Int J Clin Exp Pathol 2016;9(10):10052-10059
www.ijcep.com /ISSN:1936-2625/IJCEP0030600

Original Article

The effect of astragalus polysaccharide on the therapeutics of diabetic cardiomyopathy in diabetic rats and its mechanism

Jingyu Yang[1], Xiaoyuan Xie[2], Hui Zhao[3], Yang Li[1], Bo Dong[1]

[1]Department of Cardiology, Tianjin Chest Hospital, Tianjin, China; [2]Department of Screening, Tianjin Women's and Children's Health Center, Tianjin, China; [3]Tianjin International Travel Healthcare Center, Tianjin Ntry-Exit Inspection and Quarantine Bureau of The People's Republic of China, Tianjin, China

Received April 15, 2016; Accepted August 1, 2016; Epub October 1, 2016; Published October 15, 2016

Abstract: Diabetes mellitus was a serious chronic illness in China and diabetic cardiomyopathy was one of its complication threating people's healthy. Astragalus polysaccharide was a natural product which applied diabetes mellitus therapeutics. Diabetic cardiomyopathy sawley rats modern was treated by astragalus polysaccharide to investigate the effect and mechanism of astragalus polysaccharide on diabetic cardiomyopathy. The results showed that astragalus polysaccharide could release the symptom of diabetic cardiomyopathy in rats and the level of oxidative stress was decreased. We considered that astragalus polysaccharide could inhibit diabetic cardiomyopathy in rats through decreasing oxidative stress.

Keywords: Astragalus polysaccharide, diabetic cardiomyopathy, oxidative stress

Introduction

The incidence of diabetes mellitus shows a gradually increasing trend in China. Diabetic cardiomyopathy (DC) is one of the complications of diabetes. The main pathological changes of DC include myocardial cell proliferation, ventricular hypertrophy, extracellular matrix accumulation and fibrosis [1]. DC may lead to heart failure and death in diabetic patients. In recent years, extensive clinical studies have shown that astragalus polysaccharide (APS) can significantly reduce the mortality of cardiovascular diseases in patients with diabetes mellitus, but the molecular mechanism is still unclear [2]. Some studies in recent years indicated that oxidative stress was closely related to the occurrence and development of DC [3]. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is an important source of the reactive oxygen species (ROS) in the body and is able to catalyze the conversion from O_2 to O_2^-; accordingly, NADPH is thought to play an important role in the pathogenesis of DC. This study was designed to study the effects of APS intervention on DC in rats and explore the molecular mechanism of APS responsible for the inhibition of oxidative stress and the pathological processes of DC.

Materials and methods

Study subjects

The 60 male Sprague-Dawley rats were randomized into normal control group (N), diabetes group (D), low-dose APS group (APS-1), high-dose APS group (APS-2) and fluvastatin group (Flu), with 12 rats in each group. Each rat received an intraperitoneal injection of 55 $mg \cdot kg^{-1}$ bolus dose of streptozotocin (STZ). Then, the blood glucose was determined in 72 h; blood glucose ≥300 mg/dL was used as the criteria for successful construction of diabetic rat model. After the molding, the following doses of drugs calculated by dose/body weight ratio were administered once daily for 12 successive weeks in each group by intragastric gavage: 200 $mg \cdot kg^{-1}$ for low-dose APS group; 700 $mg \cdot kg^{-1}$ for high-dose APS group, 20 $mg \cdot kg^{-1} \cdot d^{-1}$ for Flu positive control group; equal volume of normal saline for normal control group and model group.

Table 1. The effect of astragalus polysaccharide treatment on H/B and LVMI in different groups rats

