Original Article
Inhibitory effects and related mechanisms of lycium barbarum polysaccharides on vascular lesions in type 2 diabetes mellitus

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Abstract: Objective: The aim of this study was to investigate the inhibitory effects and related mechanisms of lycium barbarum polysaccharides (LBP) on type 2 diabetes mellitus (T2DM) vascular lesions. Methods: A T2DM rat model was established by intraperitoneal injections of streptozotocin combined with a high-fat diet. Diabetic Sprague-Dawley rats were divided into a control group, T2DM group, experiment A group (group A), and experiment B group (group B). Group A and group B received 20 mg and 40 mg of LBP, respectively. The control group and T2DM group received the same amount of normal saline by gavage. The duration of the experiment was 4 weeks. At the end of the experiment, serum levels of insulin, glycosylated hemoglobin (HbA1c), triglycerides (TG), superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), low-density lipoprotein cholesterol (LDL-C), interleukin 4 (IL-4), and interleukin 6 (IL-6) were measured. H&E staining was used to detect pathological changes in vascular structures. MicroRNA and protein levels of p38 mitogen-activated protein kinase (p38MAPK) and intercellular adhesion molecule 1 (ICAM-1) in rat thoracic aortas were analyzed by qRT-PCR and Western blotting, respectively. Results: In group B, serum insulin was significantly increased after large doses of LBP but serum levels of HbA1c, LDL-C, TG, and IL-6 were significantly decreased (all P<0.05). Compared with group A, serum levels of IL-4, SOD, and GSH-PX increased significantly in group B but levels of serum HbA1c, LDL-C, TG, and IL-6 decreased significantly (all P<0.05). H&E staining showed that, compared with T2DM group, the degree of vascular lesions in group A and group B was lower. Compared with group A, mRNA and protein levels of p38MAPK were significantly increased and those of ICAM-1 were decreased in group B (all P<0.05). Conclusion: LBP reduces inflammatory response and vascular lesions induced by T2DM through regulating p38MAPK signaling pathways and increasing antioxidative capacity.

Keywords: Lcium barbarum polysaccharides, type 2 diabetes mellitus, vascular lesions

Introduction

Diabetes mellitus (DM) is a common clinical disorder of glucose and lipid metabolism. Type 2 diabetes mellitus (T2DM) is the most common type. DM has become the third most common chronic disease in humans, severely affecting life and health [1]. Vascular lesions are an important feature of patients with diabetes. They are a major cause of complications, such as hypertension and diabetic nephropathy [2]. The mechanism of diabetic vascular lesions is relatively complex. Mancia et al. found that secretion imbalance of inflammatory cytokines and oxidative stress response could lead to dysfunction of vascular endothelial cells [3]. At

present, it is considered that an increase of inflammatory reactions and excessive free radicals *in vivo* is the first step in vascular lesion genesis [4]. Studies have shown that inactivation of the p38 mitogen-activated protein kinase (p38MAPK) signaling pathways in diabetic rats can trigger oxidative stress system dysfunction, inflammatory cytokines secretion disorder, and vascular lesions [5]. DM is a chronic disease that is difficult to cure. The effects of clinical treatment of chronic complications caused by vascular lesions have not been satisfactory. As a result, more patients eventually die of diabetes-related cardiovascular disease [6]. Therefore, it is of great clinical significance to explore drugs that effectively

Table 1. Gene primer sequence

Gene	Sequence
p38MAPK	
Forward primer	5'-CTACCGATCAGCACCTCAGC-3'
Reverse primer	5'-CACTGCATGCACGTACCTAC-3'
ICAM-1	
Forward primer	5'-CCCATCTATGAGGGTTACGC-3'
Reverse primer	5'-CGTTGCGACTGCCGCATTAC-3'
β-actin	
Forward primer	5'-CTGTCCAGTCACCTACGTGC-3'
Reverse primer	5'-CTAACTACGACGTGACTCAC-3'

Note: p38MAPK, p38 mitogen-activated protein kinase; ICAM-1, intercellular adhesion molecule 1.

delay or reverse diabetic vascular lesions. Lycium barbarum polysaccharides (LBP) is water-soluble glycoprotein complex extracted from the Chinese wolfberry fruit. Animal studies have shown that LBP can lower blood sugar and enhance the body's antioxidant function [7]. The objective of this study was to analyze the mechanisms of LBP action on vascular lesions in T2DM rats and provide an experimental basis for treatment of T2DM.

