Original Article

Sparganium suppresses invasion of intestinal microvascular endothelial cells and angiogenesis induced by activated platelets

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Abstract: Objective: The aim of the current study was to investigate the effects of sparganium tablets on intestinal microvascular endothelial cells (INMECs) invasion and angiogenesis induced by activated platelets. Methods: Sparganium tablets were collected from rats. Toxicity levels of the serum which contained sparganium were measured by MTT assays, guiding the choice of suitable treatment concentrations. Isolated INMECs were divided into five groups after treatment: Normal Control (NC), Platelets, Platelets + Low 3.125 μl/mL, Platelets + Middle 6.25 μl/mL, and Platelets + High 12.5 μl/mL. Vascular endothelial growth factor (VEGF) concentrations of each group were measured using enzyme-linked immunosorbent assays. The branch point number was measured by capillary tube formation experimentation. The invasion cell number was evaluated by Transwell assays. Relative protein expression levels were measured using Western blotting and immunofluorescence assays. Results: The drug sera significantly downregulated VEGF concentrations, suppressed vascular lumen formation, and inhibited cell invasion (P < 0.05), in a dose-dependent manner. Additionally, collagen I, α-SMA, E-cadherin, p-PI3K, p-AKT, p-mTOR, and HIF-1α serum protein expression levels were significantly downregulated, in a dose-dependent manner, after sparganium treatment (P < 0.05). Activated platelets stimulated HIF-1α nuclear translocation, while sparganium treatment demonstrated opposite effects. Conclusion: Sarganium tablets suppress INMECs invasion and angiogenesis induced by activated platelets via inhibiting activation of mTOR/PI3K pathways.

Keywords: Sparganium pill, INMECs, invasion, angiogenesis

Introduction

Incidence levels of inflammatory bowel disease in China have increased three-fold. The number of patients with Crohn’s disease (CD) has especially increased in this century. However, this disease lacks specific treatment measures. It has been designated as a modern refractory disease by the World Health Organization. Intestinal fibrosis and stenosis caused by intestinal inflammation is a common complication of CD. Patients often need surgical intervention, but recurrence rates are higher [1, 2]. This disease is a chronic disease with recurrent attacks. In developed countries, such as Europe and America, incidence of this disease remains high. In China, incidence of CD is expected to continue increasing yearly [3, 4]. This trend indicates that this disease has become a serious threat in urgent need of solutions. Over 33% of CD cases have been found to cause recurrent intestinal stenosis due to fibrosis [5]. At present, modern medicine still lacks definite and effective prevention and treatment methods for intestinal fibrosis. Thus, there is an urgent need for researchers to better understand the mechanisms of intestinal fibrosis in CD, developing effective drugs with low toxicity.

Chinese Medicine has unique advantages in the treatment of CD, showing good therapeutic effects. Previous studies have revealed that Rhizoma sparganii and Radices zedoariae depress fibrosis via regulation of the epithelial-
mesenchymal transition (EMT) [6, 7]. These two herbs are the two primary components of sparganium pills used in the current study. The Traditional Chinese Medicine theory suggests that complementary treatment of diseases is better than treatment with a single prescription or component. Based on this theory and previous studies, it was inferred that sparganium tablets could improve CD via regulation of EMT in vivo.

Materials and methods

Experimental materials

Sprague-Dawley male rats (SPF level, body weight 200-220 g) were purchased from Nanjing Medical University Animal Center. RPM 11650 and fetal bovine serum (FBS) were obtained from Gibco (USA). Activated platelets, the MTT Kit, and total protein and nuclear protein extraction kits were purchased from Sigma Aldrich (USA). Vascular endothelial growth factor (VEGF) enzyme-linked immunosorbent assay (ELISA) kit, anti-α-SMA, anti-Collagen I, anti-E-cadherin, anti-VEGFR, anti-Pi3K, anti-p-Pi3K, anti-AKT, anti-p-AKT, anti-mTOR, anti-p-mTOR, anti-HIF-1α, anti-LamB1, and anti-GAPDH were purchased from Abcam (UK). Immunofluorescence anti-HIF-1α was purchased from Cell Signaling Technology (USA). Hizoma sparganii, Radices zedoariae, Chinese atractyloides, and roasted Atractylodes macrocephala were purchased from Sichuan Traditional Chinese Medicine Beverage Co., Ltd. (China).

