Journal of Ethnopharmacology 210 (2018) 434–442

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Low molecular weight fucoidan ameliorates hindlimb ischemic injury in type 2 diabetic rats☆

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ARTICLE INFO

Keywords:
Fucoidan
Hindlimb ischemia
Diabetes
Peripheral arterial disease
Foot ulceration

ABSTRACT

Ethnopharmacological relevance: Low molecular weight fucoidan (LMWF), extracted from *Laminaria japonica* Areschoug, is a traditional Chinese medicine, commonly used to alleviate edema, particularly for feet with numbness and pain.

Aim of the study: Diabetic mellitus (DM) patients are at high risk of developing peripheral arterial disease (PAD). Individuals with DM and PAD co-morbidity have a much higher risk of critical limb ischemia. LMWF showed several beneficial effects, such as anti-inflammation, anti-thrombosis, and enhancing revascularization. Therefore, we hypothesized that LMWF might be beneficial to diabetes-induced PAD, and investigated the therapeutic potential of LMWF on diabetic PAD rats.

Materials and methods: Type 2 diabetic Goto-Kakizaki (GK) rats were made PAD by injection of sodium laurate into femoral artery. LMWF (20, 40 or 80 mg/kg/day) or cilostazol (100 mg/kg/day) were given to diabetic PAD rats for 4 weeks, respectively. The effects of LMWF on foot ulceration and claudication, plantar blood flow, collateral vessel formation, endothelium morphology, gastrocnemius injury, platelet aggregation, vessel vasodilation, and the expressions of inflammation factors, VEGF, eNOS, and nitric oxide were measured.

Results: We found that LMWF markedly ameliorated foot ulceration and claudication, and improved the plantar perfusion by reversing hyperreactive platelet aggregation, ameliorating endothelium-dependent vasodilation and revascularization on diabetic PAD rats. In addition, upregulation of several inflammatory factors, such as ICAM-1 and IL-1β in the gastrocnemius muscles of ischemic hindlimb were suppressed by LMWF administration. And eNOS phosphorylation at Ser1177 and NO production were significantly enhanced in LMWF-treated diabetic PAD rats.

Conclusions: Taken together, our findings demonstrated that LMWF exhibits therapeutic effect on hindlimb ischemia in type 2 diabetic rats likely through ameliorating endothelium eNOS dysfunction and enhancing revascularization, thus, providing a potential supplementary non-invasive treatment for diabetes-induced PAD.

1. Introduction

Diabetes mellitus (DM) is a leading cause of death worldwide. Today, China has more than 114 million people suffering from DM, the highest number of any country in the world (Chan et al., 2014). What makes this condition particularly serious is that those affected by it are at a 50% higher risk of cardiovascular disease than those who don't have diabetes. Peripheral arterial disease (PAD), which narrows the arteries in the legs, limiting blood flow to the muscles, is one of the most serious vessel complications of DM (Leibson et al., 2004; Willyard, 2012). It involves small and medium size vessels of the limb's extremities that present distal ischemia and manifested as ulcers,

Abbreviations: LMWF, Low molecular weight fucoidan; DM, Diabetic mellitus; PAD, Peripheral arterial disease; GK, Goto-Kakizaki; ICAM-1, Intercellular Adhesion Molecule-1; IL-1β, Interleukin-1 beta; eNOS, Endothelial nitric oxide synthase; VEGF, Vascular endothelial growth factor; T-CHO, Total cholesterol; TG, Triglyceride; LDL-C, Low-density lipoprotein cholesterol; HDL-C, High-density lipoprotein cholesterol; IS, Ischemic; NI, Non-ischemic; APTT, Activated partial thromboplastin time

☆ Grants support: This work was supported by grants from National Natural Science Foundation (81370339, 81570206) and Beijing Innovation Promoting Project (TJSHG201510025005).