Materials and methods

Animals

This study was approved by the Animal Welfare Ethics Committee of Dongzhimen Hospital, Beijing University of Chinese Medicine. Eighty specific pathogen free (SPF) grade Sprague Dawley (SD) rats (40 males and 40 females) were purchased at 16 weeks old (License No.: SCXK (Su) 2009-0001) and randomized into control (n=10) and diabetic (n=70) groups. Rats in the control group were fed a normal diet. Those in the diabetic groups were given a high-fat diet and were injected intraperitoneally with streptozotocin at 35 mg/kg (in 0.1 mM citric acid buffer, mass fraction 5%, pH=4.5) to induce type 2 diabetic vascular lesions [8]. After 2 days, successfully modeled rats, those displaying blood glucose levels higher than 16.7 mM, were divided into the T2DM group, experiment A group (group A), and experiment B group (group B), with 10 rats per group [9]. Group A rats were given 20 mg LBP daily by gavage. Group B were given 40 mg of LBP and rats in control and T2DM group were given the same amount of normal saline for the entire 4-week duration of the experiment.
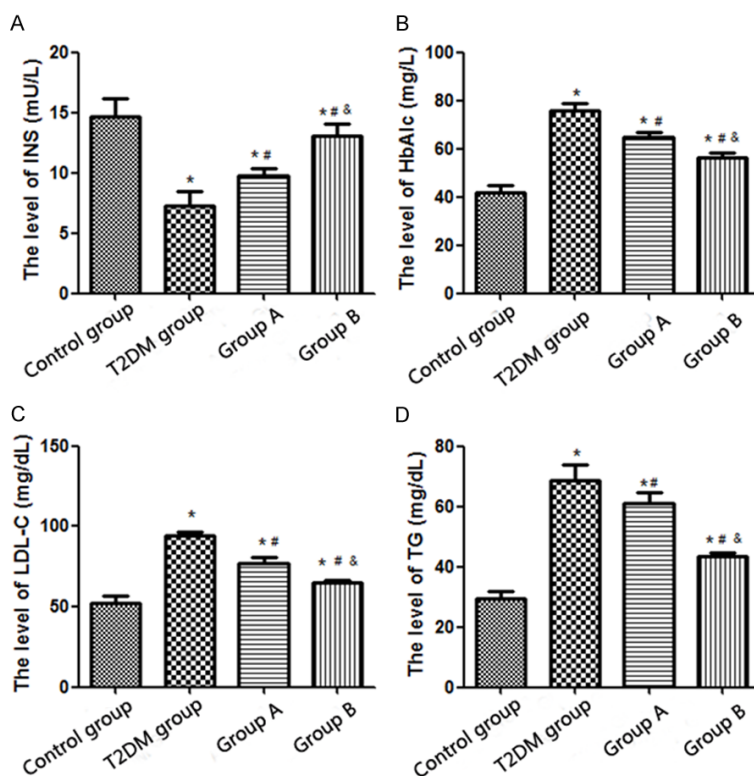
Detection of serum indices of glucose and lipid metabolism

After the last LBP dose, rats of group A and group B were fasted for 12 hours and then sacrificed. Their abdomens were incised and 6 mL of blood was collected from abdominal aortas. After centrifugation at 1,200 rpm, serum was collected and stored at -20°C. Serum triglycerides (TG) and low-density lipoprotein-cholesterol (LDL-C) levels were measured using an automatic biochemical analyzer (Olympus Corporation, Japan). Serum superoxide dismutase (SOD) levels were measured by the nitroblue tetrazolium method. Finally, serum levels of insulin, glutathione peroxidase (GSH-PX), interleukin 4 (IL-4), and interleukin 6 (IL-6) were measured by ELISA. After washing the 96-well plates, according to instructions for the ELISA kit (Nanjing Jiancheng Biotechnology Co., Ltd., Nanjing, China), the standard dilution gradient, blank controls, and sample wells were each set up in triplicate. Plates were incubated at 37°C for 30 minutes and then washed with a detergent based buffer. After adding the enzyme working solution to each well (except the blank control group), the plate was sealed with a sealing film and incubated for 30 minutes at 37°C. The colorimetric reaction was initiated with 50 μL of the color reagent and developed in the dark at 37°C for 20 minutes. After adding the stop solution, OD values of each well were measured with a microplate reader (Bio-Tek, USA) at 450 nm. Standard curve was plotted according to the OD value of the relevant standard product.

