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Original Article

Hypoxia-inducible factor-1α/vascular endothelial growth factor signaling pathway–based ulcer-healing mechanism of Astragalus Aqueous extract in diabetic foot rats

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Abstract

The main objective of this work was to investigate the mechanism of Astragalus aqueous extract ulcer healing in diabetic foot model rats through the hypoxia-inducible factor 1-alpha (HIF-1α)/vascular endothelial growth factor (VEGF) signalling pathway. Fifty specific-pathogen-free male Sprague Dawley rats were divided into blank (A), model control (B), Astragalus extract (C) and mupirocin (D) treatment groups. Group A received a regular diet, whereas the other groups received a high-fat/high-sugar diet and intraperitoneal streptozotocin injections to induce diabetes. Diabetic foot ulcers were created via skin excision. Subsequently, ulcers were debrided daily. Groups B, C and D received wet saline gauze, wet gauze with Astragalus extract and gauze with mupirocin, respectively, on the affected area. Group A received no treatment. After 14 days, the rats were assessed for ulcer healing and general condition. Immunohistochemistry was used to detect HIF-1α and VEGF levels in the dorsalis pedis artery, and ELISA was used to determine serum IL-6 and CRP levels. The results revealed that Groups C and D had significantly faster ulcer healing compared with Group B ($p < 0.01$), and ulcer healing was faster in Group C than in Group D ($p < 0.01$). Group C exhibited notably higher HIF-1α and VEGF protein expression levels compared with Groups B and D ($p < 0.01$). IL-6 and CRP expression levels in Groups C and D were significantly lower than those in Group B ($p < 0.01$). In summary, Astragalus aqueous extract effectively treats diabetic foot ulcers by up-regulating HIF-1α and VEGF expression, activating the HIF-1α/VEGF pathway, improving local tissue ischaemia and hypoxia, promoting collateral circulation and enhancing dorsalis pedis artery formation, thereby accelerating ulcer repair in diabetic rats.

Keywords: *Astragalus* aqueous extract, Diabetic foot, HIF-1α/VEGF signalling pathway, Mechanism of action.

1. Introduction

Diabetic foot (DF) is one of the most serious and costly chronic complications of diabetes mellitus (DM) [1], with 8%–20% of patients affected by foot ulcers during their lifetimes. Unlike other foot ulcers, DF ulcers carry a high amputation risk of around 15%–45% [2], encompassing 40%–70% of non-traumatic amputations [3]. Indeed, DF is the leading cause of non-traumatic amputation [4], and local ischaemic ulcer is the main factor resulting in amputation in patients with DM [5-7]. Amputation is associated with low quality of life and even increased mortality risk in these patients, emphasising the need for effective treatments to enhance both well-being and survival.

Early granulation tissue formation plays an important regulatory role in ulcer repair [8-12]. Vascular endothelial growth factor (VEGF), a critical inducer of vascular permeability during angiogenesis, enhances granulation tissue growth and thereby accelerates ulcer healing. HIF-1α regulates VEGF [13], playing a central role in oxygen metabolism and cell hypoxia stabilisation [14, 15]. Disordered and delayed angiogenesis around ulcers is a key factor in DF ulcer-healing complications [16]. Ischaemia and hypoxia in foot ulcers hinder timely HIF-1α up-regulation and cause abnormal VEGF expression, disrupting the HIF-1α/VEGF signalling pathway, and potentially impeding angiogenesis and tissue circulation. These events exacerbate tissue ischaemia and hypoxia [17, 18], often leading to limb ischaemic necrosis, amputation or death in severe cases.

Astragalus aqueous extract, derived from the active components of the traditional Chinese medicine *Astragalus membranaceus*, is renowned as an 'essential medicine for tonifying Qi' and the 'holy medicine for sores' owing to its potent effects on pus and muscle ailments. Clinically, it

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accelerates ulcer healing, revitalises necrotic tissue, boosts DF recovery rates and reduces amputation rates when used to treat DF [19-23]. The extract's therapeutic value relies on its antioxidative and immune-regulating properties, its promotion of fibroblast proliferation and its reduction of endothelial cell adhesion and related molecule expression. These attributes reduce ulcer bleeding and exudation while promoting granulation tissue growth [24].

