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Original Research Article

Schizonepeta tenuifolia Formula: Anti-hemorrhoid Activity via Anti-angiogenesis in Hemorrhoid Rats

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Sage

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Abstract

Background: Hemorrhoids are a prevalent condition characterized by anal vein dilation and prominent vascular hyperplasia, accompanied by symptoms such as rectal bleeding, itching, and discomfort. Currently, the conventional treatments possess limitations. The present research focuses on evaluating the anti-hemorrhoidal efficacy of Schizonepeta formula wash (SFW) and the underlying mechanisms.

Objectives: The present research aims to evaluate the anti-hemorrhoidal efficacy of Schizonepeta Formula Wash (SFW) and the underlying mechanisms.

Materials and Methods: Rat models of hemorrhoids were established by croton oil induction. The anti-hemorrhoid efficacy of SFW was investigated via histopathological experiments. The correlation between the anti-hemorrhoid activity and anti-angiogenic property of SFW was explored through immunofluorescence assays of vascular endothelial markers cluster of differentiation 31 (CD31) and von Willebrand factor (vWF). The impacts of SFW on the biological functions of human umbilical vein endothelial cells (HUVECs) were validated *in vitro* by employing cell counting kit-8 (CCK-8), transwell, and tube formation experiments.

Results: The efficacy of SFW treatment in reducing anorectal swelling, mucosal injury, tissue thickening, and anorectal swelling index (ASI) was comparable to that of Pilex in the hemorrhoid model. SFW treatment also improved histopathological features, including less vascular hyperplasia, restored epithelial and mucosal integrity, and reduced granulocyte and plasma cell infiltration. Mechanically, SFW treatment inhibited angiogenesis in hemorrhoidal rats, as manifested by the reduced expression of CD31 and vWF compared to the model group. The proliferation, migration, and tube formation of HUVEC were significantly inhibited by SFW *in vitro*, which suggested that SFW exerts its anti-hemorrhoidal activity by hindering angiogenesis.

Conclusion: This study investigated the efficacy of SFW treatment on hemorrhoids and found that its anti-hemorrhoidal activity was exerted through suppressing angiogenesis. Furthermore, this study provides a theoretical basis for developing alternative treatments for hemorrhoids.

Keywords

Hemorrhoids, traditional Chinese medicine, Schizonepeta formula wash, human umbilical vein endothelial cell, angiogenesis

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Introduction

Hemorrhoids, commonly known as "piles," are vascular anomalies characterized by dilated anal veins that lead to symptoms such as rectal bleeding, itching, and discomfort (Ray-Offor & Amadi, 2019). This condition is so common that up to 50% of adults will develop hemorrhoids at some point in their lives (Kibret et al., 2021). It is more common in adults, particularly those over the age of 40, where it is usually associated with a sedentary lifestyle, obesity, and

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straining during bowel movements (Kibret et al., 2021). The etiology of hemorrhoids is multifactorial, including increased intra-abdominal pressure, weakened anal sphincter muscles, and chronic constipation or diarrhea (Lohsiriwat, 2012). Such factors can lead to prolapse of the anal pads, which are the normal vascular structures that help close the anus. Current treatments for hemorrhoids range from conservative measures such as dietary modifications and increased fiber intake, as well as more invasive treatments with rubber band ligatures, infrared coagulation, and surgery (Cerato et al., 2014; Sun & Migaly, 2016). While these methods can be effective, they also have drawbacks. Invasive procedures carry risks of complications, including pain, infection, and recurrence, while conservative treatments may be insufficient for more severe cases. In this context, the exploration of herbal medicine offers a promising alternative. Traditional Chinese medicine (TCM) has a long history of using natural remedies to treat hemorrhoids. Such treatments are considered to have fewer side effects and provide a more holistic approach to treatment (Zhou et al., 2023).

Currently, there are several internationally recognized theories on the pathogenesis of hemorrhoids, including varicose vein theory, protrusion of anal cushions theory, vascular proliferation theory, bacterial infection theory, and pelvic floor dynamics theory, among which the vascular proliferation theory stands out as one of the most widely accepted (Lohsiriwat, 2012). It has been reported that hemorrhoids are intricately linked to angiogenesis, which is the formation of new blood vessels (Wang et al., 2019). For example, Chung et al. (2004) have indicated that hemorrhoidal tissue exhibited a greater microvascular density compared to that of normal anal and lower rectal tissues. Zhao et al. (2021) also found obvious vascular proliferation in hemorrhoidal tissues. Thus, excessive proliferation of blood vessels in the anal region is seen as a hallmark of hemorrhoidal development (Palumbo et al., 2023). Consequently, targeting this increased vascularization is crucial for the effective treatment of hemorrhoids.