Histopathological changes in rat aortas

After drawing blood from the rats, their aortas were dissected and washed in sterile saline to remove all traces of blood. The aortas were fixed with 4% paraformaldehyde for 20 hours. They were then dehydrated in ethanol gradient before embedding in paraffin. The paraffin blocks were then sliced into 4 m thin sections for H&E staining, according to standard protocol. Briefly, tissue sections were baked at 60°C for 15 minutes to de-paraffinize, dehydrated by ethanol gradient, washed for 5 minutes, and then placed in a saturated lithium carbonate solution for 20 seconds. The slides were then soaked in 0.5% eosin stain for 6 minutes, dehydrated, cleared of excess stain with

Figure 1. Comparison of serum glucose and lipid metabolism indices in each group. Changes in levels of insulin (A), HbA1c (B), LDL-C (C), and TG (D) in all groups are shown. *P<0.05, compared to control group; #P<0.05, compared to T2DM group; &P<0.05, compared to group A. HbA1c, glycosylated hemoglobin; LDL-C, low-density lipoprotein cholesterol; TG, triglycerides; T2DM, type 2 diabetes mellitus.

xylene for 10 minutes, and sealed with neutral gum [9].

Detection of mRNA levels of p38MAPK and intercellular adhesion molecule 1 (ICAM-1) in vascular tissues

A total of 80 mg of vascular tissue was grounded with a mortar after being frozen in liquid nitrogen. Total RNA of the tissue was extracted with TRIzol Reagent (Invitrogen, USA) and processed according to manufacturer instructions. Reverse transcription was performed using 5 μg total RNA. Sequences of p38MAPK, ICAM-1, and β-actin genes were obtained from the gene library and used for designing the corresponding primers with Prime 6.0. PCR reaction conditions were 35 cycles of 95°C for 5 minutes, 94°C for 15 seconds, 55°C for 30 seconds, and 72°C for 45 seconds, and 72°C for 6 minutes. With β-actin gene as an internal reference, relative levels of the gene of interest were calculated by $2^{-\Delta\Delta Ct}$. Primer sequences are listed in **Table 1**.

Detection of p38MAPK and ICAM-1 proteins in vascular tissue

Total protein was extracted from 0.1 g of arterial tissue and concentrations were determined. Protein sample and loading buffer (mixture of 0.6 mL of 0.5 mol/L Tris-HCl, 2 mL of 10% SDS, 5 mL of 50% glycerol, 0.5mL of 2-mercaptoethanol, and 1 mL of 1% bromophenol blue) were mixed at a ratio of 1:5 (v/v) and then denatured by boiling for 10 minutes. Protein samples (30 μL) and standard (5 μL) were loaded into 10% SDS-PAGE gel and electrophoresed for about 2 hours at 60 V. Proteins were transferred to a PVDF membrane by the wet transfer method and washed with TBST buffer containing 0.05% Tween for 2 minutes. The PVDF membrane was then blocked for 2 hours with slow shaking and incubated overnight with the diluted primary antibody at 4°C. The PVDF membrane was then washed three times (10 minutes each time) with TBST and incubated for 1.5 hours with HRP-labeled secondary antibody at 37°C. Color was developed by ECL chemiluminescence method. After photographing, relative expression levels of protein were analyzed by Quantity One software.

Statistical analysis

All data were analyzed using SPSS 19.0 software and are expressed as mean ± standard deviation ($\bar{x}$ ± sd). T-test was used for data that met normal distribution and one-way ANOVA test was used for multiple groups of data. P<0.05 is considered statistically significant.