Serum preparation

Prescription drugs and corresponding doses used in this study included Hizoma sparganii (10 g), Radices zedoariae (10 g), Chinese atractyloides (10 g), and roasted Atractylodes macrocephala (10 g). The above herbs were added to 200 mL of boiling water. They were sterilized and inactivated. The resulting extract (SLW) was stored at -20°C until use. The rats were fed SLW solution for 7 days. Blood serum was then collected from the rats for sterilization and inactivation. Serum samples were stored at -20°C for future use.

INMECs isolation and culturing [8]

Based on previous methods, intestinal microvascular endothelial cells (INMECs) were isolated from the rats. After the fourth or fifth generation, the INMECs were cultured in RPM 11650 culture medium with 10% FBS. These INMECs were used in subsequent studies.

Cell proliferation rates using MTT assays

During the logarithmic growth period, INMECs were cultured in 96-well plates until cell adherence. INMECs were, respectively, added with 1.5625 μl/mL, 3.125 μl/mL, 6.25 μl/mL, 12.5 μl/mL, 25 μl/mL, 50 μl/mL, and 100 μl/mL serum containing SLW for 4 hours of culturing. The supernatant was removed, 200 μl dimethyl sulfoxide was added to each well, and samples were subjected to oscillation at a low speed on the rocking bed for 10 minutes. Absorbance values were measured at 490 nm. Measurement of cell proliferation was also conducted, choosing suitable drug concentrations.

INMECs grouping

Using MTT assays, safe drug concentrations of 3.125 μl/mL, 6.25 μl/mL, and 12.5 μl/mL were chosen and labeled as low-dose (L), middle-dose (M), and high-dose (H), respectively. INMECs were randomly divided into five groups: Control, Platelets, and the three SLW-treated groups (L, M, and H). INMECs of different groups were cultured as 1 × 10^4 cells/well for 4 hours. Culture fluid samples of the different groups were collected after centrifugation, measuring VEGF concentrations. INMECs were then collected for subsequent experiments.

ELISA assays

After collecting the culture fluid of five groups, the samples were centrifuged at 1,000 rpm for 5 minutes at 4°C. VEGF concentrations of each sample were measured using an ELISA kit, with an absorbance of 490 nm, according to manufacturer protocol. Each experiment was repeated nine times.

Capillary tube formation assays

INMECs were injected into the 48-well culture plates at 1 × 10^5 cells/well. Matrigel matrix glue was added into each well for culturing for 30 minutes at 37°C. INMECs for each group were treated under the following conditions: (1) Control group was treated with normal serum; (2) Platelets group was treated with normal serum that contained activated platelets; (3) Low-dose SLW-treated (Platelets + SLW-L) group was treated with 3.125 μl/mL SLW based
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Modified Eagle Medium (DMEM) and diluted Matrigel (Matrigel: DMEM, 1:4, v/v). The upper chamber remained in the incubator (37°C, 5% CO₂) for 30 minutes until freezing. In the upper chamber, 100-μl suspensions of logarithmic growth period INMECs were injected, along with 600 μl serum-free DMEM. Each group had three wells. INMECs of the Control group were added to the normal culture medium. INMECs of the Platelet group were added to activate platelets. INMECs of the Platelets + SLW-L group were added to 3.125 μl/mL SLW + activated platelets. INMECs of the Platelets + SLW-M group were added to 6.25 μl/mL SLW + activated platelets. INMECs of Platelets + SLW-L group were added to 12.5 μl/mL SLW + activated platelets. The different groups were cultured for 24 hours. Afterward, the chamber was taken out and the medium was discarded. The cells were washed with phosphate-buffered saline twice. With tweezers, the filter membrane was removed and placed on the slide plate. Five fields were randomly selected under an inverted microscope, observing and counting the number of migratory cells.

