Wenyang Huazhuo Tongluo formula alleviates pulmonary vascular injury and downregulates HIF-1α in bleomycin-induced systemic sclerosis mouse model

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Abstract

Background: Vascular damage, autoimmune abnormalities, and fibrosis are the three pathological features of systemic sclerosis (SSc). However, pulmonary vascular damage is the main factor affecting the progression and prognosis of SSc. The main purpose of this study was to explore the molecular mechanism of Wenyang Huazhuo Tongluo Formula in alleviating pulmonary vascular injury in bleomycin-induced SSc mouse model.

Methods: Masson staining and H&E staining were used to analyze the degree of pulmonary vascular fibrosis and the infiltration of leukocyte cells in lung tissue of bleomycin-induced SSc mouse models treated with saline (BLM group), Wenyang Huazhuo Tongluo Formula (WYHZTL group) and HIF-1α inhibitor KC7F2 (KC7F2 group). Blood vessel exudation was determined by analyzing the cell number and albumin concentration in bronchoalveolar lavage fluid using a cell counter and ELISA assay, respectively. The degree of vascular injury was assessed by measuring the expression levels of vWF, E-selectin, ICAM-1, VCAM-1, VE-cadherin and claudin-5 in serum and pulmonary vascular endothelial cells using ELISA and immunofluorescence staining. Finally, the effect of Wenyang Huazhuo Tongluo Formula on the expression of HIF-1α was detected using immunofluorescence staining.

Results: Wenyang Huazhuo Tongluo Formula and KC7F2 significantly inhibited bleomycin-induced pulmonary vascular fibrosis and the level of perivascular inflammatory cell infiltration. The number of cells and the concentration of albumin were significantly reduced in the bronchoalveolar lavage fluid of the WYHZTL group and KC7F2 group compared with the BLM group. In addition, treatment with Wenyang Huazhuo Tongluo Formula and KC7F2 significantly downregulated the expression levels of vWF, E-selectin, ICAM-1, VCAM-1 and HIF-1α, but upregulated the expression of VE-cadherin and claudin-5 in serum and pulmonary vascular endothelial cells, compared with treatment with saline.

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Conclusions: This study reveals that Wenyang Huazhuo Tongluo Formula plays a new role in the treatment of SSc by alleviating pulmonary vascular damage. Furthermore, we found that Wenyang Huazhuo Tongluo Formula alleviates pulmonary vascular injury and inhibits HIF-1α expression.

Keywords: Wenyang Huazhuo Tongluo Formula, SSc mouse model, Pulmonary vascular injury, HIF-1α

Background
Systemic sclerosis (SSc) is an autoimmune disease characterized by vascular damage, autoimmune abnormalities, and progressive fibrosis of multiple organs [1]. Since skin and lungs are the main organs damaged by SSc, pulmonary vascular injury and pulmonary fibrosis are the main factors affecting the progression and prognosis of SSc. More than 70% of SSc patients also have pulmonary diseases, mainly interstitial lung disease and pulmonary hypertension. These two pulmonary diseases account for 60% of SSc-related deaths [2]. Early pathological changes such as microvascular injury and alveolar inflammation have been implicated in the pathogenesis of SSc-related pulmonary diseases. The inflammation and autoimmunity response induced by microvascular injury directly or indirectly stimulate the activation of fibroblasts, leading to the occurrence of fibrosis [3]. The occurrence of pulmonary microvascular endothelial cell injury has been attributed to hypoxia, inflammation, hyperphagy and endothelial-mesenchymal transition (EndoMT) [4].