Results

Effects of LBP on serum glucose and lipid metabolism indices in T2DM rats

Serum insulin level in the T2DM group was significantly lower than the control group (P<0.05).
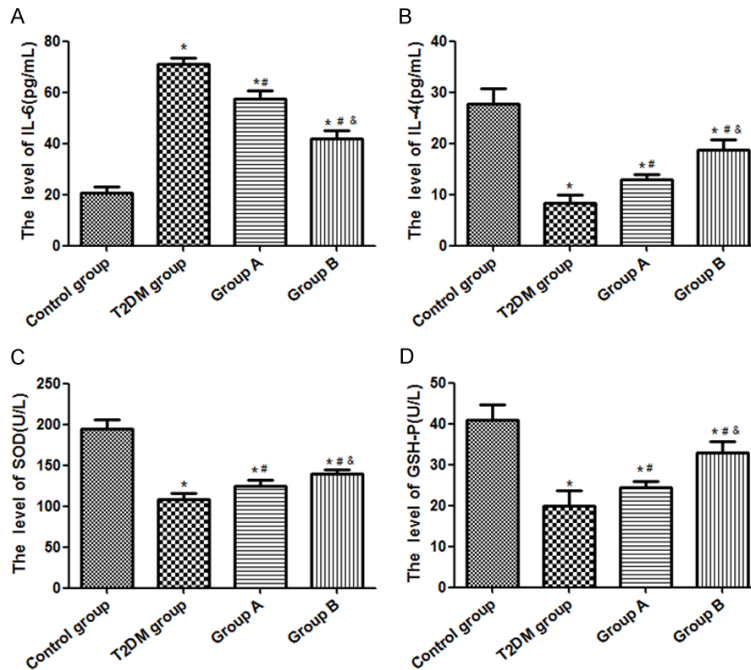
Figure 2. Comparison of serum inflammatory cytokines and oxidative stress indices in each group. Changes in serum levels of IL-6 (A), IL-4 (B), SOD (C), and GSH-PX (D) are shown respectively. *P<0.05, compared to control group; #P<0.05, compared to T2DM group; &P<0.05, compared to group A. IL, interleukin; SOD, superoxide dismutase; GSH-PX, glutathione peroxidase; T2DM, type 2 diabetes mellitus.

Serum insulin levels in group A and group B increased significantly with LBP treatment compared to the T2DM group (both P<0.05). Levels of glycosylated hemoglobin (HbA1c), LDL-C, and TG significantly increased in the T2DM group compared with the control group (all P<0.05), while those in group A and group B with LBP treatment significantly decreased compared to the T2DM group (all P<0.05) as shown in **Figure 1**.

Effects of LBP on serum inflammatory cytokines and oxidative stress indices in T2DM rats

Compared to the control group, serum IL-6 and IL-4 levels in the T2DM group were significantly increased and decreased, respectively (both P<0.05). Compared with the T2DM group, IL-6 levels in rats of group A and group B were significantly decreased after taking LBP (both P<0.05), while IL-4 levels in the rats of group A and group B, after administration of LBP, increased significantly (both P<0.05). Compared with the control group, serum SOD and GSH-PX levels in the T2DM group were signifi-

cantly decreased (both P< 0.05), while compared with the T2DM group, serum SOD and GSH-PX levels in group A and group B were significantly increased after taking LBP (all P<0.05) as shown in **Figure 2**.

Effects of LBP on vascular lesions in T2DM rats

H&E staining showed regular vascular endothelium structures in the healthy control rats, along with normal endothelial cell morphology. In the T2DM model, the intimal surface of blood vessels was rough and endothelial cells were irregularly shaped. Moreover, foam cells had accumulated and the arrangement of smooth muscle cells was disorderly. These vascular lesions improved significantly in group A and group B after administration of LBP (**Figure 3**).

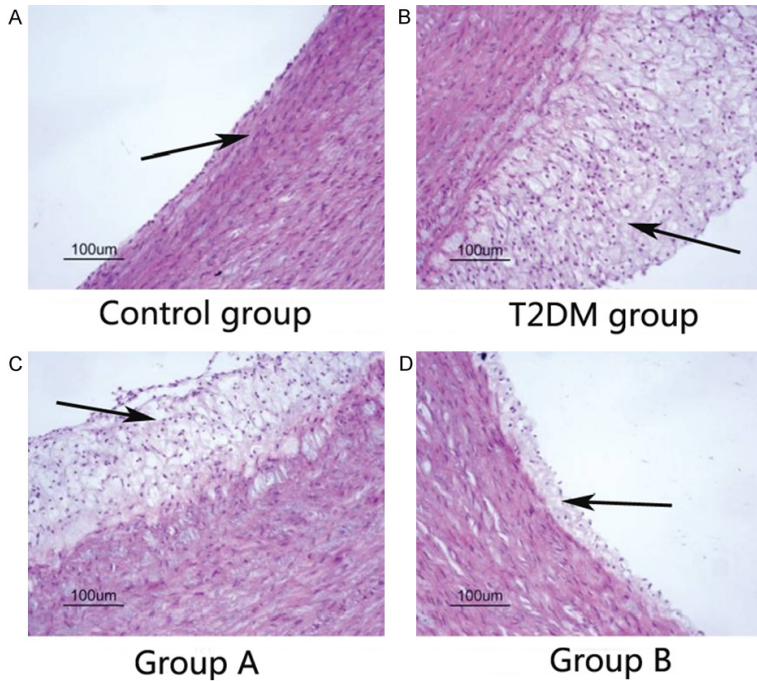*Effects of LBP on p38MAPK and ICAM-1 mRNA levels in rats with T2DM*