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http://dx.doi.org/10.1016/j.jep.2017.09.014
Received 1 June 2017; Received in revised form 25 August 2017; Accepted 12 September 2017
Available online 14 September 2017
0378-8741/

claudication, rest pain and finally gangrene.

Using sodium laurate injection into femoral artery to induce PAD is a common experimental model, which is supposed to damage femoral artery endothelial cells, causing platelets aggregation in peripheral vascular beds, and further leads to thrombus formation followed by plaque rupture (Ashida et al., 1980; Ogawa et al., 2009; Uemura et al., 2006). With the development of thrombus and ischemic stimulation, angiogenesis is activated, which is important for promoting neovascularization and against ischemia in patients affected by PAD (Biscetti et al., 2009). Among all the growth factors in angiogenesis, vascular endothelial growth factor (VEGF) is the most crucial one and the major target of pro-angiogenic therapy for PAD (Ji et al., 2007).

Brown seaweed *Laminaria japonica,* which also be referred to as Haidai in China, is one of the Traditional Chinese Medicine commonly used to resolve hard lump and alleviate edema. According to Chinese literature (Zou, 2016), L. *japonica* is widely used for the treatment of "weak feet", which shows the symptoms like feet numbness, edema and thrombosis in lower extremity. L. *japonica* is also traditionally used as a beneficial food for anti-thrombosis in Japan (Ren et al., 2013). Fucoidans, a class of L-fucose-enriched sulfated polysaccharides extracted from L. *japonica*, have been characterized by a wide variety of bioactivities, including anti-aggregation and anti-inflammation, which all seem to be beneficial for ameliorating diabetes complications (Cumashi et al., 2007). Several investigations have demonstrated that the clinical use of fucoidan stimulated the expression of prostacyclin, a potent inhibitor of platelet aggregation (Ren et al., 2013). In addition, fucoidan administration could decrease low-density lipoprotein cholesterol and ameliorate insulin resistance in obese adults (Hernándezcorona et al., 2014). As previously described, low molecular weight fucoidan (LMWF) with the number-average molecular weight of 5300 Da and weight-average molecular weight of 6500 Da, was extracted from *Laminaria japonica* J. E. Areschoug (1851) (Laminariaceae), which was cultured at the coast of Rongcheng (China), collected in August 2015, authenticated by Prof. Lanping Ding and stored as a voucher specimen (No. 83) in the Jerbarium of the Algal Chemistry Department, Institute of Oceanology, Chinese Academy of Sciences, Qingdao, China (Cui et al., 2014; Liang et al., 2016). Over the past years, our lab found that LMWF is beneficial in the treatment of thrombosis, and profoundly ameliorated hypertension and hyperlipidaemia in STZ-induced type 1 and Goto-Kakizaki (GK) type 2 diabetic rats (Cui et al., 2014; Liang et al., 2016). In addition, LMWF has been suggested to facilitate angiogenesis in a PAD rat model (Luyt et al., 2003), and greatly assist endothelial colony forming cells or mesenchymal stem cells in enhancing angiogenesis *in vivo* (Sarlon et al., 2012; Han et al., 2015; Lee et al., 2016). Although the beneficial effects of LMWF on angiogenesis have been reported in PAD, the effect of LMWF on diabetic PAD (the vast majority of PAD populations) and its underlying mechanism remains largely elusive. Thus, we conducted our experiments in GK rats to evaluate the *in vivo* effects of LMWF on diabetic PAD and compared it with the effect of cilostazol, a widely used drug in clinical to ameliorate hindlimb ischemia through anti-thrombosis and angiogenesis. We found that LMWF profoundly protects against hindlimb ischemic injury in diabetic PAD rats probably through the promotion of vasodilation and angiogenesis in peripheral arteries.