Group	n	BW (g)	HW (g)	LVM (g)	H/B (10^{-3})	LVMI (10^{-3})
N	12	423±18	1.22±0.13	0.85±0.11	2.89±0.062	2.01±0.041
D	12	327±13$^{*,\Delta}$	1.83±0.15$^{*,\Delta}$	1.23±0.09$^{*,\Delta}$	5.560±0.073$^{*,\Delta}$	3.76±0.062$^{*,\Delta}$
APS-1	12	345±15$^{*,\Delta}$	1.54±0.07$^{*,\Delta}$	1.11±0.07$^{*,\Delta}$	4.46±0.046$^{*,\Delta}$	3.22±0.055$^{*,\Delta}$
APS-2	12	372±15$^{*,\Delta,\bigstar}$	1.42±0.07$^{*,\Delta,\bigstar}$	0.98±0.06$^{*,\Delta,\bigstar}$	3. 82±0.038$^{*,\Delta,\bigstar}$	2.63±0.048$^{*,\Delta,\bigstar}$
Flu	12	383±17$^{*,\Delta}$	1.31±0.07$^{*,\Delta}$	0.93±0.06$^{*,\Delta}$	3.42±0.032$^{*,\Delta}$	2.43±0.045$^{*,\Delta}$

$^{\Delta}$P<0.05 compared with normal group; *P<0.05 compared with diabetes mellitus group; $^{\bigstar}$P<0.05 compared with the astragalus polysaccharide low dose group.

Instruments and drugs

The male Sprague-Dawley rats (with body weights of 200 g±10 g) were purchased from Shanghai SLAC Laboratory Animal (License No.: SCXK (Shanghai) 2003-0003); Streptozotocin (STZ) and dihydrorhodamine-123 were purchased from Sigma; APS was purchased from Tianjin Cinorch Pharmaceutical; fluvastatin was purchased from Novartis Pharmaceutical; SOD activity, GSH-px activity and GSH content test kits were purchased from Beyotime Biotechnology; nucleoprotein extraction kit was purchased from Pierce; P22Phox, p47phox, NF-κb, FN, Col III and p38MAPK primary antibodies were purchased from Santa Cruz; Trizol reagent was purchased from Invitrogen; RNA reverse transcription kit was purchased from Fermentas; Multiskan MK3 microplate reader was purchased from Thermo, Calibur flow cytometer was purchased from BD.

Calculation of rat heart/body ratio and left ventricular mass index

The rats were sacrificed 12 weeks after the administration of APS and weighed. Under sterile conditions, the blood samples were collected and the left ventricular apex was excised and weighed. Then, the heart was removed and the heart weight (HW) was measured. After removing the atria and the right ventricular free wall, the left ventricular mass (LVM) was measured. The heart/body weight ratio (H/B) was calculated by formula H/B = HW/BW; the left ventricular mass index (LVMI) was calculated by formula LVMI = LVM/BW.

Determination of ROS expression in rat myocardial tissue

Rat myocardial tissue was chopped into pieces and filtered through a 300 mesh sieve; the dissociated cells were collected in DMEM medium containing 1 μmol·L^{-1} dihydrorhodamine-123 and 10% fetal bovine serum and cultured under 37°C and 5% CO_2 condition for 2 h; the cells were collected through centrifuging at 300 g/5 min and washed once with cold PBS. ROS expression in rat myocardial tissue was determined with flow cytometry (excitation at 488 nm, emission at 525 nm).

Determination of SOD and GSH-px activity in rat serum

Blood samples were collected with anticoagulant tubes and mixed evenly through inversion; after centrifuging at 4°C and 600 g/10 min, the supernatant was used to determine the intracellular SOD and GSH-px activity with reference to the kit instructions.

Determination of GSH expression in rat myocardial tissue

Rat myocardial tissue was chopped into pieces and filtered through a 300 mesh sieve to collect the cells; the cells were collected through centrifuging at 300 g/5 min, protein removal agent at 3-fold volume of the cell pellet was added, followed by rapid freezing and thawing twice in liquid nitrogen and 37°C water bath, ice bath for 5 min, and centrifuging at 4°C, 10,000 g/10 min. The supernatant was collected to determine GSH content according to the kit instructions.

p22Phox, p47phox, NF-κb, FN, Col III and p-Akt protein expression in rat myocardium

Rat myocardial tissue was chopped into pieces and grinded; the cells were lysed and the lysate was centrifuged at 4°C, 10,000 g/10 min; total tissue proteins were extracted from the supernatant. Rat myocardial tissue was chopped into