Traditional Chinese medicine has potential in the treatment of SSc. For example, Tripterygium wilfordii significantly alleviates the forced vital capacity of patients with SSc-related interstitial lung disease, in a similar manner to cyclophosphamide, but its side effects, such as the inhibition of white blood cells, are weaker than cyclophosphamide [5]. In addition, recent studies have found that Tanshinone IIA, an extract derived from Salvia miltiorrhiza, can significantly inhibit SSc-induced skin and lung fibrosis caused by collagen deposition, reverse the bleomycin-induced EndoMT by inhibiting the Akt/mTOR/p70S6K pathway in vivo and in vitro, and alleviate SSc-induced vessels damage [6]. Geniposide derived from gardenia can inhibit bleomycin-induced EndoMT of endothelial cells through the mTOR signaling pathway, thereby improving SSc fibrosis in vivo and in vitro [7]. Most studies on the use of traditional Chinese medicine (TCM) in treating SSc have mainly focused on skin fibrosis, with very few studies exploring their effect on pulmonary vascular injury.

Wenyang Huazhuo Tongluo Formula was invented by our team and has been widely used in the clinical treatment of SSc. Our previous studies have shown that Wenyang Huazhuo Tongluo Formula regulated immune imbalance and exerts anti-fibrosis effects in the treatment of SSc. Wenyang Huazhuo Tongluo Formula exerts its anti-fibrosis effects by regulating the fibroblast cell cycle, inhibiting its proliferation, and reducing collagen synthesis by inhibiting the Wnt/β-catenin signaling pathway [8–10]. Wenyang Huazhuo Tongluo Formula can also inhibit the expression of type I and III collagen by inhibiting the TGF-β1/Smad signaling pathway in SSc fibroblasts. In addition, Wenyang Huazhuo Tongluo Formula can also improve fibrosis by upregulating MMP-9, inhibiting the expression of TIMP-1, and redressing the imbalance of MMP-9/TIMP-1 [11]. Wenyang Huazhuo Tongluo Formula regulates immune imbalance by alleviating the Th17/Treg cells imbalance in SSc [12]. In clinical treatment, we found that Wenyang Huazhuo Tongluo Formula could significantly ameliorate the hypoxic symptoms of SSc through unknown mechanisms.

The main purpose of this study was to explore the therapeutic effects of Wenyang Huazhuo Tongluo Formula against pulmonary vascular injury during SSc treatment. In addition, we also demonstrated that Wenyang Huazhuo Tongluo Formula alleviates pulmonary vascular injury and regulates HIF-1α expression.

Methods
Construction of bleomycin-induced SSc mouse model and measurement of skin thickness
Male C57/BL6N mice (purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd.) aged 8 weeks and weighing 17-20 g were used to construct a bleomycin-induced SSc mouse model as previously described [8]. Briefly, 100 ul of bleomycin (200 μg/ml) was subcutaneous injected to the restricted area of the upper back each day, which lasted for 3 weeks. PBS was used as a negative control during the bleomycin treatment. The study protocol was approved by the Ethics Committee of Nanyang Institute of Technology. A skin caliper was used to measure the skin thickness of the lesions of each group.

Drug preparation and experiment grouping
The ingredients and dosage of Wenyang Huazhuo Tongluo Formula are presented in Table 1. Wenyang Huazhuo Tongluo Formula was prepared at the Affiliated Hospital of Nanyang Institute of Technology, as...
previously described [8]. Briefly, all of the ingredients of WYHZTL were prepared as crude slices and boiled twice (90 min per time) with ultrapure water as the doctor’s directions. The water extracts were combined, filtered and evaporated under reduced pressure to a final concentration of 1.5 g/mL based on the equivalent amount of the crude drugs. Fifty C57/BL6 mice were randomly divided into 5 groups of 10 mice each. The 5 groups included a no treatment group (labeled as the Normal group), a group of mice injected subcutaneously with 100ul PBS solution (labeled as the PBS group), and three groups of mice used to construct the bleomycin-induced SSc mouse model (labeled as BLM group). The drug treatment plan is: normal group mice, PBS group mice and one of the BLM group received daily intragastric administration of normal saline (1 ml/day), another group received intragastric administration of Wenyang Huazhuo Tongluo Formula (47 g/kg/day, labeled as WYHZTL group), and the last group received subperitoneal injection of KC7L2 (dissolved in PBS solution, 10 mg/kg/day, labeled as KC7L2 Group). All treatment started concurrently with bleomycin and lasted for 4 weeks, and then the mice were anesthetized with an intraperitoneal injection of a mixture of ketamine (150 mg/kg) and acepromazine (15 mg/kg). Finally, mice were euthanized by cervical dislocation under anesthesia. Bronchoalveolar lavage fluid (BALF), serum, skin and lung tissue were then collected as previously described [13]. The serum was stored at -80 °C, while the skin and lung tissue were immediately fixed in formalin solution.

