rats.

Baicalin regulates SIRT1/NF-κB signaling in rat IVC tissues with thrombosis

Increasing evidence highlights the pivotal functions of SIRT1/NF-κB signaling in the pathophysiology of DVT (Yao et al., 2019; Tang et al., 2020, 2023). In this part, the protein expression of factors implicated in the SIRT1-NF-κB pathway was evaluated via western blotting. Compared to rats in the control group, thrombotic rats displayed a reduction in SIRT1 protein expression and an increase in the ratio of p-p65 to p65 in IVC tissues, denoting the downregulation of SIRT1 and activation of NF-κB signaling in DVT. These changes were recovered by baicalin administration, and SIRT1 and p-p65/p65 levels were close to those of the control (Fig. 4A-C). In summary, baicalin upregulates SIRT but inactivates the NF-κB pathway in rat IVC tissues

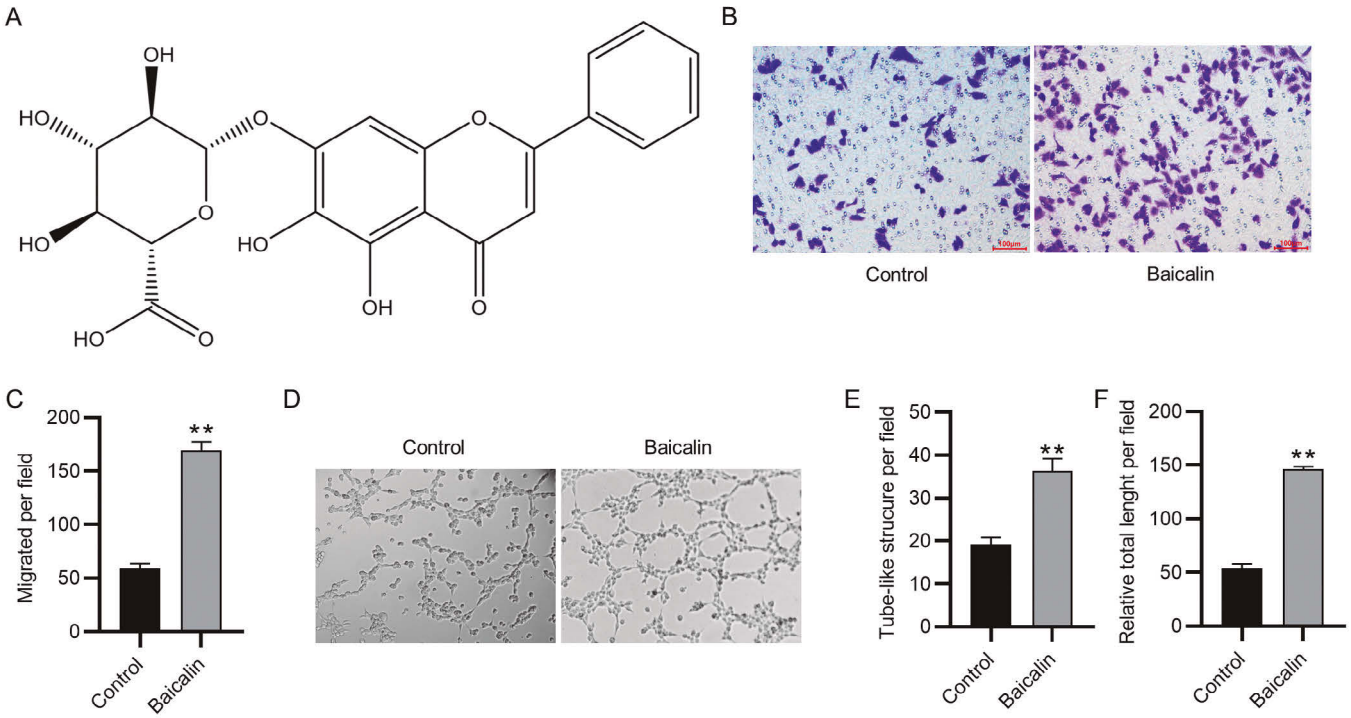

Fig. 2. Baicalin enhances the migration and angiogenesis of EPCs. **A.** The chemical structure of baicalin is provided. **B, C.** Transwell assays were performed to test the migratory capability of EPCs treated with or without baicalin. n=3. **D-F.** Tube formation assays were performed to test the angiogenic ability of EPCs in the presence or absence of baicalin. n=3. $^{**}p<0.01$.

containing thrombi.