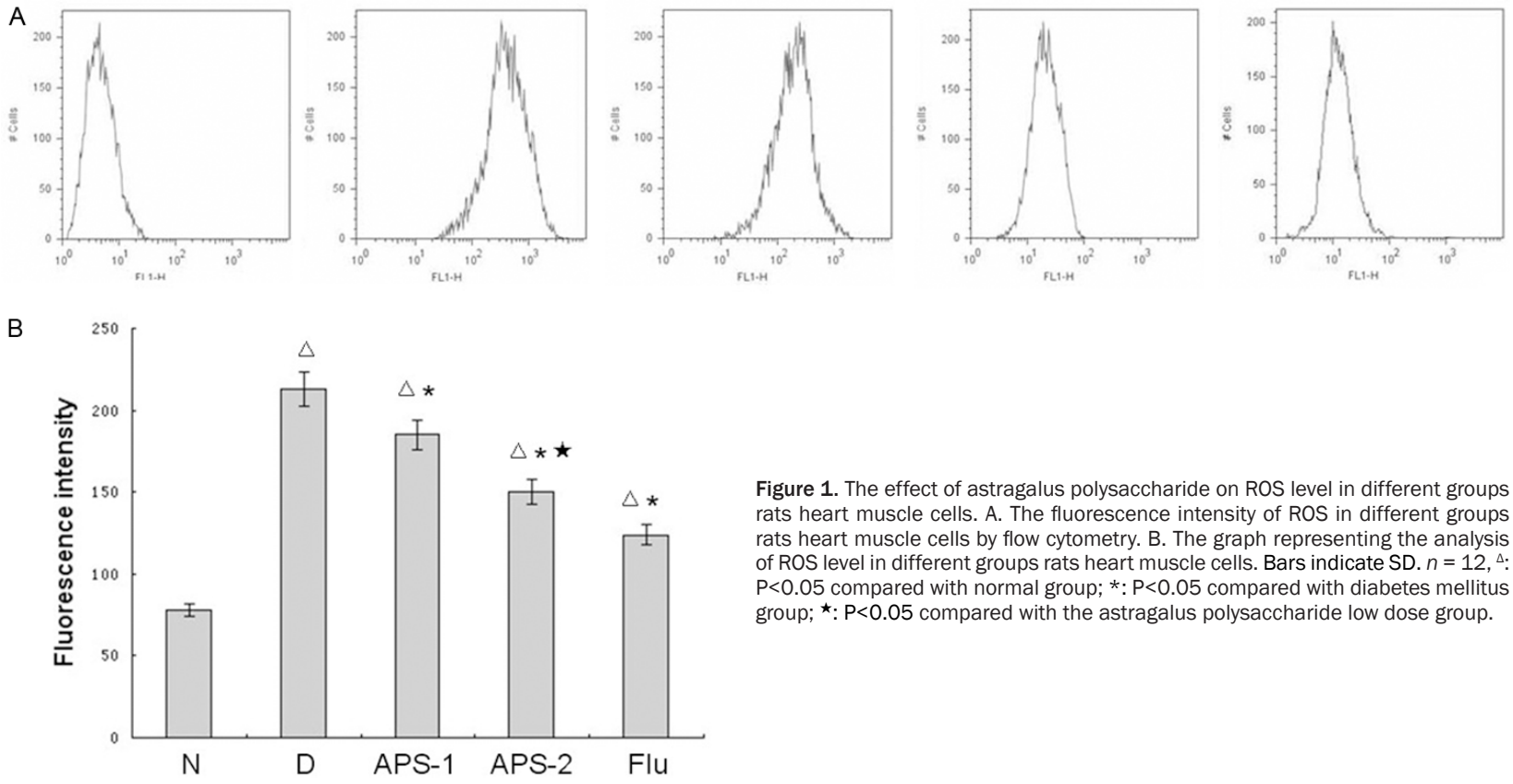

Figure 1. The effect of astragalus polysaccharide on ROS level in different groups rats heart muscle cells. A. The fluorescence intensity of ROS in different groups rats heart muscle cells by flow cytometry. B. The graph representing the analysis of ROS level in different groups rats heart muscle cells. Bars indicate SD. $n = 12$, $^{\Delta}$: $P<0.05$ compared with normal group; *: $P<0.05$ compared with diabetes mellitus group; ★: $P<0.05$ compared with the astragalus polysaccharide low dose group.