2. Materials and methods

2.1. Reagents and chemicals

LMWF produced in October 2015 was supplied by the Institute of Oceanology, Chinese Academy of Sciences, Qingdao, China. The chemical composition and molecular weight of LMWF were shown in our previous study (Liang et al., 2016). LMWF was dissolved in distilled water before intragastric administration to the rats. Cilostazol tablets were purchased from Dazhong Pharmaceutical Company (Zhejiang, China). Rat nitric oxide (NO), total cholesterol (T-CHO), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C) and activated partial thromboplastin time (APTT) ELISA kits were bought from Nanjing Jiancheng Chemical (China). Chloralhydrate and sodium laurate were purchased from Sinopharm Group (China). Adenosine diphosphate, thrombin, acetylcholine and phenylephrine were bought from Sigma Aldrich (Germany). Primary antibodies against CD31, VEGF, ICAM-1, IL-1β and α-tubulin were purchased from Santa Cruz (USA). Primary antibodies against p-eNOS (p-Ser1177), and total-eNOS were purchased from Abcam (UK).

2.2. Experimental animals and groups

This study was approved by the Capital Medical University Animal Care and Use Committee (AEEI-2015-045), and all studies were conducted in accordance with 'Guide for the Care and Use of Laboratory Animals' adopted by the Beijing government and 'Guide for the Care and Use of Laboratory Animals' published by the US National Institutes of Health (publication No. 85–23, revised 1996).

GK rats and Wistar rats were purchased from SLAC Laboratory Animal (Shanghai, China) and reared in a standard experimental animal laboratory with normal food and water given *ad libitum*. Five groups of twenty-week-old male diabetic GK rats (n = 7–12 per group) were studied: DM group (treated with distilled water), DM-F20 group (treated with 20 mg/kg LMWF), DM-F40 group (treated with 40 mg/kg LMWF), DM-F80 group (treated with 80 mg/kg LMWF), and DM-Cilo100 group (treated with 100 mg/kg cilostazol). Additionally, age-matched Wistar rats (n = 9) were used as the N-Model control group (N-Model, treated with distilled water). The drugs were given by intragastric administration for 4 weeks, once a day instantly after injection of laurate.

2.3. Laurate injection-induced PAD model and the measurement of plantar perfusion

Sodium laurate was injected into the left femoral artery to induce unilateral hindlimb ischemia in rats as previously described (Uemura et al., 2006). Briefly, all animals were anesthetized with the injection of chloralhydrate. The femoral artery blood flow was interrupted with an artery clamp. 0.5 mg sodium laurate solution was injected into the left femoral arteries of rats over 20 s. After injection, some surgical binding agent (0.5 ml; Beijing Fuailetechnology Co. Ltd., Beijing) was applied to the arterial wound to prevent from bleeding, and the clamp was removed to restart vascular flow at 5 min after injection. The surgical skin wound was closed with 5-0 sutures. Before rats awake, the hindlimb blood flow was measured with a Full-field Laser Perfusion Imager system (moorFLPI, Moor Inc), immediately after surgery and then at 1-week intervals, until the end of the research, for a total follow-up of 4 weeks.

2.4. Foot ulcers and claudication in diabetic PAD rats

To determine the progress of the lesion, the area of ulceration in the hindlimb was observed and classified 2 weeks after surgery as follows: Grade 1, normality; Grade 2, local ulceration; Grade 3, diffuse ulceration; Grade 4, necrosis affecting digits; Grade 5, severe necrosis, or loss of foot necrosis (Sasaki et al., 2012; Uemura et al., 2006).

Treadmill apparatus (FT-200; Chengdu technology & Market Co. Ltd., China) were located in the same room as the rats were housed, and all tests were conducted in the dark at 10 A.M. or 2 P.M. (belt speed of 6 m/min, and duration of 1 min above). The severity of gait disturbance was measured 2 weeks after surgery. Then, the claudication of the hindlimb was observed, graded and scored as follows: Grade 1, normality; Grade 2, imbalanced walk and the ischemic plantar can't be seen from the back; Grade 3, seriously imbalanced walk; Grade 4, drag, necrosis or defluxion.