Compared to the control group, mRNA levels of ICAM-1 in the blood vessels of T2DM rats increased (P<0.05). Compared to the T2DM group, expression levels of ICAM-1 mRNA in group A and group B were decreased after administration of LBP (both P<0.05). Compared to the control group, mRNA levels of p38MAPK were decreased in the T2DM group (P<0.05). Compared to the T2DM group, mRNA levels of p38MAPK in group A and group B were increased significantly after administration of LBP (both P<0.05) as shown in **Figure 4**.

Effects of LBP on expression of p38MAPK and ICAM-1 proteins in vascular tissues of T2DM rats
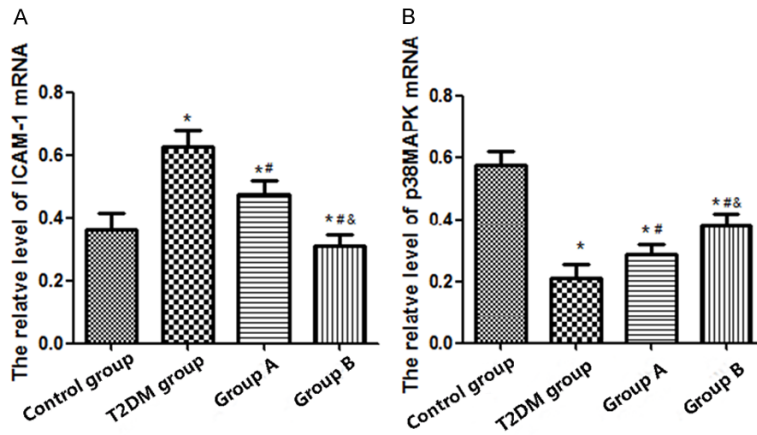
Compared to the control group, expression of p38MAPK in the blood vessels of T2DM rats was decreased (P<0.05). Compared to the T2DM group, expression levels of p38MAPK in group A and group B were both increased after administration of LBP (both P<0.05). Compared

Figure 3. H&E staining of vascular lesions in each group (200×). T2DM, type 2 diabetes mellitus.

Figure 4. Comparison of mRNA levels of p38MAPK and ICAM-1 in the blood vessels in each group. Expression levels of mRNAs of vascular ICAM-1 (A) and p38MAPK (B). *P<0.05, compared to control group; #P<0.05, compared to T2DM group; &P<0.05, compared to group A. p38MAPK, p38 mitogen-activated protein kinase; ICAM-1, intercellular adhesion molecule 1; T2DM, type 2 diabetes mellitus.

Discussion

Vascular lesions are a complication commonly found in patients with T2DM. They cause greater damage to the cardiovascular system and can increase the risk of other diseases, such as hypertension, diabetic nephropathy, etc. [10]. One study hypothesized that glucose and lipid metabolism disorders in T2-DM patients were the cause of vascular lesions, although the exact mechanisms were not yet known [11]. Hyperglycemia can also stimulate excessive free radical production, resulting in oxidation product accumulation in blood vessel walls, leading to structural and functional abnormalities [12]. SOD and GSH-PX are potent antioxidant enzymes that can effectively reduce levels of oxygen free radicals in the body [13]. This study found lower serum levels of SOD and GSH-PX in T2DM rats, which could result in an imbalance of the redox system, as shown previously [14]. Serum SOD and GSH-PX levels increased significantly in group A and group B after administration of a certain amount of LBP, suggesting that LPS could effectively reduce oxidative stress in T2DM rats. H&E staining showed that LBP reduced the extent of vascular lesions in T2DM rats. Therefore, it is of great clinical value to study the inhibitory effects of LBP on vascular lesions associated with T2DM.

to the control group, expression of ICAM-1 in blood vessels of T2DM group was increased (P<0.05). Compared to the T2DM group, expression of ICAM-1 was significantly decreased in both group A and group B after administration of LBP (both P<0.05) as shown in **Figure 5**.

