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Effect and mechanism of fucoidan on VEGF in atherosclerotic oxidative stress signal pathway

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Abstract: **To investigate the effects and mechanisms of fucoidan sulfate (Fucoidan) on VEGF in atherosclerotic (AS) oxidative stress signaling pathway of apolipoprotein E deficient mice. The healthy male Kunming mice (n=10) and absence of ApoE gene (ApoE-/-) mice (30) were chosen and randomly divided into 4 groups: blank control group, Fucoidan treatment group, (ApoE-/-) negative AS group and the (ApoE-/-) the negative control group. The effects of Fucoidan on lipid metabolism and AS plaques in model animals were observed by using ELASA kit which helps to detect blood biochemical indexes in the peripheral blood of the AS model. Using HE staining, oil red O staining, and immunohistochemistry methods to detect the expression of vascular endothelial growth factor (VEGF) in oxidative stress signal pathway. The blank control group, Fucoidan treatment group, ApoE (-/-) AS model group were compared with the negative control group. 4 group animal blood biochemical indexes in peripheral blood were tested. The total cholesterol, triglyceride and other indexes of Fucoidan treatment group was compared with the AS model control group (P<0.05). Low density lipoprotein cholesterol levels increased significantly (P<0.05). HE staining and oil red O staining showed that the size of atherosclerotic plaques in the Fucoidan treatment group was significantly smaller than that of the ApoE (-/-) AS model group (P<0.05). Immunohistochemistry result showed that the level of Fucoidan in the treatment group with the ApoE VEGF was significantly higher than that of AS model group (P<0.05). Fucoidan in brown fat can decrease total cholesterol and triglyceride levels in the peripheral blood, improve the level of LDL cholesterol, reduce the formation of atherosclerotic plaque, enhance oxidative stress signaling pathways in the related factor, protein expression.**

Keywords: Fucoidan; Atherosclerosis; Oxidative stress; Vascular endothelial growth factor

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1. Introduction

Atherosclerosis (AS) is the most common and important type of arteriosclerotic vascular disease. It is the main cause of coronary heart disease, cerebral infarction and peripheral vascular disease[1]. So far, the pathogenesis of AS is not yet clear. There are many theories related to many risk factors, which lead to the lack of effective targeted drugs. Studies have confirmed that oxidized low density lipoprotein (ox-LDL) can promote the development of AS through a variety of ways, eventually leading to plaque rupture and acute cardio cerebral vascular disease[2-3]. Inhibition and treatment of cardiovascular and cerebrovascular diseases of AS has not yet found effective prevention and treatment ways. To find a safe and effective, non-toxic side effects and good social benefits, and can effectively alleviate the lipid metabolic disorder in patients with AS, reduce AS and vascular complication incidence of new drugs is very important.

Fucoidan sulfate (Fucoidan) has a similar anticoagulant activity with heparin, it can effectively inhibit platelet aggregation and thrombosis[4]. Fucoidan is a natural macromolecule polymer in brown alga, and has many biological activities, such as antioxidant, hypolipidemic, anticoagulant, anti adhesion, anti thrombotic, anti-inflammatory, antiviral, anti mutation, anti-tumor and immunomodulation. In this study, the effects and mechanisms of Fucoidan on atherosclerosis were explored by establishing a model of atherosclerotic mice.

2. Materials and Methods

2.1. Establish AS model

This experiment adopts the SPF 8 weeks old male 10 Kunming mice (weighing 46-59G) and ApoE (-/-) 30 mice (body weight 25-33g). Kunming mice were purchased from Beijing Hua Fukang Biological Technology Services Company, number 11401300014365. At room temperature =22+2℃, humidity =60+5%, under natural light conditions, free feeding drinking water. The AS animal model was induced with high fat diet (including 21% fat and 0.15% cholesterol), and the success rate of the model was 80%.

2.2. Experimental grouping and drug delivery method

The mice were kept under the standard of experimental condition. Mice in each cage were grouped as follows: A: blank control group: 10 male Kunming mice were fed with ordinary feed. B: ApoE (-/-) negative control group: ApoE (-/-) 10 mice were

fed with normal diet. C: ApoE (-/-) AS (-/-) ApoE model group: 10 mice fed with high fat diet. D: Fucoidan group: ApoE (-/-) mice 10, high fat diet, 200 mg/kg Fucoidan solution gavage. After a week of adaptive feeding, all the mice were treated with the above concentration at morning 08:00-10:00 every two days. The drug was given for 10 weeks.