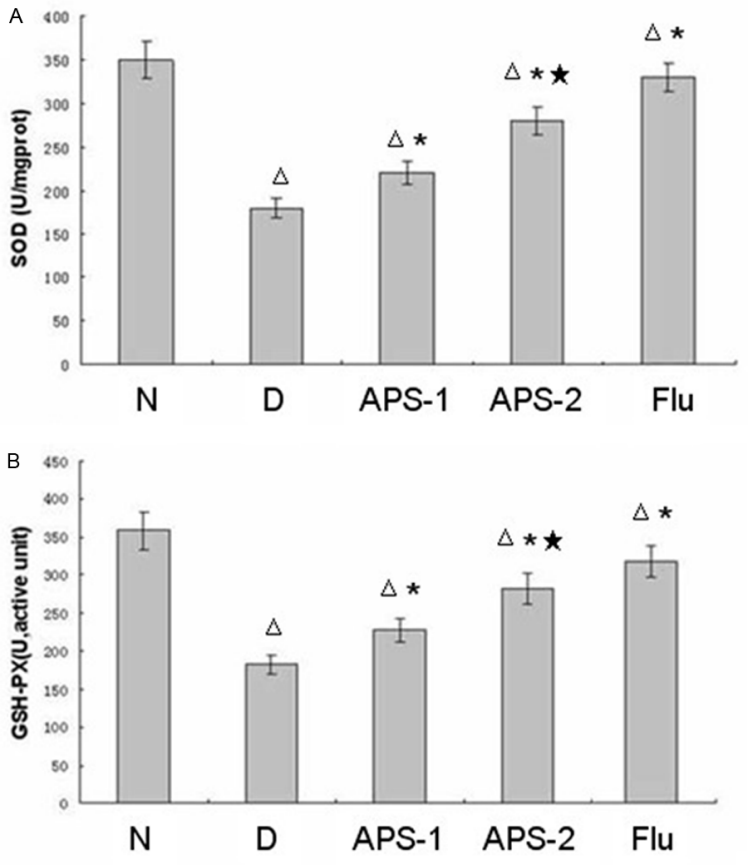

Figure 2. The effect of astragalus polysaccharide on the activity of SOD and GSH-px in different groups rats. The effect of astragalus polysaccharide on the activity of SOD in different groups rats (A) The effect of astragalus polysaccharide on the activity of GSH-px in different groups rats (B). Bars indicate SD. *n* = 12, △: P<0.05 compared with normal group; *: P<0.05 compared with diabetes mellitus group; ★: P<0.05 compared with the astragalus polysaccharide low dose group.

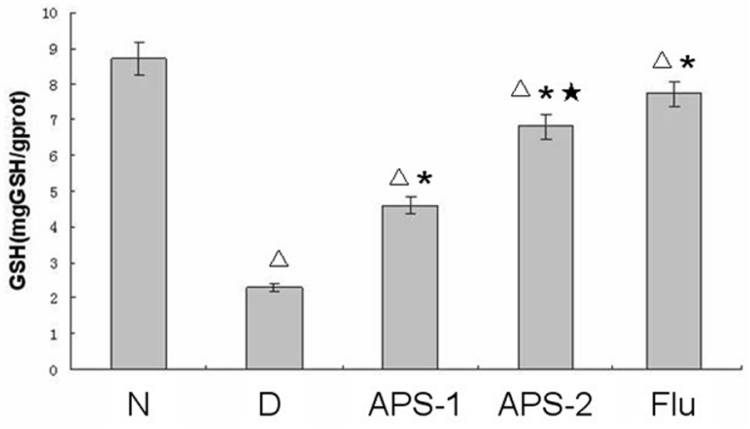

Figure 3. The effect of astragalus polysaccharide on the expression of GSH in different groups rats. Bars indicate SD. *n* = 12, △: P<0.05 compared with normal group; *: P<0.05 compared with diabetes mellitus group; ★: P<0.05 compared with the astragalus polysaccharide low dose group.