From the perspective of TCM, the etiology and pathogenesis of hemorrhoids are primarily attributed to dampness, heat, and stasis (Zhang, 2017). The fundamental pathogenic mechanism is the stagnation of qi and blood in the meridians, which aligns with the general pathogenic mechanisms of surgical diseases characterized by "stagnation of qi and blood" and "ulcers and sores are caused by heat toxins" (Zhai, 2012). Based on these characteristics, the Department of Proctology at Shandong Provincial Hospital of TCM has developed a compound Schizonepeta fumigation wash (Jingjie Fang, SFW), which has the effects of activating blood circulation, relieving pain, detoxifying, and reducing swelling. The composition of the compound Schizonepeta fumigation wash mainly includes Schizonepeta, Saposhnikovia, Euphorbia, *Sophora flavescens*, Aconite, Cimicifuga, Ranunculus, Portulaca oleracea, Sappan wood, Honeysuckle, Forsythia, and Licorice, which can dispel wind, activate blood, remove dampness, and detoxify. Currently, this wash has been used to treat the initial symptoms of diseases such as mixed hemorrhoids, external hemorrhoids, and perianal abscesses, including local redness, swelling, heat, and pain. It has been applied clinically for many years with excellent therapeutic outcomes. We investigated the anti-hemorrhoidal activity of SFW and the underlying mechanisms in this study.

Materials and Methods

Animals

In the present study, rat models were established using croton oil-induced inflammation to mimic hemorrhoids, according to previous research (Yu et al., 2024). The study utilized healthy Sprague–Dawley (SD) rats, aged 8–10 weeks and weighing between 210 and 230 g. For the induction of hemorrhoids, a croton oil mixture was prepared by combining distilled water, pyridine, ether, and 6% croton oil in the ratio of 1:4:5:10, respectively. A cotton ball soaked with 0.16 mL of croton oil mixture was inserted into the anus of 6-week-old rats for 10 s. To explore the anti-hemorrhoid activity of SFW, the experiments were conducted with the following groups: (a) control group-no treatment; (b) Model group-hemorrhoids were induced using croton oil; (c) Model placebo group-orally administered 1% Tween 80 (HY-Y1891, MCE, Shanghai, China) at a dosage of 5 mL/kg once a day for 7 consecutive days; (d) SFW treatment group-rats were given a 10-minute sitz bath with 500 mL of SFW starting at 6 hours post-modeling, and this treatment was repeated daily for 7 consecutive days. (e) Pilex ointment treatment group (positive control, Himalaya, Bangalore, India)-6 h post-modeling, the ointment was applied topically to the anal area for 10 min, continued daily for 7 days. SFW was prepared uniformly by the Affiliated Hospital of Shandong University of Traditional Chinese Medicine.

Anorectal Swelling Index (ASI)

After euthanizing the animals, a 20 mm segment of the rectum was immediately excised starting from the anal verge, and the surrounding connective tissue was trimmed. The wet weight of the rectal segment was then measured. Subsequently, the samples were placed in an 80°C drying oven for 16 h to obtain the dry weight. The severity of rectal and anal swelling was represented by the rectal and ASI, which was calculated as follows:

ASI = (wet weight of the rectum − dry weight of the rectum)/wet weight of the rectum × 100%.

Hematoxylin–Eosin (HE) Staining

The tissue samples were initially fixed with 4% paraformaldehyde. Subsequently, the samples were then dehydrated and embedded in paraffin. Thin slices of 4 μm thickness were carefully cut with a paraffin slicer and adhered to glass slides.

After dewaxing, the ethanol gradient dehydration was carried out. Next, the tissue sections were immersed in a hematoxylin staining solution at room temperature for 5 min. Subsequently, 1% hydrochloric acid ethanol was applied for 30 s to facilitate differentiation. To restore the color to blue, light ammonia water was added and left for 1 min. The sections were then thoroughly rinsed in distilled water for 5 min.