Western blot assays

INMECs of the five groups were collected. Total proteins or nuclear proteins were extracted using appropriate kits, according to manufacturer instructions. Protein concentrations were measured using the bicinchoninic acid method. A 50-μg protein sample was used for electrophoresis using 12% SAD-PAGE, membrane, sealing, film washing, adding primary antibodies (α-SMA 1:1000; collagen I 1:1000; E-cadherin 1:2000; VEGFR 1:1000; PI3K 1:1000; p-PI3K 1:1000; AKT 1:1000; p-AKT 1:1000; mTOR 1:1000; p-mTOR 1:1000; HIF-1α 1:1000; LamB1 1:2000; and GAPDH 1:500) for culturing at 4°C overnight. They were washed three times with PBS, adding horseradish peroxidase-marked second antibody (1:2000) for culturing for 1 hour. Next, electrochemiluminescence liquid was added for coloring. Relative gray values were analyzed by Image J software (GAPDH or LamB1 were used as reference controls in this experiment).

Immunofluorescence staining

The cells migrated into the orifice cells. Corresponding drugs were added at 12 hours.
Next, PBS washing was conducted and cells were fixed with 2% polyoxymethylene. The cells were then washed again with PBS. They were permeated by 1% TritonX-100, using 5% bovine serum albumin to close, adding the HIF-1α for culturing at 4°C overnight. They were washed again with PBS, adding fluorescein isothiocyanate (FITC) remarked fluorescent second antibody for culturing at room temperature for 2 hours. After using DAPI to stain the nucleus, buffered glycerol was used to close. Nucleation of HIF-1α was then observed under a laser confocal microscope for 1 hour.

Statistical analysis

Experimental data are presented as mean ± standard deviation. SPSS 22.0 software was used to analyze the data with one-way ANOVA and post-hoc Dunnett’s t-test to determine differences between experimental groups. P-values less than 0.05 indicate statistical significance.

Results

Different SLW concentrations affect INMECs cell proliferation

According to results of MTT assays, no significant differences were found between the 0 µl/mL, 1.5625 µl/mL, 3.125 µl/mL, 6.25 µl/mL, and 12.5 µl/mL SLW concentration groups (P > 0.05). However, INMECs proliferation rates of the 25 µl/mL, 50 µl/mL, and 100 µl/mL SLW concentrations groups were significantly lower than that of the 0 µl/mL SLW concentration group (P < 0.01). Results indicate that 0 µl/mL, 1.5625 µl/mL, 3.125 µl/mL, 6.25 µl/mL, and 12.5 µl/mL SLW concentrations were safe concentrations for INMECs in vitro. Relative data are shown in Figure 1.

Measuring VEGF concentrations using ELISA assays

VEGF concentrations in the Platelets group were significantly higher than those of the Control group. However, with SLW treatment, VEGF concentrations of the Platelets + SLW-M and Platelets + SLW-H groups were significantly lower than those of the Platelets group (P < 0.05 and P < 0.001). Relative data are shown in Figure 2.

Branch point numbers

The branch point number of the Platelets group was significantly higher than that of the Control group, indicating that activated platelets promoted INMECs to angiogenesis. How-
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However, branch point numbers of SLW-treated groups were significantly lower than those of the Platelets group (P < 0.05), with SLW concentrations that were dose-dependent (P < 0.05). Relative data are shown in Figure 3.

**Figure 5.** α-SMA, Collage I, E-cadherin, and VEGFR protein expression of different groups. #: P < 0.05, compared with Control (untreated) group; *: P < 0.05, compared with Platelets group; **: P < 0.05, compared with SLW-L treated group; ***: P < 0.05, compared with SLW-M treated group.

**Evaluation of migratory cell numbers**

According to results of Transwell assays, the number of invaded INMECs in the Platelets + SLW-M and Platelets + SLW-H groups were sig-
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Significantly lower than that in the Platelets group (P < 0.05 or P < 0.01). Relative data are shown in Figure 4.

Protein expression levels of α-SMA, collagen I, E-cadherin, and VEGFR

According to Western blot assays, protein expression levels of α-SMA, collagen I, and E-cadherin were significantly higher in the Platelets group than the Control group (P < 0.05). With SLW treatment, protein expression levels of α-SMA, collagen I, and E-cadherin were significantly lower, in a dose-dependent manner, than those of the Platelets group without SWL treatment (P < 0.05, P < 0.01, and P < 0.001, respectively). However, no significant differences in VEGFR protein expression levels were found between the five groups. Relative data are shown in Figure 5.