pieces and washed with cold PBS; after centrifuging at 4°C, 300 g/5 min, the cells were lysed with Cytoplasmic Lysis Buffer; then, the nucleoproteins were extracted with Nuclear Extraction Buffer. The proteins were electrophoresed in 12% SDS-polyacrylamide gel and transferred onto a PVDF membrane, followed by blocking in 5% skim milk at 4°C overnight; then, the respective primary antibody (P22Phox, 1:2500; p47phox, 1:2500; NF-κb, 1:800; FN, 1:2500; Col III, 1:2500; p-Akt, 1:1200; Akt, 1:2500) and β-actin (1:5000) were added to incubate at 4°C overnight; the membrane was incubated with HRP-labeled IgG (1:2000) at room temperature for 1 h, followed by washes and color development.

P22Phox, p47phox, NF-κb, FN and Col III mRNA expression in rat myocardium

Rat myocardial tissue was chopped into pieces and grinded; total RNA was extracted with TRIZOL reagent; using GAPDH as an internal reference; the total reaction volume was 20 μL. The following sequences were used for amplification: P22 Phox upstream primer sequence: 5'-CTCTATTGTTGCAGGAGTGC-3', downstream primer sequence: 5'-TCACACGACCTCATCTGTCAG-3'; p47 phox upstream primer sequence: 5'-GCTCACCGAGTACTTCAACA-3', downstream primer sequence: 5'-GCCTTCTGCAGATACATGGA-3'; NF-κb upstream primer sequence: 5'-GAAGAAGCGAGACCTGGAG-3', downstream primer sequence: 5'-TCCGGAACACAATGGCCAC-3'; FN upstream primer sequence: 5'-CAGTTTGTGGAAGTGACCGA-3', downstream prim-

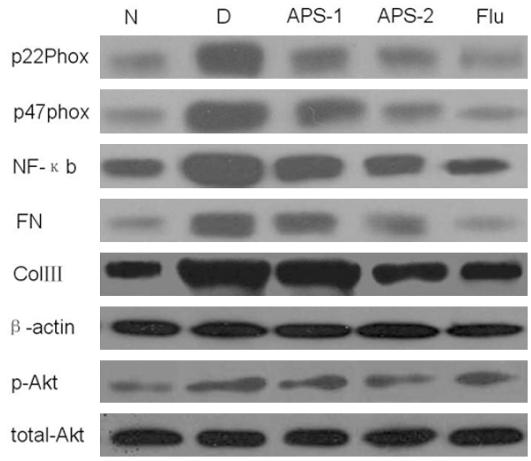

Figure 4. The effect of astragalus polysaccharide on the protein expression of p22Phox, p47phox, NF-κb, FN, Col III and p-Akt in different groups rats. The protein expression of p22Phox, p47phox, NF-κb, FN, Col III and p-Akt in different groups rats was measured by western blot assay. β-actin or total-Akt was used as an internal control for loading.

er sequence: 5'-TGGAGGTTAGTGGGAGCATA-3'; Col III upstream primer sequence: 5'-TCTGCAGGGGACCTTACAGT-3', downstream primer sequence: 5'-GGTCTTCCTGGAAGTAGAAC-3'; GAPDH upstream primer sequence: 5'-ACCACAGTCCATGCCATCTA-3'; downstream primer sequence: 5'-TCCACCACCCTGTTGCTGAC-3'. The 94°C 5 min was followed by 94°C 30 s, 65°C 45 s and 72°C 49 s, totally 40 cycles. The melting curves of PCR products were analyzed.

Statistical analysis

SPSS 11.0 software was used for one-way ANOVA; $P<0.05$ indicated statistically significant differences; each experiment was repeated 3 times.

Results

Effects of APS on rat H/B and LVMI

When compared with normal control group, both H/B and LVMI increased significantly in diabetes model group ($P<0.05$). After intervention with APS and Flu, both H/B and LVMI decreased significantly ($P<0.05$). When compared with low-dose APS group, the decrease in the high-dose APS group was more significant (**Table 1**), suggesting that APS was able to reduce myocardial hypertrophy of the whole heart and the left ventricle in diabetic rats in a dose-dependent manner.