Discussion

EPCs contribute to the recanalization of DVT, and RNAs or natural products enhancing EPC migration and angiogenesis have recently attracted attention (Yu et al., 2023; Lyu et al., 2024). The present study revealed that baicalin promoted rat EPC migration and angiogenesis *in vitro* and inhibited thrombosis in the rat model of DVT via SIRT1/NF-κB signaling. Compared with the previous article revealing that baicalin has relatively

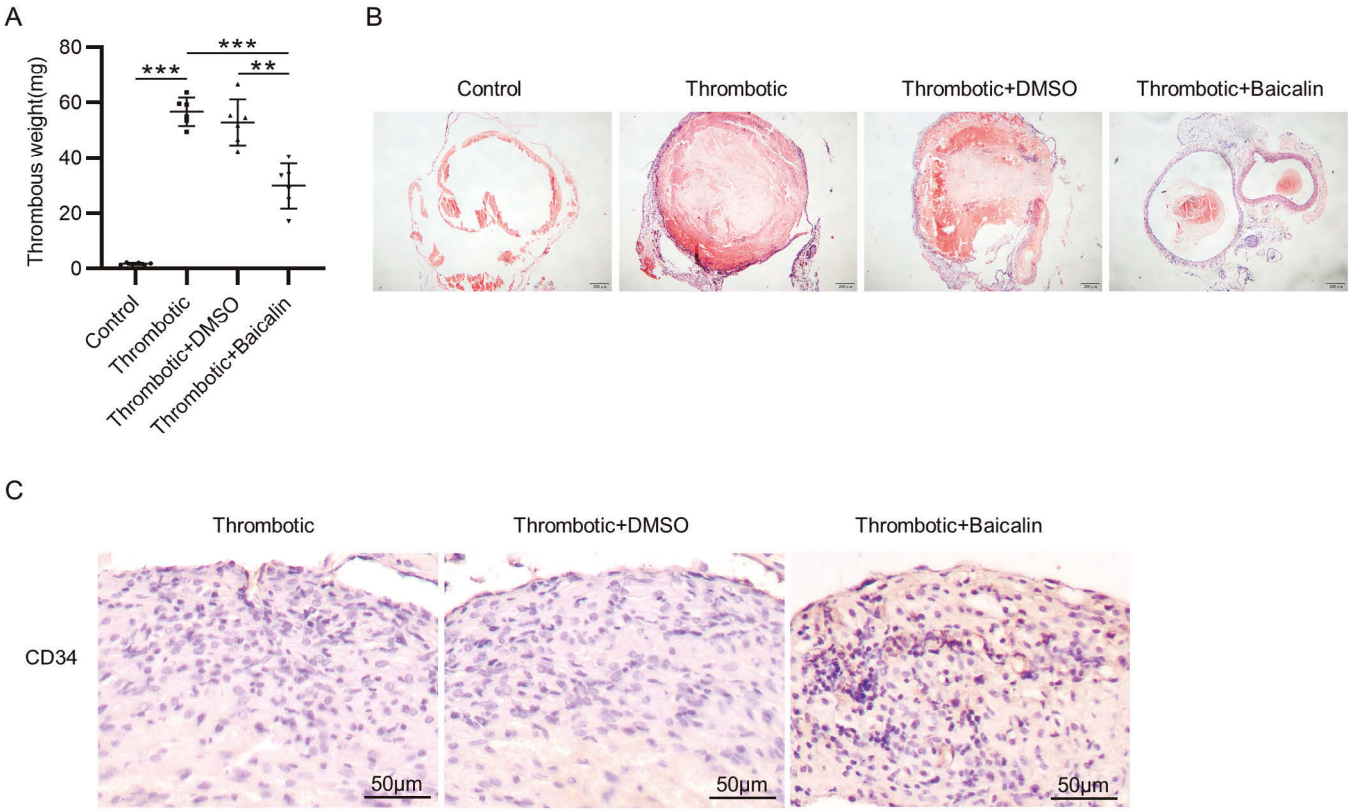

Fig. 3. Baicalin prevents thrombus formation in DVT rats. **A.** The thrombus weight of rats from the four groups was recorded. n=6. **B.** H&E staining of rat IVC tissues was performed to observe pathological changes. **C.** Immunohistochemistry was performed to measure the expression of endothelial cell marker CD34 in thrombi. n=6. ***p*<0.01, ****p*<0.001.