In the present study, to investigate the mechanism underlying *Astragalus* aqueous extract's promotion of DF healing, DF model rats were established using applications of extract-infused gauze to affected areas. Using this approach, effects on ulcer-healing time, HIF-1α and VEGF expression in the dorsalis pedis artery and serum interleukin-6 (IL-6) and CRP levels were assessed. By exploring the mechanism of *Astragalus* extract in DF treatment, this research provides insights into clinical DF management.

2. Materials and methods

2.1. Experimental animals

Fifty specific-pathogen-free male Sprague Dawley rats with an average weight of approximately 180 ± 20 g were purchased from Changsha Tianqin Biotechnology Co., Ltd. [licence number: SCXK (Xiang) 2019-0014].

2.2. Animal diet

Rats in the blank group received a standard diet throughout the experiment. Model group rats were subjected to a high-fat/high-sugar diet during the modelling phase and a standard diet during treatment. Feed was purchased from Changsha Tianqin Biotechnology Co., Ltd. [licence number: SCXK (Xiang) 2019-0013].

2.3. Experimental substances and reagents

The experimental materials included Astragalus aqueous extract [potency: 10 ml/ampoule, containing 20 g of A. membranaceus; Chiatai Qingchunbao (approval number: Guo Yao Zhun Zi Z33020179)]; mupirocin ointment [potency: 5 g/each; Tianjin Shike Pharmaceutical Co., Ltd. (approval number: Guo Yao Zhun Zi H10930064)]; streptozotocin (STZ; Sigma, USA; batch number: S0130-500MG); primary antibodies targeting HIF-1α (Beijing Bioss Biotechnology Co., Ltd.; batch number: bs-0737R) and VEGF (Beijing Bioss Biotechnology Co., Ltd.; batch number: bs-1313R); and test kits for hypersensitive C-reactive protein (hs-CRP) [ELISA Lab (Wuhan) Technology Co., Ltd.; batch number: JYM0253Ra/GR2020-07] and IL-6 [ELISA Lab (Wuhan) Technology Co., Ltd.; batch number: JYM0646Ra/GR2020-07].

2.4. Developing the DF rat model

Following one week of adaptive feeding, rats were exposed to a high-fat/high-sugar diet for three weeks. After a 12 h fast, an intraperitoneal injection of 1% STZ (35 mg/kg) was administered. After three days, random blood glucose (RBG) was measured, and successful model creation was confirmed when RBG exceeded 16.7 mmol/L. Continuing on a high-fat/high-sugar diet for one week, diabetic rats underwent a 12 h fast before intraperitoneal injection of 10% chloral hydrate (0.3 mL/100 g) for anaesthesia. After skin preparation, a 2.5 × 2.0 cm rectangular area was demarcated on the rat foot dorsum. Following disinfection, the foot's skin was incised down to the fascia, establishing the DF rat model.

2.5. Grouping and drug application

Upon successful modelling, 37 DF model rats were randomly allocated into the model control (B) group, Astragalus aqueous extract (C) group and mupirocin (D) group. Group A served as the control. Groups B, C and D were subjected to daily applications of wet saline gauze, wet gauze with Astragalus aqueous extract and gauze with mupirocin ointment, respectively. No treatment was administered to Group A rats. After 14 days of continuous treatment, rats were euthanised, and pertinent outcomes were assessed.

2.6. Outcome assessment and techniques

2.6.1 Serum indexes

Serum IL-6 and hs-CRP levels were analysed following ELISA kit instructions.

2.6.2 Dorsalis pedis artery

Dorsal pedis arteries from affected limbs were collected, processed as per kit guidelines and subsequently underwent dehydration, trimming, embedding, sectioning, staining, mounting and microscopic examination, adhering to relevant Standard Operating Procedures for pathological analysis.

2.7. Statistical analysis

Data were statistically evaluated using SPSS 26.0. Outcomes were presented as means ± standard deviations (x ± S). Normally distributed measurement data were analysed through one-way analysis of variance, whereas pre-treatment and post-treatment data were compared using t-tests. Non-normally distributed measurement data were analysed using the Kruskal–Wallis test for multiple dataset comparisons and the Nemenyi test for pairwise comparisons. Differences were considered statistically significant where p-values were <0.05.

3. Results

3.1. Modelling success rate and mortality in rats

Throughout the modelling period, two rats died, and three exhibited RBG levels below 16.7 mmol/L, leading to their exclusion from the experiment. This yielded 37 successfully modelled rats, equating to an 88.10% success rate. During treatment, nine rats died, leaving 36 surviving rats at the experiment's conclusion. These survivors comprised eight rats in Group A, eight in Group B, eleven in Group C and nine in Group D (Table 1).