2.2.1. General Indicators

Weight, food intake, appearance and signs, behavior, hair status, mental status and death status were observed and recorded in each group during administration.

2.2.2. Blood Specimens

Second days after the end of the treatment, 10% chloral hydrate was injected into the mice by intraperitoneal injection, and the blood was collected from the heart by 1-2ml. 3H and 4000 rpm were centrifuged for serum and preserved at 4℃.

2.2.3. Tissue Specimens

After taking blood, 10 rats in each group were taken, and the tissues of mice heart, liver and aorta were removed. After liquid nitrogen was frozen rapidly, the samples were stored at -80℃for further experiments. In the rest of the mice, the heart, liver and aorta were removed and kept in 4% polycondensation Formaldehyde Solution at 4℃.

2.2.4. Frozen Sections

The tissues fixed by 4% polyformaldehyde were placed in 20% sucrose solution, 30% sucrose solution in turn, and statically dehydrated at 4 centigrade. The completely dehydrated tissue was embedded in OCT, frozen in liquid nitrogen quickly, the frozen slice machine was sliced continuously, the thickness was 10 um, and dried at 4℃ naturally.

2.3. Detection index and experimental method

Detection of serum biochemical indexes: the automatic biochemical analyzer (CX-7, Beckman) was used to test the blood biochemical index: total cholesterol, triglyceride, low-density lipoprotein and oxidized low density lipoprotein, high density lipoprotein, apolipoprotein A, B and E, calculate the blood lipid index and body fat content in AS. Aortic frozen oil red O staining: 4% aortic poly formaldehyde fixed double distilled water rinse, 60% isopropyl alcohol soaking, dry filter paper specimens surface moisture in oil red O stain min 2, 60% isopropanol to differentiate into interstitial clear, distilled water, hematoxylin dye 1.5min 1% hydrochloric acid alcohol differentiation of 10s distilled water, flushing, glycerogelatin mounting, AS mice aortic plaque location under a microscope, and calculate the plaque size and area. Hematoxylin eosin (HE) staining: frozen sections of distilled water rinse 2min, hematoxylin staining in 5 min 30 s, tap water, wash away the excess dye; 1% hydrochloric acid alcohol differentiation of 5 s, 15 min back to blue, eosin staining 90 s, gradient dehydration, xylene transparent, neutral resin under observation the plaque area under light microscope, morphology. Immunohistochemical staining: the frozen section was operated according to the instructions of the hypersensitive two step kit of Zhonghujin Bridge. VEGF was incubated at 4℃ for the night. After washing, TBS was labeled with streptomycin, horseradish enzyme (SABC), labeled with two antibodies, incubated at 37℃ for 20min. DAB was positive, and the positive cells were brown. Under the optical microscope, each slice was randomly selected 5 fields of vision and counted the positive cells, and the mean value was taken. The protein expression level was expressed in the positive cell index (number of positive cells/cells).

2.4. Statistical analysis

The results of the experiment were analyzed with the SPSS software version 19.0. One-way-anova was used in the comparison between the two groups. $P<0.05$ was significant difference.

3. Results

3.1. Test results of serum biochemical indexes in each group

Triglyceride in serum biochemical indices (triglycerides, TG), total cholesterol (total, cholesterol, TC), high density lipoprotein cholesterol (high density lipoprotein cholesferol, HDL-C), low density lipoprotein cholesterol (low density lipoprotein cholestorol, LDL-C) were compared between ApoE group and ApoE (-/-) AS (-/-) ($P<0.05$). ApoE (-/-) negative control group was compared with the control group, ($P<0.05$). Fucoidan treatment group and ApoE (-/-) AS model group, TG, TC, LDL-C in the index, the Fucoidan treatment group was significantly lower than that in ApoE (-/-) AS model group ($P<0.05$). The HDL-C in the Fucoidan treatment group was higher than that of the AS model control group ($P<0.05$). Comparison of apolipoprotein A1 and apolipoprotein B in ApoE (-/-) group and ApoE (-/-) AS model was compared to the negative control group ($P<0.05$). ApoE (-/-) negative control group was compared with the control group ($P<0.05$), (Table 1).