Detection of the cell number in BALF
The cell number and albumin concentration in BALF was determined immediately after collection. The BALF was centrifuged at 1500 rpm/min for 10 min, and the cell pellet was resuspended in 200ul PBS solution, and then detected with LUNA-II cell counter (Logos Biosystems, Korea).

Enzyme-linked immunosorbent assay (ELISA)
The concentrations of albumin in BALF as well as vWF, SELE, ICAM-1 and VCAM-1 in the serum were determined using ELISA assays. The ELISA kits were purchased from Cusabio Biotech, and the assays were carried out in accordance with the kit instructions.

Hematoxylin–eosin (H&E) staining and analysis of lung injury
The skin and lung tissues were fixed in formalin, and then embedded in paraffin for sectioning. The tissue sections were stained with the H&E staining kit (Solarbio Technology Co., LTD, Beijing) according to the manufacturer’s instructions. The stained lung tissue sections were used to assess lung injury under a microscope according to the following scoring criteria: ① Edema: 1 point represents non-existent, 2 point represents mild (10% alveolar involvement), 3 point represents moderate (10–50% alveolar involvement) or 4 point represents severe (50% alveolar involvement); ② Inflammation: 1 point represents none, 2 point represents mild (10 inflammatory cells/each high power field), 3 point represents moderate (10–50 inflammatory cells/each high power field), or 4 point represents severe (50 inflammatory cells/each high power field); ③ Hyaline membrane: 1 point represents absence, 2 point represents presence. The assessment of lung injury was carried out by three pathologists in accordance with the double-blind principle.

Masson staining and analysis of lung fibrosis
Lung tissue sections were also stained with masson stain according to the manufacturer’s instructions. Collagen appears blue after masson staining and can be used to assess the degree of lung fibrosis. The degree of pulmonary fibrosis is measured using the Ashcroft score, which uses the following criteria: 0 represent normal lung tissue; 1 represents slight alveolar septum or bronchial wall thickening; 2 to 3 represent moderate alveolar wall or bronchial wall thickening, but no lung structure damage; 4 to 5 represent fibrous tissue hyperplasia with obvious lung structural damage or fibrous tissue mass formation; 6 to 7 represent severe lung structural damage and large-scale fibrous tissue formation, honeycomb lung; 8 represent observation of fibrous tissue in the full view under observation. The above results were analyzed separately by three pathologists in accordance with the double-blind principle.

Table 1 The herbal ingredients and dosage of Wenyang Huazhuo Tongluo Formula

| number | herbal ingredients                  | dosage (g) |
|--------|-------------------------------------|------------|
| 1      | Radix Astragali membranacei         | 30         |
| 2      | Radix Codonopsis pilosulae          | 15         |
| 3      | Radix Dioscoreae oppositae         | 12         |
| 4      | Herba Epimedii                      | 12         |
| 5      | Radix Rehmanniae praeparata         | 15         |
| 6      | Ramulus Cinnamomi cassiae           | 9          |
| 7      | Herba Glechomae longitubae          | 15         |
| 8      | Semen Sinapis ibae                  | 9          |
| 9      | Capparis zeylanica Linn             | 15         |
| 10     | Fasciculus vascularis Lufae         | 9          |
**Immunofluorescence staining**

Immunofluorescence staining was used to detect the expression levels of vWF, SELE, ICAM-1, VCAM-1, HIF-1α, VE-cadherin and claudin-5 in pulmonary vascular tissues, as previously described [14]. The nuclei were stained using DAPI staining, while vWF, SELE, ICAM-1, VCAM-1, HIF-1α, VE-cadherin and claudin-5 proteins were labeled with green fluorescence. The antibodies against vWF, SELE, ICAM-1, VCAM-1, HIF-1α, VE-cadherin and claudin-5 were purchased from Affinity Biosciences Company. According to the previous study, the quantitative analysis of immunofluorescence staining was performed using image J [15].