Effects of APS on ROS expression in rat myocardial tissue

When compared with normal control group, ROS expression in rat myocardial tissue increased significantly in diabetes model group ($P<0.05$). After intervention with APS and Flu, ROS expression in rat myocardial tissue decreased significantly ($P<0.05$). When compared with the low-dose APS group, the decrease in high-dose APS group was more significant (**Figure 1**), suggesting that APS was able to reduce ROS expression in myocardial tissue of diabetic rats in a dose-dependent manner.

Effects of APS on SOD and GSH-px activity in rat serum

When compared with the normal control group, SOD and GSH-px activity in rat serum decreased significantly in diabetes model group ($P<0.05$). After intervention with APS and Flu, SOD and GSH-px activity in rat serum increased significantly ($P<0.05$). When compared with low-dose APS group, the increase in the high-dose APS group was more significant (**Figure 2**), suggesting that APS was able to increase SOD and GSH-px activity in the serum of diabetic rats in a dose-dependent manner.

Effects of APS on GSH expression in rat myocardial tissue

When compared with the normal control group, GSH expression in rat myocardial tissue decreased significantly in diabetes model group ($P<0.05$). After intervention with APS and Flu, GSH expression in rat myocardial tissue increased significantly ($P<0.05$). When compared with the low-dose APS group, the increase in the high-dose APS group was more significant (**Figure 3**), suggesting that APS was able to increase GSH expression in myocardial tissue of diabetic rats in a dose-dependent manner.

Effects of APS on p22Phox, p47phox, NF-κb, FN, Col III and p-Akt protein expression in rat myocardial tissue

When compared with normal control group, p22Phox, p47phox, NF-κb, FN, Col III and p-Akt protein expression in rat myocardial tissue increased significantly in diabetes model group ($P<0.05$). After intervention with APS and Flu, expression of the above proteins in rat myocar-

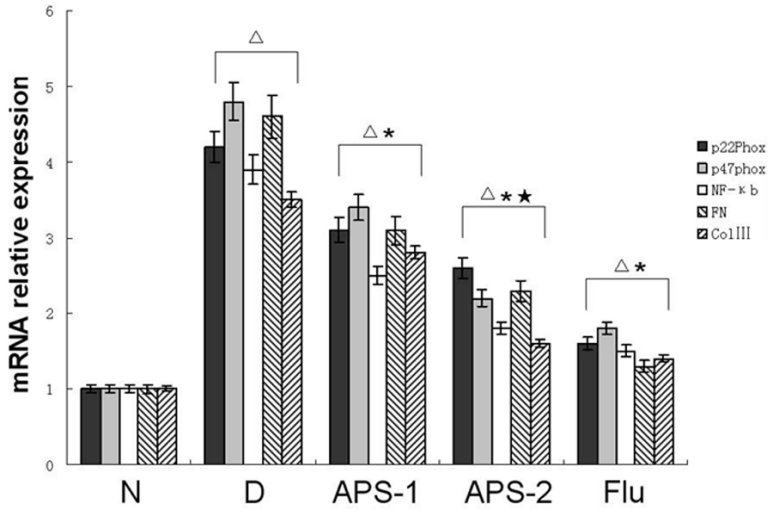

Figure 5. The effect of astragalus polysaccharide on the mRNA expression of p22Phox, p47phox, NF-κb, FN and Col III in different groups rats. The mRNA expression of p22Phox, p47phox, NF-κb, FN and Col III in different groups rats was measured by real time assay. GADPH was used as an internal control for loading. △: P<0.05 compared with normal group; *: P<0.05 compared with diabetes mellitus group; ★: P<0.05 compared with the astragalus polysaccharide low dose group.

dial tissue decreased significantly (P<0.05). When compared with the low-dose APS group, the decrease in high-dose APS group was more significant (**Figure 4**), suggesting that APS was able to reduce p22Phox, p47phox, NF-κb, FN, Col III and p-Akt protein expression in myocardial tissue of diabetic rats in a dose-dependent manner.