3.2. Body weight and blood glucose

3.2.1. Body weight

Rats in Groups C and D showed no significant variance in body weight compared with Group B ($p > 0.05$). Moreover, no statistically significant differences in body weight were observed before and after treatment in each group ($p > 0.05$). This implies that Astragalus aqueous extract had

Table 1. Number of rats before and after treatment in each group.

Groups	Before treatment	after treatment	Death
Group A	8	8	0
Group B	13	8	5
Group C	12	11	1
Group D	12	9	3

no substantial impact on the body weight of treated DF rats.

3.2.2. Blood glucose

Blood glucose levels in Groups C and D showed no significant difference compared with Group B levels ($p > 0.05$). Furthermore, no significant differences in blood glucose before and after treatment were observed within any group ($p > 0.05$), indicating that external application of Astragalus aqueous extract did not lead to substantial hypoglycaemic effects on DF rats.

3.3. Ulcer-healing time

Ulcer-healing time in Groups C and D was significantly shortened compared with that in Group B ($p < 0.01$), and ulcer healing occurred significantly faster in Group C than in Group D ($p < 0.05$). These findings indicate that applying the aqueous extract externally accelerated ulcer healing in DF rats, with Group C showing superior efficacy to Group D (Table 2).

3.4. HIF-1α expression in dorsal pedis arteries

A mildly expressed HIF-1 alpha and VEGF. Compared with A, B and D, the expression of HIF-1 and VEGF in group C was significantly increased ($P < 0.01$ =; (See Table 3).

3.5. Microscopic imaging of HIF-1α and VEGF in dorsal pedis arteries

Negatively stained cells were visualised as blue, the substrate was white and positively stained cells were yellow or brown. HIF-1α–positive products were mainly distributed in the cytoplasm and intercellular matrix.; (See Table 4).

3.6. Serum IL-6 and CRP levels

Levels of IL-6 and CRP in Groups B, C and D were significantly increased compared with Group A levels ($p < 0.01$). Furthermore, IL-6 and CRP levels in Groups C and D displayed significant reductions relative to Group B ($p < 0.01$). Notably, no significant disparities were observed in IL-6 and hs-CRP levels between Group C and Group D ($p > 0.05$) (Table 5).

4. Discussion

DF, a concurrent symptom also known as ‘gangrene of the toe’ in traditional Chinese medicine, has historically yielded diverse physician interpretations of its aetiology and pathogenesis. Currently, diabetes and gangrene are generally perceived as being rooted in ‘deficiency in origin and excess in superficiality, and stasis due to deficiency’. Prolonged diabetes results in Yin and Qi depletion, causing an insufficiency of both. Hindered by Qi deficiency, normal blood circulation falters, resulting in blood stasis. Yin deficiency depletes blood, while dry heat diminishes fluids, leading to dense, viscous blood. Hindered blood circulation leads to stasis, vein obstruction and muscle and joint stiffness. Qi deficiency, weak blood flow and Yin depletion all slow circulation, impeding the pulse and Qi flow. Yin deficiency influencing Yang and deficient warmth in Yang yield cold and stagnant blood, undersupplying limbs and malnourished skin and muscle. Thus, Qi deficiency leading to blood stasis is a pivotal factor fostering gangrene progression.

Table 2. Ulcer-healing time (days; $\bar{x} \pm S$)

Groups	n	Healing time
Group A	8	--
Group B	8	13.33 ± 0.82
Group C	11	9.28 ± 1.99*#
Group D	9	10.29 ± 0.95*

Note: * $p < 0.01$, versus Group B; # $p < 0.05$, versus Group D.

Table 3. Mean optical densities (ODs) of HIF-1α in dorsal pedis arteries (OD; $\bar{x} \pm S$).

Groups	n	HIF-1α ($\bar{x} \pm S$)	VEGF ($\bar{x} \pm S$)
Group A	8	0.2093 ± 0.0099	0.1926 ± 0.069
Group B	8	0.2134 ± 0.0061	0.1996 ± 0.0094
Group C	11	0.2393 ± 0.0065*#▲	0.2529 ± 0.0175*#▲
Group D	9	0.2138 ± 0.0057	0.2282 ± 0.0166

Note: * $p < 0.01$, versus Group A; # $p < 0.01$, versus Group B; ▲ $p < 0.01$, versus Group D.