Following this, the eosin staining solution was added at room temperature for 2 min. After that, the samples were rinsed in distilled water again for 2 min. Ethanol gradient decolorization was then performed. Subsequently, xylene was used for permeation for 2 min. Ultimately, the glass slides were sealed using neutral gum.

Immunofluorescence

Anorectal tissue specimens from rats were fixed and then subjected to routine paraffin embedding. Immunofluorescence assays on tissues were conducted using antibodies specific for cluster of differentiation 31 (CD31) (ab222783, Abcam, Shanghai, China) and von Willebrand factor (vWF) (ab287962, Abcam, Shanghai, China) with incubation overnight. Subsequently, secondary antibodies conjugated with Alexa Fluor® 488 or 594 (obtained from Abcam, Shanghai, China) were applied for 1 h, followed by DAPI nuclear staining. The samples were examined using an SP5 confocal laser scanning microscope (Leica) 24 h post-mounting.

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were obtained from Procell (Wuhan, China). HUVEC cells were propagated in a medium specifically designed for HUVEC. These cells were maintained at a temperature of 37°C with a 5% CO_2 atmosphere, and the culture medium was refreshed every 2 days.

Cell Counting Kit-8 (CCK-8) Assay

CCK-8 was applied to examine the optimal concentration of SFW for the treatment of HUVECs. First, to prepare the SFW extract, the medicinal herbs in the formula are wrapped in gauze, brewed with boiling water for 30 min, and then mixed with warm boiled water to make the total volume reach 2,000 mL. Subsequently, the mixture is filtered through four layers of gauze to remove the dregs. The filtrate is concentrated to one-tenth of its original volume under reduced pressure at 50°C using a rotary evaporator and then lyophilized to obtain a dry powder. When in use, the dry powder is formulated into the required concentrations of 5%, 10%, 20%, 40%, and 60% with the culture medium. About 100 μL of HUVEC cell suspension (containing 6,000 cells per well of the 96-well plate) was added to each well. The cells were cultured and subjected to SFW treatment with varying concentrations of SFW: 5%, 10%, 20%, 40%, and 60%, ensuring that the volume of the drug did not exceed one-tenth of the cell suspension volume. Following the treatment, 10 μL of CCK-8 solution (G4103, Servicebio, Wuhan, China) was added to each well. After the addition of CCK-8, the cells were incubated for an additional 4 h. The absorbance was then measured at 450 nm using a microplate reader.

Migration Assay

Transwell was used to examine the migration of HUVEC cells. First, a serum-free cell suspension was prepared, and the cell count was adjusted to 60,000 cells per well in a 24-well plate. The cells were then treated according to the experimental groups. About 200 μL of the cell suspension was added to the upper chamber, while 500 μL of culture medium containing 30% FBS was added to the lower chamber. The chambers were incubated in a 37°C incubator for 24 h. The inserts were then inverted onto absorbent paper to remove the medium, and any non-migrated cells in the upper chamber were gently wiped off with a cotton swab. The lower surface of the membrane was stained with crystal violet staining solution (CB0331, Sangon, Shanghai, China) for 15 min to visualize the migrated cells. Microscopic imaging was performed by randomly selecting fields of view in each transwell insert and capturing three photographs.

In Vitro Angiogenesis Assay

For the *in vitro* angiogenesis assay, 50 μL of Matrigel (1343001, Corning, Shanghai, China) was added to each well of a pre-cooled 96-well plate. Subsequently, the 96-well plate was placed in a 37°C incubator for 30–60 min to allow the matrix to solidify. HUVECs at a density of 3×10^4 cells per well were added to the 96-well plate. The 96-well plate was gently tapped to ensure even distribution of the cells. The cells were treated according to the experimental groups: a control group and a treatment group with SFW. Observations and photography were conducted during a 6-h cell culture period, with photographs taken under a microscope at regular intervals.

Statistical Analysis

Triplicates were conducted for the cell experiments, with results expressed as the mean ± standard error (SE) where specified. For statistical analysis, Student's *t*-test was employed to assess the significance of differences between two groups, while one-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test, was utilized for comparisons among multiple groups. A p value less than .05 was considered statistically significant, indicating notable differences between the groups under investigation.