Studies have shown that inflammatory factors damage insulin receptors and cause insulin resistance, leading to disorders in glucose and lipid metabolism [15]. Helfre et al. suggested that anti-inflammatory cytokine IL-4 and pro-inflammatory cytokine IL-6 are important in mediating inflammatory response [16]. In addi-

A

B

C
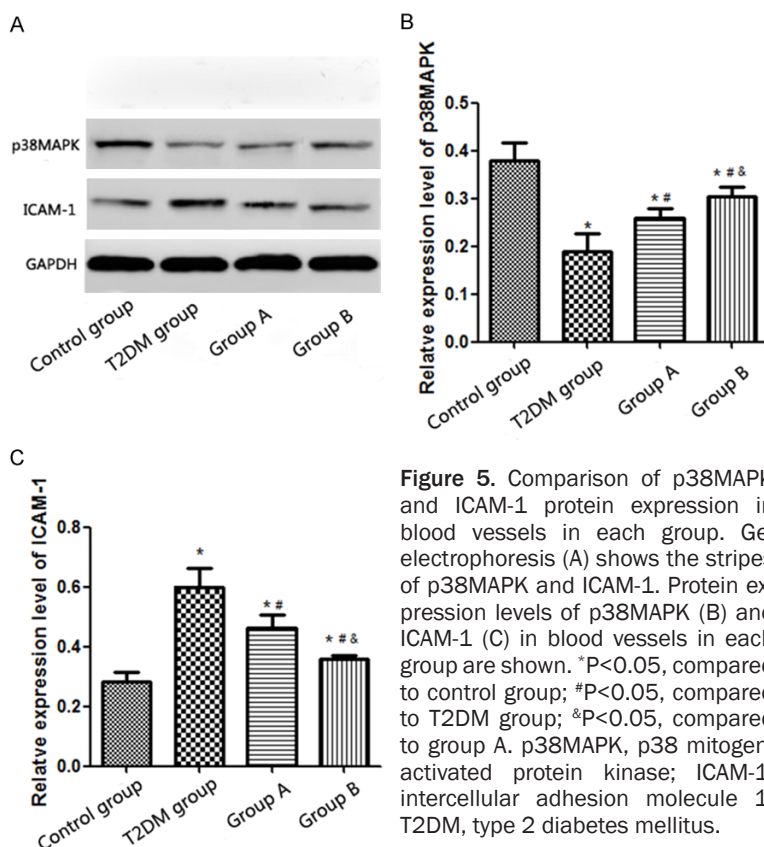
Figure 5. Comparison of p38MAPK and ICAM-1 protein expression in blood vessels in each group. Gel electrophoresis (A) shows the stripes of p38MAPK and ICAM-1. Protein expression levels of p38MAPK (B) and ICAM-1 (C) in blood vessels in each group are shown. *$P<0.05$, compared to control group; #$P<0.05$, compared to T2DM group; &$P<0.05$, compared to group A. p38MAPK, p38 mitogen-activated protein kinase; ICAM-1, intercellular adhesion molecule 1; T2DM, type 2 diabetes mellitus.

significantly increased. However, after administration of TPA polysaccharides, expression levels of ICAM-1 were significantly decreased. This result suggests that LBP can effectively increase expression of p38MARk protein kinase and reduce the level of adhesion factor, thereby inhibiting monocyte adhesion to the vascular wall. This is consistent with existing research [20]. T2DM vascular lesions are a complex physiological process. The present research only studied the effects of LBP on some related indicators or cytokines. Therefore, further studies concerning the mechanisms of LBP action, using genomics or the proteomics approach, are necessary.

In summary, LBP can effectively reduce inflammatory response, enhance antioxidant capacity, and regulate the activity of MAPK signaling pathways, thereby reducing T2DM vascular lesions.

Disclosure of conflict of interest

None.

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tion to aggravating the inflammatory cascade by a feedback regulation, pro-inflammatory cytokines can disrupt the cellular redox balance and aggravate vascular endothelial cell damage. Vlad et al. found a strong correlation between serum inflammatory cytokines and vascular lesions in T2DM rats [17]. In this study, it was found that LBP effectively reduced serum IL-6 levels and increased IL-4 levels in T2DM rats, thereby improving the balance between pro-inflammatory and inhibitory cytokines.

As an adhesion factor, ICAM-1 can induce many monocytes to adhere to blood vessel walls and further accelerate the cascade of inflammatory cytokines. This is the pathogenic basis of atherosclerotic plaque [18]. MAPK signaling pathways modulate this process via the p38MARK protein kinase which phosphorylates several downstream targets, ultimately transferring extracellular signals to vascular endothelial cells and inhibiting ICAM-1 overexpression [19]. It was found that levels of p38MARK protein were decreased in vascular tissues of T2DM rats and levels of ICAM-1 were

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