**Statistical analysis**

The experimental results were expressed as mean±standard deviation. Graphpad Prism 7.0 was used for statistical analysis. Differences among groups were analyzed using one-way ANOVA followed by Tukey’s post-test. P<0.05 was considered to be statistically significant.

**Results**

**Wenyang Huazhuo Tongluo formula significantly alleviates skin and lung pathological changes in the bleomycin-induced SSc mouse model**

The results of H&E staining and masson staining showed a significant increase in the skin thickness and the collagen deposition of the BLM group compared with the PBS group (Fig. 1A-B). In addition, quantitative evaluation of pathological changes in lung tissue showed that lung injury and fibrosis in the BLM group were more serious and mainly manifested as pulmonary edema, inflammatory cell infiltration, and thickening of alveolar septum caused by collagen deposition, and even the disappearance of alveolar structure, compared with the PBS group (Fig. 1C-E). These results indicated that the bleomycin-induced SSc mouse model had been successfully constructed.

**Wenyang Huazhuo Tongluo formula alleviates the structural abnormalities of the pulmonary vessels in the bleomycin-induced SSc mouse model**

Vascular injury is an early event in the pathogenesis of SSc that appears before the development of fibrosis and immune abnormalities [16]. In this study, we assessed vascular fibrosis in the lung tissue of the SSc mouse model using masson staining, and found that the pulmonary vessels of the mice in the BLM group exhibited significant fibrosis and reduction in the diameter of the vessels compared with the PBS group (Fig. 2A). Treatment of mice with Wenyang Huazhuo Tongluo Formula and KC7F2 significantly alleviated the pulmonary vascular fibrosis observed in the SSc mouse models (Fig. 2A).

SSc-induced pulmonary vascular injury is characterized by the gradual loss of capillaries caused by the necrosis of vascular endothelial cells, which ultimately leads to tissue hypoxia and activation of dermal fibroblasts. Necrosis of endothelial cells can be evaluated using specific protein markers in serum, such as von Willebrand factor, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin (SELE) [17–19]. In this study, the concentrations of vWF, SELE, ICAM-1 and VCAM-1 in the serum of the BLM group mice were significantly higher than those in the PBS group (Fig. 2B-E). These results were a reflection of serious vascular damage in the bleomycin-induced SSc mouse model. More importantly, Wenyang Huazhuo Tongluo Formula and KC7F2 significantly reduced the concentrations of vWF, SELE, ICAM-1 and VCAM-1 in serum (Fig. 2B-E), suggesting that Wenyang Huazhuo Tongluo Formula and KC7F2 can protect vascular endothelial cells and alleviate vessel damage in SSc.
were significantly reduced, while Wenyang Huazhuo Tongluo Formula and KC7F2 upregulated their expression levels in pulmonary vascular endothelial cells (Fig. 3B, C). These results indicated that Wenyang Huazhuo Tongluo Formula and KC7F2 can alleviate pulmonary vascular leakage by regulating the expression levels of VE-cadherin and claudin-5.

Wenyang Huazhuo Tongluo formula alleviates the functional abnormalities of the pulmonary vessels in the bleomycin-induced SSc mouse model

During the occurrence and development of SSc, the activation of endothelial cells is the main manifestation of vascular endothelial cell dysfunction, which is mainly characterized by increased expression of adhesion molecules on the surface of endothelial cells.
[16]. Cell adhesion molecules such as vWF, ICAM-1, VCAM-1 and SELE located on the surface of vascular endothelial cells are necessary for the adhesion of platelets and leukocytes [22]. In this study, H&E staining was used to investigate the infiltration of lungs by inflammatory cells, especially around the pulmonary vessels. The results showed that there was a large number of infiltrating inflammatory cells around the pulmonary vessels in the BLM group, a condition that was significantly inhibited by treatment with Wenyang Huazhuo Tongluo Formula and KC7F2 (Fig. 4A, B). We also analyzed the number of cells in the BALF, and found that the results were consistent with H&E staining (Fig. 4C).