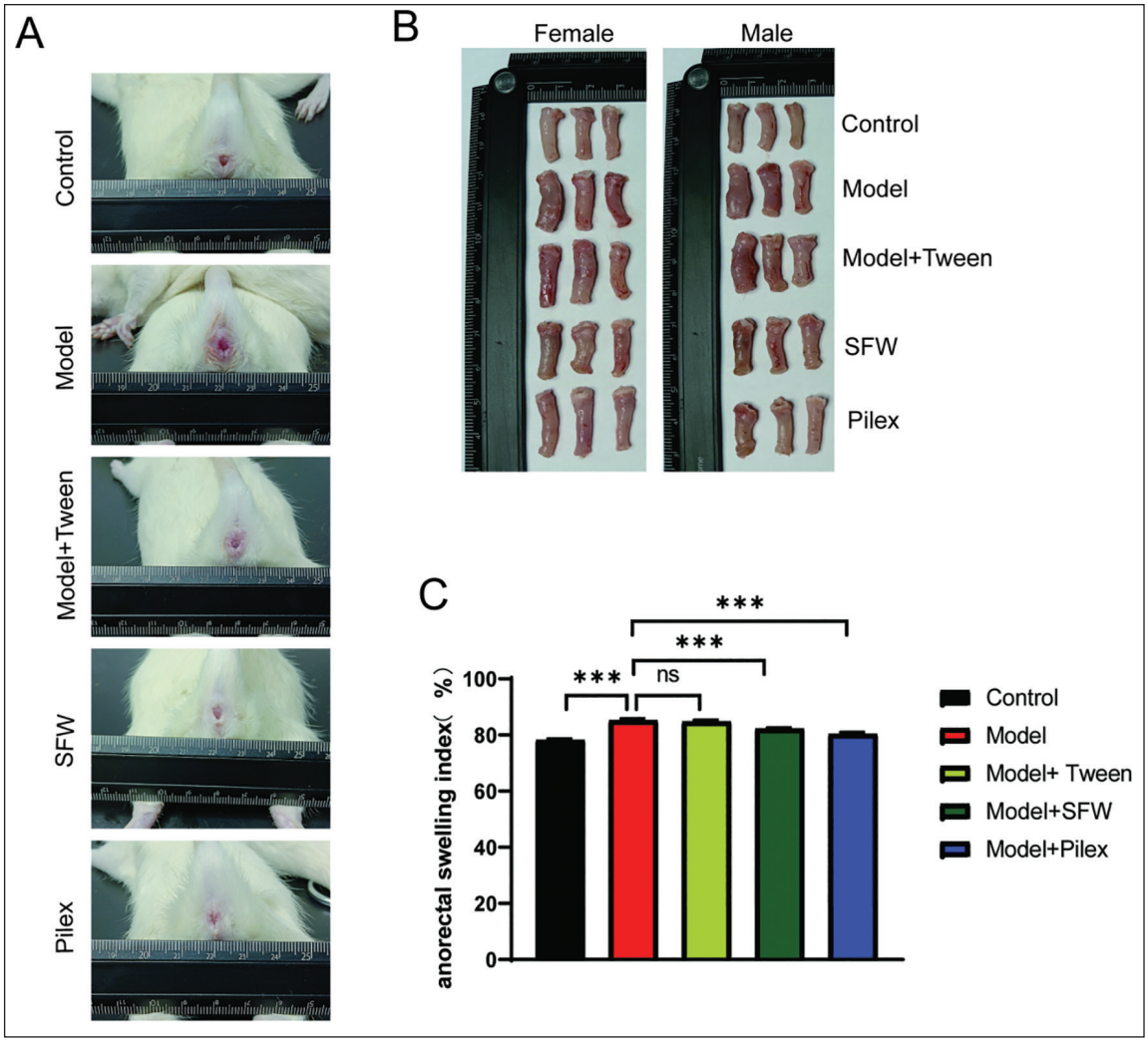


Figure 1. Schizonepeta Formula Wash (SFW) Decreased the Degree of Anorectal Swelling in Hemorrhoid Rats.

Notes: (A) Photos of rat anuses in different groups; (B) the anorectal tissues of rats in different groups; (C) the effects of SFW on anorectal swelling index (ASI). $n = 6$, ***$p < .001$, ns, not significant.