Table 4. Microscopic imaging of HIF-1α and VEGF in dorsal pedis arteries.

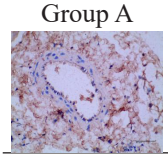
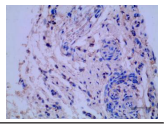
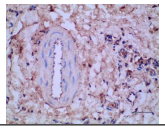
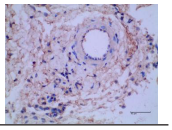
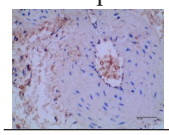
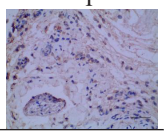
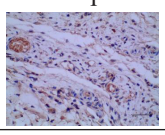
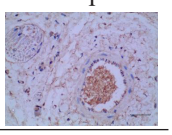
HIF-1α			
Group A	Group B	Group C	Group D
			
VEGF			
Group A	Group B	Group C	Group D
			

Table 5. Serum IL-6 and hs-CRP levels (pg/mL; $\bar{x} \pm S$).

Groups	n	IL-6	hs-CRP
Group A	8	68.12 ± 5.25	2930.68 ± 419.70
Group B	8	88.23 ± 3.33*	5508.02 ± 206.09*
Group C	11	76.36 ± 4.90*#	4070.60 ± 365.18*#
Group D	9	75.52 ± 6.59*#	3996.74 ± 583.35*#

Note: * $p < 0.01$, versus Group A; # $p < 0.01$, versus Group B.

Astragalus membranaceus, also known as Beiqi and Radix Astragali, is a leguminous plant with a sweet taste. It is slightly warming in nature and is associated with the spleen and lung meridians. It is mainly produced in Hebei, Heilongjiang and Inner Mongolia, as well as some other regions in China. In clinical practice, it is valued for Qi tonification, Yang elevation, sweat reduction, muscle support, treating sores and oedema alleviation. The plant’s potent effects on pus and muscles have earned it the titles ‘essential medicine for tonifying Qi’ and ‘holy medicine for sores’. Thus, it is valuable for treating Qi and blood-deficient gangrene. Astragalus aqueous extract, which is more stable and purer than traditional decoctions, is extensively used in clinical applications. Leveraging this, in the present study, Astragalus aqueous extract was studied to elucidate the ulcer-healing mechanism associated with A.

membranaceus.

DF is the main cause of amputation and DM-related disability, exhibiting challenging traits that include recalcitrance, high amputation rates and poor prognosis, with a nearly 40% 5-year mortality rate [25]. Inflammatory factors are closely associated with DF development and progression, although its multifaceted pathogenesis has not been fully elucidated. Clinical DF treatment is arduous; thus, studies on inflammatory factors are required to identify potential therapies. The results of the present study showed that IL-6 and hs-CRP levels in the model control group and all treatment groups were significantly increased compared with those in the normal group, implying IL-6 and hs-CRP involvement in DF development. Foot damage triggers immune responses, potentially elevating IL-6 and hs-CRP expression levels. Furthermore, exposed feet may be subjected to aggravated infections, exacerbating inflammatory factor levels and complicating ulcer healing, potentially increasing rat mortality. In the present study, IL-6 and hs-CRP levels in Groups C and D were significantly decreased compared with those in the model control group, potentially influenced by routine ulcer debridement, a practice tempering inflammation and infection, thereby reducing serum inflammatory factor expression. However, Group B exhibited the highest inflammatory factor expression level and slowest wound healing, despite basic disinfection, possibly attributed to prolonged ulcer healing in Group B, intensifying IL-6 and hs-CRP expression.