Furthermore, we analyzed endothelial cell adhesion molecules which were closely related to inflammatory
cell exudation. As expected, bleomycin significantly upregulated the expression levels of vWF, SELE, ICAM-1 and VCAM-1 in endothelial cells compared with PBS, while Wenyang Huazhuo Tongluo Formula and KC7F2 significantly reversed the bleomycin-induced upregulation of vWF, SELE, ICAM-1 and VCAM-1 (Fig. 5A-D). These findings show that Wenyang Huazhuo Tongluo Formula and KC7F2 can inhibit the activation of pulmonary vascular endothelial cells and alleviate SSc pulmonary vascular dysfunction.

Wenyang Huazhuo Tongluo formula alleviates pulmonary vascular endothelial cell injury in bleomycin-induced SSc mouse model and regulates HIF-1α

KC7F2 is widely used as the inhibitor of HIF-1α [23–25]. In this study, we demonstrated that KC7F2 can significantly alleviate pulmonary vascular injury, an indication that inhibiting HIF-1α is one of the effective measures to alleviate pulmonary vascular injury. Therefore, we explored the effects of Wenyang Huazhuo Tongluo Formula on HIF-1α in the bleomycin-induced SSc mouse model using immunofluorescence. The results showed
that the expression levels of HIF-1α in the pulmonary vascular endothelial cells of the BLM group were significantly higher than those in the PBS group, and that Wenyang Huazhuo Tongluo Formula significantly reversed the upregulation of HIF-1α in BLM group (Fig. 6). These results indicated that the Wenyang Huazhuo Tongluo Formula may alleviate SSc pulmonary vascular injury by regulating the expression of HIF-1α.

Discussion
The correlation between SSc vascular injury and hypoxia is attributed to the loss of capillaries and the remodeling of arterioles during the occurrence and development of SSc, which results in severe hypoxia of tissue cells, including vascular endothelial cells. Hypoxia further aggravates vascular damage by directly causing dysfunction of vascular endothelial cell and inducing cell necrosis through oxidative stress and other mechanisms, which forms a vicious circle [26]. Therefore, breaking the vicious circle between vascular injury and hypoxia can be one of the strategies for the treatment of SSc.

The SSc-induced vascular injury includes two aspects: structural abnormalities and dysfunction of vascular endothelial cell [27, 28]. The mechanisms resulting in structural abnormalities and dysfunction of vascular endothelial cell are closely related to hypoxia [29]. vWF, VCAM-1, ICAM-1 and SELE are mainly synthesized in vascular endothelial cells and distributed in cells or between cells. However, damage factors can induce endothelial cell necrosis, leading to the release of these proteins from endothelial cells into the blood, resulting in an increased concentration in the serum. Many studies have reported a significant increase in the serum levels of vWF, SELE, ICAM-1 and VCAM-1 in SSc patients, which is closely related to the disease progression [30–32]. In this study, we demonstrated that
Fig. 5 (See legend on previous page.)
Wenyang Huazhuo Tongluo Formula can significantly reduce the concentrations of vWF, SELE, ICAM-1 and VCAM-1 in serum, suggesting that Wenyang Huazhuo Tongluo Formula can protect vascular endothelial cells and alleviate SSc-induced structural abnormalities.