Table 1. Comparison of serum biochemical indexes in each group

Group	TG	TC	HDL	LDL	ApoA1	ApoB
Blank control group	2.532	3.865	1.256	0.584	0.010	0.028
Fucoidan	1.613#	22.867*	4.027*#	6.119*	-0.007	0.028
ApoE(-/-)AS	2.324*	23.461*	3.184*#	6.273*	-0.008	0.022
ApoE(-/-) negative	1.796#	10.666#	2.324#	3.929#	0.001	0.054

Note: * ApoE (-/-) and negative control group; # and ApoE (-/-) AS model group

3.2. The results of Hematoxylin Eosin (HE) staining in each group

The volume of atherosclerotic atherosclerotic plaque in the model mice showed that there was no atherosclerotic plaque in the blank control group. The atherosclerotic plaque volume of blank control group, negative control group (ApoE-), ApoE (-/-) AS model group and Fucoidan treatment group were (218744 + 36728), (217986 + 87463), (268744 + 88974), (292892 + 93874) m3 respectively. The aortic atherosclerotic plaque volume of ApoE (-/-) AS model group was significantly greater than the control group (P<0.05). The aortic atherosclerotic plaque volume of ApoE (-/-) negative control group was compared with the control group (P>0.05). The aortic atherosclerotic plaque volume of Fucoidan group was compared with the control group (P>0.05), (Figure 1).

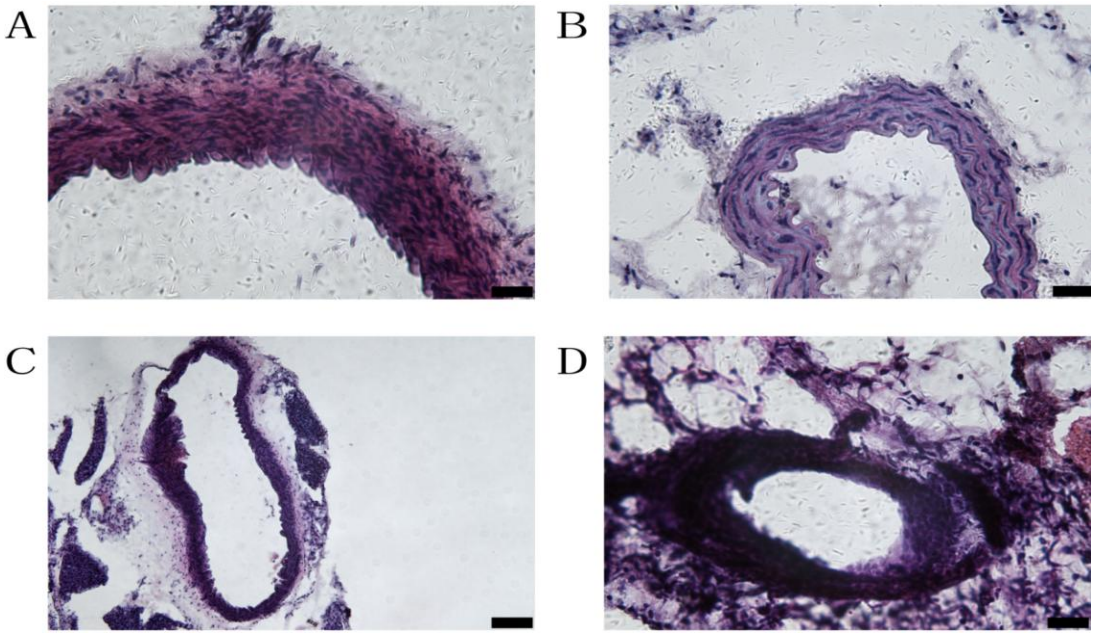

Figure 1. Hematoxylin eosin (HE) staining (200X). (A) Blank control group HE staining showed that the arterial wall was thicker and conformed to the normal structure. (B) ApoE (-/-) negative control group, HE staining showed that the artery wall was thin, with the pathological changes of artherosclerosis. (C) ApoE (-/-) AS model group, HE staining showed that the artery wall was thin, with the pathological changes of artherosclerosis. The HE staining in the (D) Fucoidan treatment group showed that the arterial wall was thicker than the AS group.

3.3. The result of Oil Red O staining in frozen section of aorta

The volume of atherosclerotic atherosclerotic plaque in the model mice showed that there was no atherosclerotic plaque in the blank control group. The atherosclerotic plaque volume of blank control group, negative control group (ApoE-), ApoE (-/-) AS model group and Fucoidan treatment group were (165541 + 41973), (168893 + 52918), (278947 + 67453), (290874 + 45622) m3 respectively. The aortic atherosclerotic plaque volume of ApoE (-/-) AS model group was significantly greater than the control group (P<0.05). The aortic atherosclerotic plaque volume of ApoE (-/-) negative control group was compared with the control group (P>0.05). The aortic atherosclerotic plaque volume of Fucoidan group was compared with the control group (P>0.05) (Figure 2).