PI3K/AKT/mTOR pathways and HIF-1α protein expression levels

According to results of Western blot assays, no significant differences in protein expression levels of PI3K, AKT, and mTOR were found among Control, Platelets, Platelets + SLW-L, Platelets + SLW-M, and Platelets + SLW-H groups (P > 0.05). However, protein expression levels of p-PI3K, p-AKT, p-mTOR, and HIF-1α in the Platelets group were significantly upregulated, compared with those of the Control group (P < 0.05). With SLW added, protein expression of p-PI3K, p-AKT, p-mTOR and HIF-1α in the SLW-treated groups was significantly suppressed.
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Compared with that of the Platelets group, in a dose-dependent manner (P < 0.05). Relative data are shown in Figure 6.

**Nuclear HIF-1α and LamB1 protein expression levels**

Compared with those in the Control group, HIF-1α protein expression levels in the Platelets group were significantly upregulated in the nucleus (P < 0.05). HIF-1α protein expression levels in SLW-treated groups were significantly lower than those in the Platelets group, in a dose-dependent manner (P < 0.05), in the Platelets + SLW-L, Platelets + SLW-M, and Platelets + SLW-H groups. No significant differences in GAPDH protein expression was observed between the five groups. However, GAPDH protein expression in all five groups was low. This result indicates that proteins were from the nucleus. Relative data are shown in Figure 7.

**HIF-1α protein nuclear translocation by immunofluorescence**

According to results of immunofluorescence staining, HIF-1α protein expression increased with activated platelets. However, SLW also suppressed HIF-1α protein expression and depressed HIF-1α protein nuclear translocation in vitro. Relative data are shown in Figure 8.

**Discussion**

CD is defined as chronic non-specific granulomatous inflammation [9]. Its cause remains unclear, even though it is considered to be associated with immune factors. CD often causes severe symptoms, including intestinal stenosis [10]. Due to the repeated irritation of inflammation to the intestinal wall, an entire layer of intestinal fibrosis is formed, causing stricture and even obstruction of the intestines. Many patients with CD are forced to be treated with surgical interventions, resolving these complications. Common drugs used to treat CD include mesalazine, as well as immunosuppressive agents and biological agents. Many Western medicines have toxic side effects, including drugs used to treat CD. In the present study, suitable SLW concentrations were chosen, according to MTT assays. In subsequent cell experiments, this study examined the effects and mechanisms of SLW treatment in a CD cell model. Results showed that SLW improved EMT (angiogenesis and invasion) induced by activated platelets in vitro, in a dose-dependent manner.

Recent studies have found that angiogenesis is closely correlated with fibrosis [11-13]. High expression of VEGF has been shown to play a key role in angiogenesis development [14-16]. The current study found that VEGF concentrations were depressed with SLW supplement. Based on these results, it was concluded that SLW has anti-fibrosis effects by depressing VEGF expression. In addition, this study aimed to explain the relevant mechanisms of action and signal pathways involved. Thus, the current study evaluated relative protein expression of VEGF, finding no significant differences in VEGFR among the groups. This suggests that
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![Immunofluorescence staining for nuclear translocation of HIF-1α. Red indicates HIF-1α.](image)

**Figure 8.** Immunofluorescence staining for nuclear translocation of HIF-1α. Red indicates HIF-1α.

Stimulated VEGF was not correlated with VEGFR expression. Intensive investigations have found that SLW supplement suppresses PI3K/AKT/mTOR pathways via phosphorylation in vitro. Previous studies have shown that PI3K/AKT/mTOR pathway phosphorylation stimulates the upregulation of VEGF concentrations [17, 18].

EMT is another important step that leads to fibro-genesis [19]. E-cadherin and α-SMA are biomarkers of EMT. When overexpressed, they could stimulate EMT development [20, 21]. In the current study, SLW depressed protein expression of E-cadherin and α-SMA. This may indicate improved EMT, leading to fibrosis. Hypoxia inducible factor-1α (HIF-1α) is a transcription factor that widely exists in mammals, including humans, under hypoxic conditions. HIF-1α nuclear translocation and subsequent activation promotes angiogenesis and EMT [22, 23]. Furthermore, HIF-1α is an important downstream marker of PI3K/AKT/mTOR pathways [24]. According to results of Western blot assays and immunofluorescence staining, SLW treatment depressed HIF-1α expression in the nucleus. Results also suggest that SLW improved fibrosis-induced platelet activation (CD cell model) by depressing angiogenesis and EMT, in a dose-dependent manner, in vitro.

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