Cell metabolism suffers due to ischaemia and hypoxia within foot tissue, leading to disrupted and delayed ulcer-associated angiogenesis [26] and hindered DF healing. HIF-1α is pivotal in regulating body oxygen metabolism, modulating hypoxic conditions and achieving cell stability [27, 28]. It can regulate VEGF, inducing vascular endothelial cell migration and proliferation, as well as heightened permeability, thereby promoting microvascular development and collateral circulation. This improves ulcer hypoxia and enhances local tissue perfusion [29, 30]. Under normoxic conditions, HIF-1α is barely expressed. Hypoxia inhibits proline residues in HIF-1α, impeding degradation and leading to cellular accumulation, subsequent nuclear entry and increased HIF-1α levels under hypoxic conditions [31]. VEGF is crucial for vascular permeability in vessel growth. During early-stage ulcer healing, particularly capillary formation, VEGF enhances plasma protein extravasation and vascular endothelial cell division, inducing angiogenesis and safeguarding vascular endothelial cell migration and angiogenesis, which is critical for angiogenesis. Overall, this enables robust blood supply, promoting fibroblast proliferation, granulation tissue growth and expedited ulcer healing [32-35]. Although a multifaceted approach to DF treatment is required, optimal treatment encompasses blood glucose control, enhanced tissue oxygenation and blood supply reconstruction. Given this foundation, it is surmised that activating the HIF-1α/VEGF signalling pathway is pivotal in promoting ulcer healing in DF rats.

In the present study, low HIF-1α and VEGF expression was low in Group A rats, suggesting balanced HIF-1α and VEGF levels under normal conditions. This likely resulted from activation of the HIF-1α/VEGF pathway in DF rats treated with Astragalus aqueous extract. This intervention improved foot ischaemia and hypoxia status, augmented pro-angiogenic factor expression and hastened rat ulcer healing. Expression levels of VEGF and HIF-1α in dorsalis pedis arteries were higher in Groups B and D than in Group A, although they were lower than Group C levels. This discrepancy may have arisen from delayed HIF-1α/VEGF activation due to insufficient pro-angiogenic factor expression in Groups B and D. Consequently, ulcer healing in Groups B and D lagged that in Group C.

In this study, Group C rats exhibited the shortest healing time, followed by Group D, whereas Group B had the longest healing time after 2 weeks of treatment. The variance in rat healing time among these groups post-intervention prompts further inquiry. The most protracted healing time occurred in Group B. Some rats in this group had unresolved ulcers by the end of the study, and Group B had the highest mortality rate. This suggests that unchecked ulcer expansion, aggravation and potential necrosis occur when DF is not promptly treated. In such instances, rat ulcers might endure extended unhealed periods, elevating mortality risks. Conversely, Group C rats showed the quickest ulcer healing. Previous studies have highlighted the efficacy of Astragalus in muscle regeneration and pus drainage. In Traditional Chinese Medicine, the primary pathology of DF is Qi deficiency–induced blood stasis. Thus, Qi tonification is vital. Qi flow ensures blood circulation, yielding smooth pulse vessel flow and dispersing stagnant blood, promoting new blood generation as well as neovascularisation. Consequently, the efficacy of Astragalus aligns with DF treatment principles. Modern research has revealed that Astragalus is abundant in flavonoids, polysaccharides and glycosides, which are effective in detoxification, pus drainage, cell regeneration, haemostasis and muscle regeneration. Moreover, it reduces blood viscosity, inhibits inflammation and curbs infection in patients with DF [36]. Previous studies have demonstrated the capacity of Astragalus to enhance epidermal, granulation tissue and capillary regeneration, as well as improve skin microcirculation and hasten ulcer recovery and healing time [37-39]. These findings are consistent with those from the present study, i.e. that Astragalus extract expedites ulcer healing, reinforcing its notable efficacy in treating DF.

5. Conclusion

In summary, Astragalus aqueous extract accelerates ulcer healing in DF rats, potentially through up-regulation of the pro-angiogenic factors HIF-1α and VEGF, activation of HIF-1α/VEGF signalling, enhancement of local tissue ischaemia, mitigation of hypoxia and promotion of collateral circulation for quicker healing. However, the limited observation period in this study focused only on ulcer-healing speed, pro-angiogenic factors and inflammatory factors, omitting any exploration of the antibacterial effects of Astragalus aqueous extract on ulcer secretions. Consequently, forthcoming research will investigate such antibacterial effects, and the results may further improve the extract's potential for clinical application.

Conflict of Interests

The authors declare no conflict of interest.

Consent for publications

The author read and approved the final manuscript for publication.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

All authors contributed equally to this research. Xuxu Jia conducted the experiments and wrote the manuscript. Yujie Yu performed the data analysis. Jingrun Dai and Jiang Mei conducted data collection. Qian Guo and Juan Yang designed the study and amended the paper. All authors have read and agreed to the published version of the manuscript.

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