Results

Impacts of SFW on Anorectal Swelling

First, we utilized the degree of anorectal swelling to assess the severity of hemorrhoids. As displayed in Figure 1A and 1B, rats in the control group exhibited normal anorectal morphology, with no evidence of mucosal bleeding, edema, or ulceration. In contrast, rats in the model group displayed anorectal bleeding, edema, and a lightened coloration, with significant distension and deformation of the colon, and hyperplastic thickening of the rectum, which indicated impaired rectal and anal sphincter function. These findings confirmed the successful establishment of a hemorrhoid model in rats. However, after intervention with SFW, an obvious reduction in mucosal damage, a decrease in edema, and alleviation of tissue thickening were observed in the anorectal tissues of rats compared to the model group. Moreover, the data indicated that the therapeutic effect of SFW on hemorrhoids was comparable to that of Pilex.

Effects of SFW on ASI

Next, it was assessed for ASI in order to enhance the analysis, with higher index values indicating a greater degree of tissue inflammation and edema. As exhibited in Figure 1C, the ASI of the model group was higher than that of the control group. And there was no significant change between model + Tween and model groups (Figure 1C). Similar to the results in Figure 1A and 1B, after SFW and Pilex treatments, ASIs were obviously lower than those of the model + Tween group. The results indicated that SFW could obviously alleviate the swelling degree of hemorrhoids.

Influence of SFW on the Histopathological Characteristics of Anorectal Tissues

To assess the effect of SFW on histopathological changes within anorectal tissue, the anorectal specimens from rats were harvested and then processed for HE staining. Figure 2A–2E shows the integrity of the epithelial cell layer and the mucosal layer in the control group. In contrast, within the model group, the epithelial cell layer and the mucosal layer were markedly damaged with significant granulocyte and plasma cell infiltration (Figure 2A–2E). Moreover, conspicuous vascular proliferation was discernible, and the vascular lumens were congested with erythrocytes (Figure 2A–2E). Following the treatment with SFW or Pilex, a remarkable improvement in vascular hyperplasia was observed, and the epithelial tissue layer tended to be intact. The results indicated that SFW exhibited excellent anti-hemorrhoid activity.

Inhibition of Angiogenesis in Hemorrhoidal Rats by SFW Treatment

In order to verify the impact of SFW on vascular hyperplasia within hemorrhoid tissues, immunofluorescence assays were performed to detect the vascular endothelial markers CD31 and vWF in anorectal tissues. The results demonstrated that, in contrast to the control group, the expression of CD31 and vWF was markedly enhanced in the model group (Figure 3). However, the treatment with SFW or Pilex successfully reversed this trend (Figure 3). Consequently, the beneficial effect of SFW in the treatment of hemorrhoids might be achieved through the suppression of vascular hyperplasia within anorectal tissue.

Suppression of Function of HUVEC Cells by SFW Treatment

Given the above results indicating that SFW treatment alleviated hemorrhoids and the vascular proliferation, *in vitro* experiments were conducted to confirm whether SFW exerted its anti-hemorrhoidal activity by inhibiting angiogenesis. The effects of SFW on the function of HUVEC were examined. First, to prepare the SFW extract, SFW was pulverized and subjected to boiling water extraction, followed by filtration and concentration under reduced pressure, and finally sterile filtration. Initially, the CCK-8 assay was employed to explore the optimal concentration of SFW for the treatment of HUVECs (Figure 4A). Specifically, the concentrated SFW solution was used to formulate solutions at concentrations of 5%, 10%, 20%, 40%, and 60%, respectively. According to the data presented in Figure 4A, the inhibition rate of 20% SFW on HUVECs was around 0.7. Hence, 20% SFW was chosen for the subsequent cellular experiments. Transwell migration assay revealed that SFW obviously inhibited the migration of HUVEC cells (Figure 4B). In addition, the tube formation assay was utilized to examine the effects of SFW on angiogenesis, as Figure 4C displays that SFW obviously hinders the formation of lumens and branches. Therefore, SFW exerted its anti-hemorrhoidal activity by inhibiting the function of HUVECs.