HIF-1α is the core factor in hypoxic stress response [33] that is up-regulated in SSc, especially in SSc patients with vascular lesions [34]. Under hypoxic conditions, HIF-1α plays a key role in extensive vascular remodeling, ultimately leading to the occurrence of pulmonary hypertension, which is one of the important causes of death in SSc patients [35]. The results of a recent study showed that HIF-1α gene polymorphism was not associated with susceptibility to SSc, but was significantly associated with the severity of PAH, suggesting that HIF-1α may be involved in vascular damage in SSc [36]. In the present study, we observed the upregulation of HIF-1α in the SSc mouse model. In addition, KC7F2 can significantly improve lung tissue fibrosis and vascular injury in SSc mouse model, suggesting that HIF-1α plays an important role in SSc-induced lung injury and may be a potential target for the treatment of SSc. In this study, we found for the first time that Wenyang Huazhuo Tongluo Formula has an effect similar to KC7F2 in improving lung tissue fibrosis and vascular injury in SSc mouse model.

Dysfunction of vascular endothelial cells is also one of the important pathogenesis of SSc [37]. Injury factors induce changes in the expression levels of cell adhesion molecules, chemokines, cytokines and growth factors in vascular endothelial cells, resulting in the activation and enhanced infiltration of inflammatory cells into the perivascular area. Microvascular thrombosis, which is also common in SSc, is closely related to the expression of the adhesion molecule vWF on the surface of endothelial cells. The intensity and nature of inflammation are largely regulated by cell adhesion molecules expressed on endothelial cells. Uncontrollable inflammation causes tissue damage and promotes fibrosis [38]. Therefore, inhibiting the activation of SSc vascular endothelial cells is another strategy for the treatment of SSc. Studies have also confirmed that HIF-1α can regulate the expression levels of vWF, VCAM-1, ICAM-1, and SELE [39–41]. In our study, we found that Wenyang Huazhuo Tongluo Formula inhibited the expression levels of HIF-1α, vWF, VCAM-1, ICAM-1, and SELE in pulmonary vascular endothelial cells. Therefore, we speculated that Wenyang Huazhuo Tongluo Formula could downregulate vWF, VCAM-1, ICAM-1, and SELE by inhibiting the expression of HIF-1α, thereby alleviating SSc-induced pulmonary vascular injury.

**Conclusions**

In summary, this study is part of a series of studies on the treatment of SSc using Wenyang Huazhuo Tongluo Formula. Our previous studies demonstrated that Wenyang Huazhuo Tongluo Formula has anti-fibrosis effects and regulates immune imbalance. In this study, we further demonstrated that Wenyang Huazhuo Tongluo Formula alleviates SSc-induced pulmonary vascular damage and inhibits HIF-1α. Our findings provide sufficient evidence to support the clinical application of Wenyang Huazhuo Tongluo Formula, and more importantly, a useful reference for the development of new drugs for SSc.
Abbreviations
SSC: Systemic Sclerosis; ROS: Reactive Oxygen Species; EndoMT: Endothelial-Mesenchymal Transition; TCM: Traditional Chinese Medicine; BALF: Bronchoalveolar Lavage Fluid; ELISA: Enzyme-Linked Immunosorbent Assay; H&E: Hematoxylin-Eosin; vWF: Von Willebrand Factor; VCAM-1: Vascular Cell Adhesion Molecule-1; ICAM-1: Intercellular Adhesion Molecule-1; SELE: E-selectin; HIF-1α: Hypoxia-inducible Factors-1a.

Supplementary Information
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Additional file 1. The original images of H&E staining, massonstaining and immunofluorescence staining.

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Not applicable.

Authors’ contributions
KL and QW carried out the molecular biology assay and drafted the manuscript. HB provided the WYHZTL formula, designed the study and helped to draft the manuscript. QL, KG and LH constructed mouse model, PD and YD helped the in vivo study. All authors read and approved the final manuscript.

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Availability of data and materials
The data used to support the findings of this study are available from the corresponding author upon request.

Declarations
Ethics approval and consent to participate
The study protocol is approved by the Ethics Committee of Nanyang Institute of Technology (the ethics approval code: NYSTIRB2020-002). All animal experiment procedures have been performed in accordance with the relevant guidelines and regulations. Also, it is confirmed that the study was carried out in compliance with the ARRIVE guidelines.

Consent for publication
Not applicable.

Competing interests
The authors declare no potential conflict of interest.

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