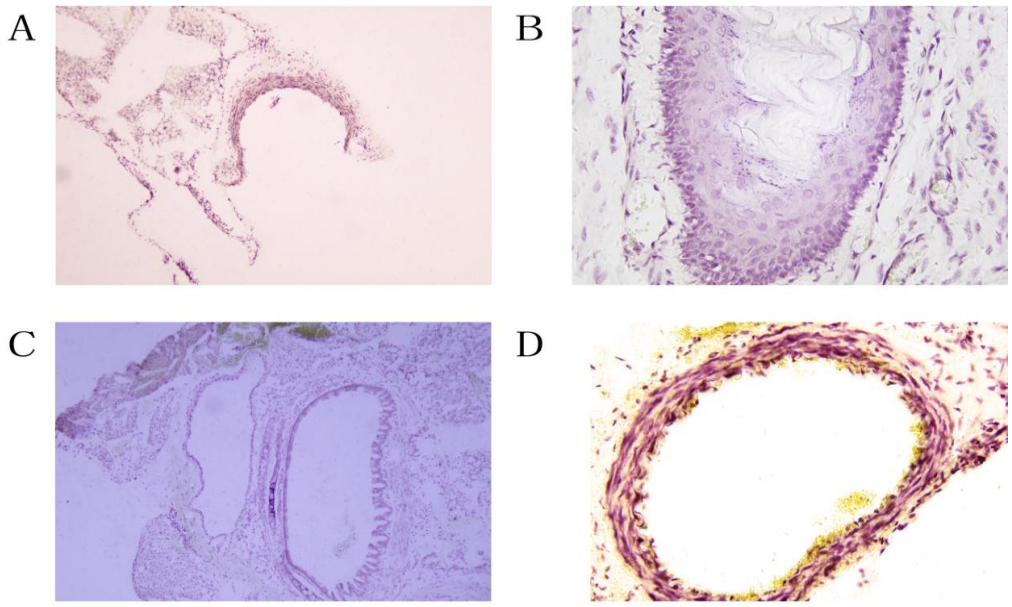

Figure 2. Staining results of frozen section with oil and red O (200X). (A) Blank control group oil red O staining showed that the arterial wall was thicker and conformed to the normal structure. (B) ApoE (-/-) negative control group, oil red O staining showed that the artery wall was thin, with the pathological changes of artherosclerosis. (C) ApoE (-/-) AS model group, oil red O staining showed that the artery wall was thin, with the pathological changes of artherosclerosis. The results of oil red O staining in the (D) Fucoidan treatment group showed that the arterial wall was thicker than the AS group.

3.4. The results of immuno histochemical analysis in each group

Immunohistochemistry results showed that the brown yellow positive particles were mainly expressed in the membrane of the aortic endothelial cells of the model mice. No VEGF protein expression was found in the blank control group. ApoE (-/-) was significantly greater than the control group the expression of VEGF protein AS in the model group ($P<0.05$). There was no significant difference between aortic expression of VEGF protein in ApoE-/- negative control group and control group ($P>0.05$). There was no significant difference of expression of VEGF protein between the blank control group and Fucoidan treatment group ($P>0.05$), (Figure 3).