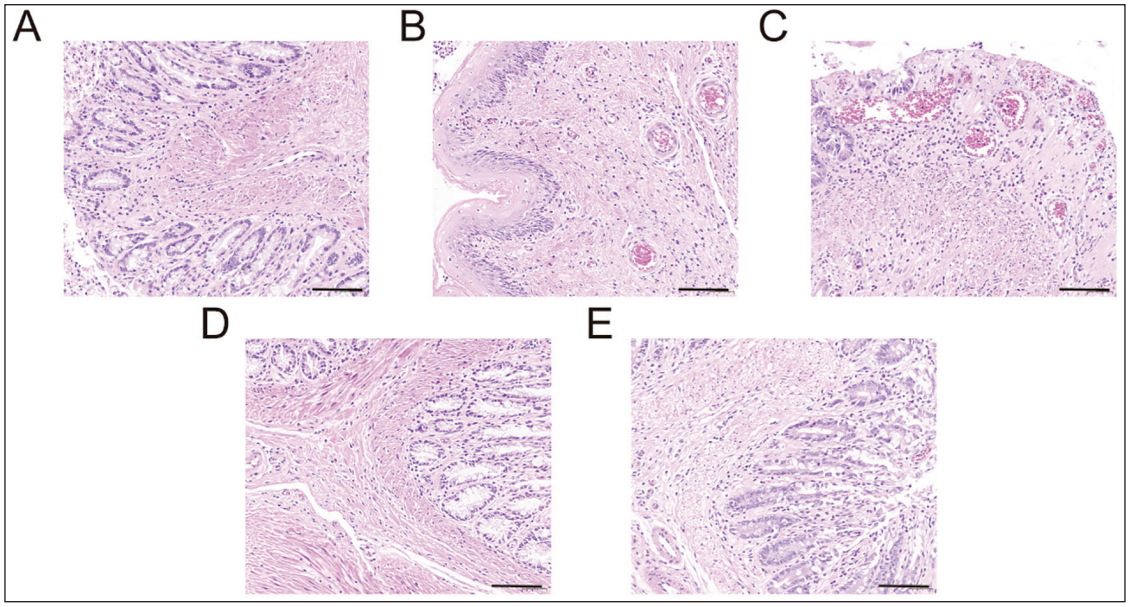


Figure 2. Hematoxylin–Eosin (HE) Staining Assays Were Utilized to Examine the Influence of Schizonepeta Formula Wash (SFW) on the Histopathological Characteristics of Anorectal Tissues.

Note: Scale bar: 100 μm.

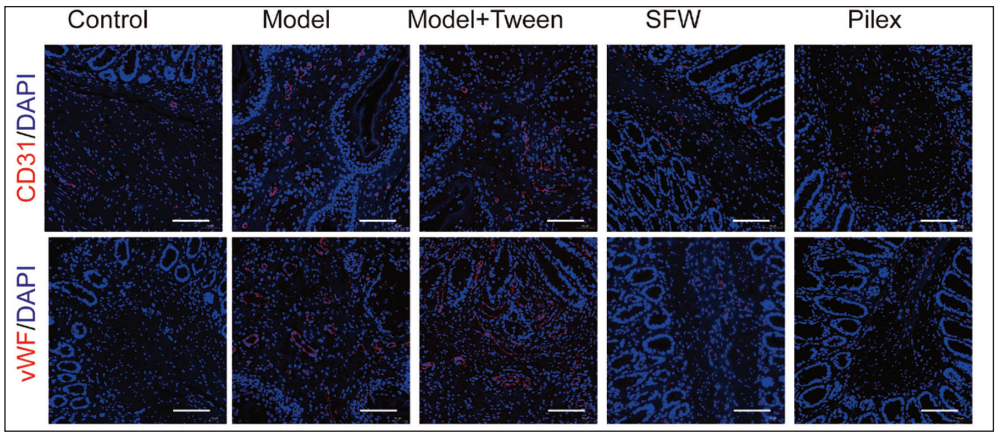


Figure 3. Immunofluorescence Assays Were Performed to Detect the Vascular Endothelial Markers Cluster of Differentiation 31 (CD31) and von Willebrand factor (vWF) in Anorectal Tissues in Different Groups.

Note: Scale bar: 100 μm.