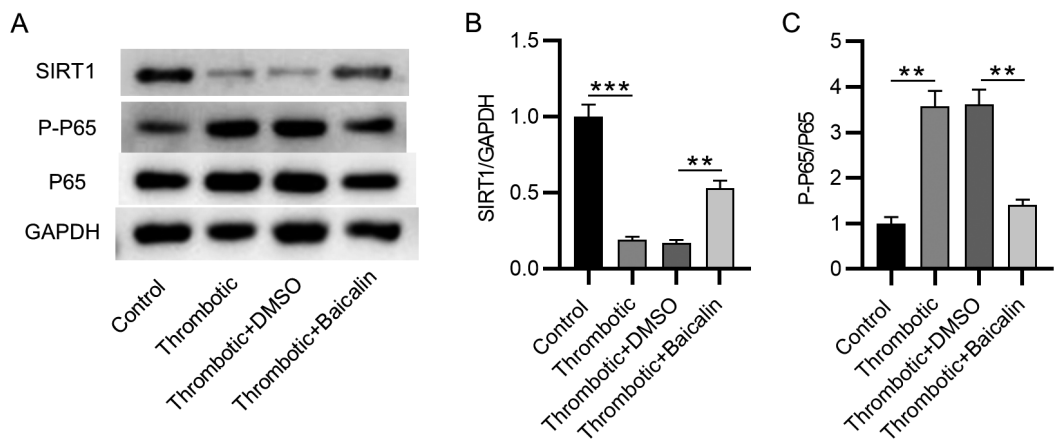

Fig. 4. Baicalin regulates the SIRT1/NF-κB pathway in rat IVC tissues with thrombosis. **A.** Western blotting was performed to measure protein levels of SIRT1, p-p65, and p65 in rat IVC tissues of the above-mentioned groups. n=6. **B.** The ratio of SIRT1/GAPDH or p-p65/p65 was quantified using Quantity One software. n=6. ***p*<0.01, ****p*<0.001.

high antithrombotic activity in ischemic brain injury (Liu et al., 2021), the current study not only verified its antithrombotic properties but also explained the underlying mechanism.

SIRT1 is a member of the Sirtuin family and affects various cell activities, such as metabolism, senescence, differentiation, inflammation, and antioxidant balance (Tang, 2016; Chen et al., 2020). The present study showed the downregulation of SIRT1 and the increase in the ratio of p-p65/p65 in thrombi of model rats without baicalin administration. Baicalin, in this study, exerted its antithrombotic activity by upregulating SIRT1 and reducing the phosphorylation of p65. These findings were consistent with conclusions from previous studies that revealed the involvement of the SIRT1/NF-κB pathway in DVT (Liu et al., 2019; Yao et al., 2020). Furthermore, SIRT1 was reported to restrain apoptosis of oxidative stress-stimulated EPCs (Wang et al., 2015) and reduce the replicative senescence of EPCs (Lamichane et al., 2019). The current work concluded that baicalin can promote EPC migration and angiogenesis and thereby hamper DVT progression via the SIRT1/NF-κB pathway, highlighting the role of EPCs in angiogenesis and vascular disorders via SIRT1 signaling, being consistent with previous articles (Wang et al., 2015; Lamichane et al., 2019).

Baicalin also has anti-inflammatory properties by regulating various signaling pathways or cell surface proteins. For example, baicalin mitigates lung inflammation induced by lipopolysaccharide (LPS) via inhibition of NF-κB and MAPK pathways (Shen et al., 2023). Baicalin activates Nrf2 and PI3K signaling pathways to ameliorate renal inflammation (Ning et al., 2023). Baicalin inhibits LPS-stimulated inflammation in macrophages and mice by inhibiting the expression of CD14, which is a cell surface protein found on many TLR4-expressing cells (Fu et al., 2021). The current work demonstrated that the expression of CD34 (endothelial cell marker) was reduced in rats with thrombi, and this downregulation of CD34 was rescued by baicalin. The findings indicated the protective effect of baicalin on EPCs by upregulating the endothelial cell marker, which is a novelty of the present study.

Overall, the current study validated the protective effects of baicalin against DVT. Baicalin accelerates the migration and angiogenesis of EPCs but prevents thrombus formation via regulating SIRT/NF-κB signaling in IVC stenosis-induced DVT rat models. The study may provide an effective ingredient for the management of DVT. However, there are several limitations in this study. Since the inflammatory response is closely linked to SIRT/NF-κB signaling and DVT etiology, the impact of baicalin on the inflammatory response in DVT should be a future research direction. Additionally, other regulatory molecules modulated by baicalin in DVT also deserve further investigation.

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Conflicts of interest. The authors declare no conflict of interest.

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