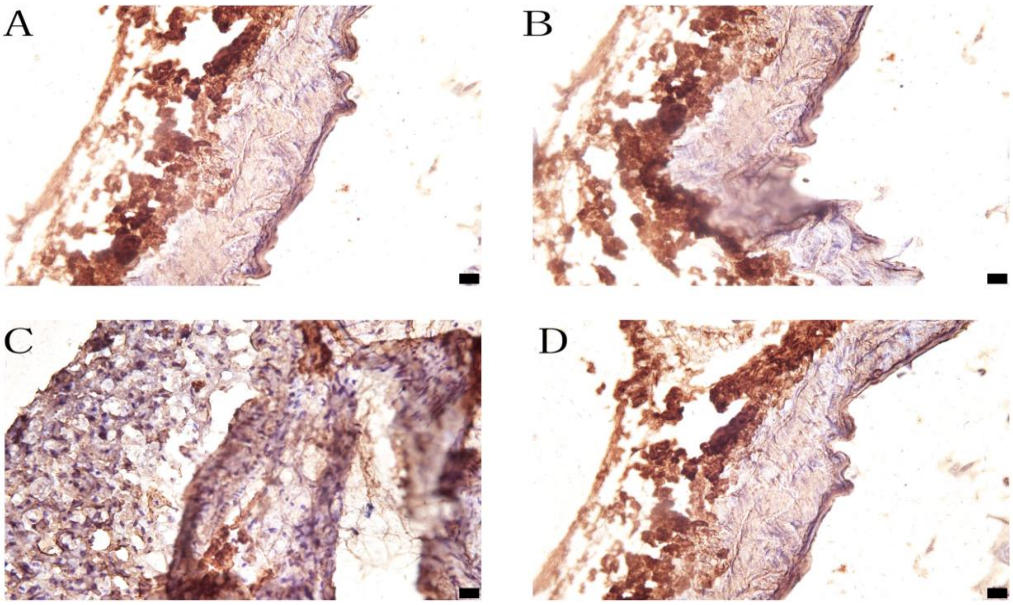

Figure 3. Results of immunohistochemical analysis (400X). (A) Blank control group. (B) ApoE (-/-) negative control. (C) ApoE (-/-) AS group. (D) Fucoidan treatment group.

4. Discussion

Atherosclerosis is a gradual change process, which starts with the lesions of endothelial cells, and then closely related to lipid deposition and inflammatory cell infiltration[5-7]. Fucoidan is a natural macromolecule polymer in brown algae. It is mainly composed of highly sulfated alpha -L- fucose and D-galactose, and a small amount of D- mannose, D-xylose, D- rhamnose, D- glucose, D- Arabia sugar and uronic acid. Fucoidan has anticoagulant activity similar to heparin, which can effectively inhibit platelet aggregation and form thrombus and produce antithrombotic effect[8-11]. Fucoidan is also a natural antioxidant. In this paper, the mechanism of Fucoidan's action on atherosclerosis and the mechanism of endoplasmic reticulum stress are discussed [12-14].

TG, TC, HDL-C, and LDL-C are the primary factors leading to atherosclerosis and all kinds of cardiovascular and cerebrovascular diseases. Recently, low density lipoprotein (LDL) is the only lipoprotein that can bring cholesterol into vascular endothelial cells, and it is the key material to promote atheromatous plaque formation. ApoE (-/-) AS group and ApoE (-/-) compared with the negative control group were significantly different ($P<0.05$). The experimental atherosclerosis model was constructed successfully. Fucoidan treatment group and ApoE (-/-) AS group, in TG, TC, LDL-C index, Fucoidan treatment group was significantly lower than that in ApoE (-/-) AS group. It also indicates that the development process of Fucoidan on atherosclerosis has obvious therapeutic effect and inhibition. The HDL-C in the Fucoidan treatment group was higher than that in the AS model control group. The Fucoidan has a certain inhibitory and therapeutic effects on hyperlipidemia and atherosclerosis. It is mainly caused by HDL-C. ApoE (-/-) AS (-/-) ApoE group and positive control group of apolipoprotein A1 and apolipoprotein B group, further illustrates the successful construction of the experimental atherosclerosis. HE staining and oil red staining showed that the aorta atherosclerotic plaque volume in the model mice was compared in the blank control group and no atherosclerotic plaque was found in the blank control group. The aortic atherosclerotic plaque volume of ApoE (-/-) AS group was significantly greater than that in the control group. The further evidence of the successful construction of atherosclerosis model in this experiment showed Fucoidan has obvious inhibitory effects on atherosclerosis.

To explore whether Fucoidan is related to endoplasmic reticulum stress in the mechanism of atherosclerosis, we used immunohistochemical method to compare the expression of endoplasmic reticulum stress marker protein VEGF in the above four groups. No obvious expression of VEGF protein was found in the blank control group. ApoE (-/-) was significantly greater than the control group the expression of VEGF protein in the AS group, which proves that the model is successfully constructed. The blank control group with statistically significant difference between the expression of VEGF protein in the aorta in Fucoidan treatment group shows that Fucoidan can inhibit the expression of VEGF protein, which can prove that Fucoidan marker protein VEGF the role of endothelial cells in arteries by endoplasmic reticulum stress, thereby inhibiting atherosclerosis.

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