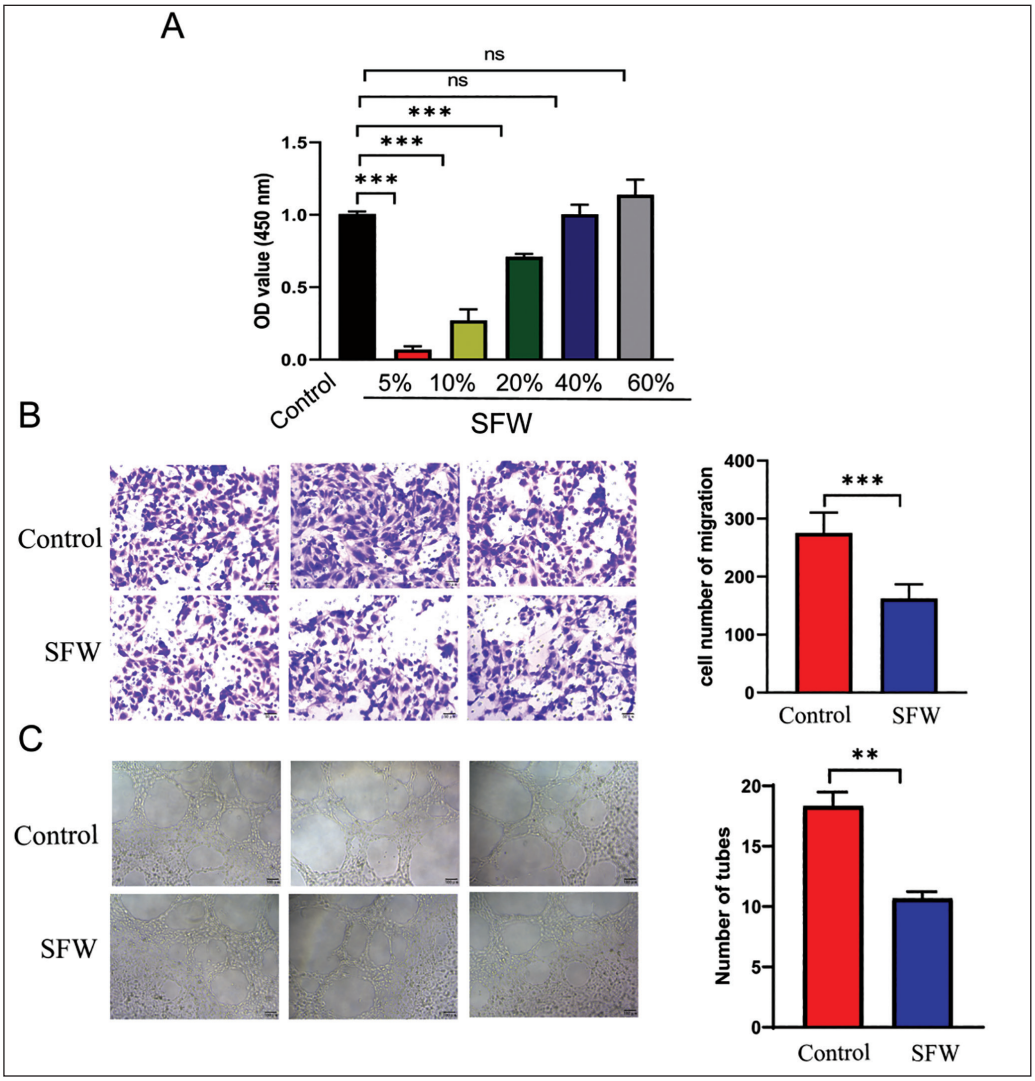


Figure 4. Schizonepeta Formula Wash (SFW) Exerted Its Anti-hemorrhoidal Activity by Inhibiting Angiogenesis *In Vitro*.

Note: (A) Cell counting kit-8 (CCK-8) assays were applied to investigate the optimal concentration of SFW for the treatment of human umbilical vein endothelial cells (HUVECs); (B) transwell migration assay was applied to explore the effects of SFW on the migration of HUVECs; (C) tube formation assay was applied to explore the effects of SFW on the angiogenesis of HUVECs.

Discussion

Currently, the frequently employed animal models for hemorrhoids primarily consist of the hemorrhoid model induced by croton oil mixture, acetic acid injection, carrageenan, or surgical ligation of the rectal vein (Azeemuddin et al., 2014; Ke et al., 2023). All these models are designed to mimic the pathological characteristics. Among the aforementioned procedures, the most commonly adopted approach is to locally stimulate the rectal mucosa using inflammatory agents. Croton oil, as a commonly utilized inflammatory agent, is capable of stimulating tissues to release leukotrienes, bradykinin, and prostaglandins, thereby triggering tissue inflammation (Ke et al., 2023; Mao et al., 2024; Pegoraro et al., 2021). Consequently, the croton oil-induced hemorrhoid model was adopted in the present study. The swelling degree of anorectal tissue and HE staining results displayed the successful construction of rat model of hemorrhoids. Pilex is a well-known ointment, which is commonly used in the treatment of hemorrhoids (Azeemuddin et al., 2014; Dhiman et al., 2023). In the present research, SFW demonstrated a curative effect on hemorrhoids, which was substantially similar to that of Pilex. This may be the result of the combined effect of the active ingredients in SFW. For example, its main components, such as *Schizonepeta tenuifolia, Saposhnikovia divaricata*, and *S. flavescens*, possess potent anti-inflammatory, analgesic, and antioxidant effects and have already been used in the treatment of various inflammatory diseases (Jeon et al., 2019; Li et al., 2020; Yang et al., 2020; Zhang et al., 2020).

Hemorrhoids are characterized by distinct pathological features, such as vascular hyperplasia and dilation, inflammatory response, tissue fibrosis, and changes in mucosal and epithelial tissues, with vascular hyperplasia being a prominent aspect (Loder et al., 1994; Romano et al., 2021). Microscopically, in the affected anorectal tissues, there is a significant increase in the number and size of blood vessels (Aigner et al., 2006). The endothelial cells lining these vessels show signs of active proliferation, leading to the formation of a denser vascular network (Díaz-Flores et al., 2018). This abnormal vascular growth disrupts the normal tissue architecture and homeostasis. The hyperplastic blood vessels often exhibit enlarged lumens and thickened walls. The increased vascularity not only contributes to the characteristic swelling and protrusion of hemorrhoidal tissues but also alters the local microcirculation (Chung et al., 2004). This can result in congestion and stasis of blood within the affected area, further exacerbating the pathological condition. Additionally, the presence of a large number of proliferating blood vessels is associated with enhanced permeability, which may lead to the leakage of plasma proteins and subsequent edema formation (Han et al., 2005). Such vascular changes are accompanied by an inflammatory response, with the infiltration of various immune cells such as granulocytes and lymphocytes, which further modulate the progression of the disease and the overall pathological phenotype of hemorrhoids (Han et al., 2005). In the present study, both the HE staining and immunofluorescence results demonstrated a significant augmentation of angiogenesis within the model group, while the treatment with SFW effectively reversed this abnormal phenomenon. Moreover, *in vitro* experiments revealed that SFW obviously suppressed the proliferation, migration, and tube formation of HUVEC cells, indicating the anti-angiogenic activity of SFW. Zhang et al. (2013) have demonstrated that *S. flavescens*, one of the main components of SFW, exerts anti-angiogenic activity in tumor.

From the TCM perspective, hemorrhoid pathogenesis relates to "dampness, heat, and stasis." SFW's "activating blood circulation" effect links to its anti-angiogenic and anti-inflammatory properties in this study. SFW may enhance anorectal microcirculation and reduce stasis by promoting blood circulation. This inhibits excessive blood vessel proliferation. In addition, SFW's anti-inflammatory and detoxifying effects also mitigate "dampness-heat"-related inflammation, improving hemorrhoid symptoms.

Although this study confirmed the efficacy of SFW in rat hemorrhoid models, several limitations exist. Human dosing cannot be directly extrapolated from rat experiments and requires further research. The bioavailability of its active components in humans remains unknown, and the formulation stability also needs attention. Future studies should address these issues to promote clinical application.

Conclusion

Within this research, a rat model of hemorrhoids was constructed via croton oil induction. The anti-hemorrhoid activity of *S. tenuifolia* formula was identified, and such activity was manifested through its anti-angiogenic function. This study provides new ideas for the research of hemorrhoid pathophysiology, facilitating the discovery of new biomarkers and therapeutic targets. Clinically, it paves a new way for hemorrhoid treatment, increasing treatment options. It also promotes the research and development of traditional Chinese medicine, enhancing the integration of traditional and modern medicine and facilitating the internationalization of traditional Chinese medicine.

Abbreviations

ASI: Anorectal swelling index; CCK-8: Cell counting kit-8; HE: Hematoxylin–eosin; HUVECs: Human umbilical vein endothelial cells; SE: Standard error; SFW: Schizonepeta formula wash; TCM: Traditional Chinese medicine.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

The animal experiments were approved by the Animal Ethics Committee of the Affiliated Hospital of Shandong University of Traditional Chinese Medicine (No. SDSZYYAWE20240408002).

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Informed Consent

Not applicable.

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