Effects of APS on p22Phox, p47phox, NF-κb, FN and Col III mRNA expression in rat myocardial tissue

When compared with the normal control group, p22Phox, p47phox, NF-κb, FN and Col III mRNA expression in rat myocardial tissue increased significantly in diabetes model group (P<0.05). After intervention with APS and Flu, mRNA expression of the above genes in rat myocardial tissue decreased significantly (P<0.05). When compared with the low-dose APS group, the decrease in the high-dose APS group was more significant (**Figure 5**), suggesting that APS was able to reduce p22Phox, p47phox, NF-κb, FN and Col III mRNA expression in myocardial tissue of diabetic rats in a dose-dependent manner.

Discussion

The incidence of diabetes mellitus shows a gradually increasing trend in China. DC, a common chronic complication of diabetes mellitus, is one of the primary causes of heart failure and sudden death in diabetic patients.

DC is mainly characterized by myocardial cell hypertrophy, myocardial interstitial fibrosis and myocardial cytopenia. The clinical symptoms of DC include diastolic dysfunction, ventricular systolic dysfunction and eventual occurrence of congestive heart failure [4]. Given the lack of effective treatment of DC at the present time, finding effective treatment drugs for DC become a focus of contemporary research.

Studies have shown that high sugar and high fat can cause oxidative stress; the amplified oxidative stress through chain reactions can result in myocardial cell injuries and apoptosis. Therefore, reducing the effects of oxidative cell injuries is an important measure to protect the myocardial cells in diabetic patients. Activation of reduced NADPH oxidase is thought to be one of the main reasons for ROS generation in vivo; inhibiting NADPH oxidase activity can effectively reduce oxidative injuries of myocardial cells in diabetic patients [5]. The p22phox and p47phox subunits are important NADPH oxidase subunits; the increased expression may be one of the important mechanisms responsible for elevated ROS expression in the body.

In addition to direct injuries of myocardial cells caused by oxidative reactions, higher ROS expression in the body can also increase the consumption of SOD, GSH-px and GSH [6]. Increased ROS and reduced SOD, GSH-px and GSH contents can disrupt the dynamic equilibrium between oxidative injury and anti-oxidative injury; this will result in the activation of a series of signal transduction pathways, subsequent Akt phosphorylation, NF-κb activation, and increased FN and Col III expression, leading to myocardial cell hypertrophy and myocardial interstitial fibrosis, and eventual the occurrence of DC [7].

Astragalus is believed to have the efficacy of "nourishing qi to invigorate spleen and promoting fluid production to quench thirst" since ancient times. It is also thought to be able to effectively improve insulin resistance and lower blood glucose. Because studies have proven that APS can reduce blood glucose, improve glucose tolerance and increased insulin sensitivity in rats with type 2 diabetes, this natural product is thought to be able to treat diabetes mellitus effectively [8]. Since it has been confirmed that Flu can effectively alleviate myocardial hypertrophy caused by DC in rats [9, 10], we selected Flu as a positive control to examine the therapeutic effects of APS on DC in rats.

In this study, APS was able to not only reduce blood glucose and lipid in rats with type 2 diabetes (data not shown) but also reduce H/B and LVMI, demonstrating its ability to improve myocardial hypertrophy and fibrosis in diabetic rats. During the exploration of the mechanism of APS in the treatment of DC in rats, we found in rat myocardial tissue that APS was able to reduce ROS expression, increase SOD and GSH-px activity, increase GSH content, restore the dynamic equilibrium between oxidative injuries and anti-oxidative injuries in the cells, and reduce NADPH oxidase subunits p22phox and p47phox at the same time. Therefore, in our opinions, one of the mechanisms of APS in the treatment of DC is to reduce the expression of NADPH oxidase subunits p22phox and p47phox, consequently reducing intracellular oxidative stress and inhibiting oxidative injuries of the myocardial cells.

We further investigated the signal transduction pathways involved in APS effects on DC. We found that APS was able to reduce Akt phosphorylation and consequently inhibit the activation of NF-κb. NF-κb is an important transcription factor in the body and able to regulate inflammatory and immune responses, which is thought to be closely related to a variety of physiological responses. The activated NF-κb can bind to DNA containing specific gene transcription sequences in the nucleus to promote FN and Col III expression, resulting in accumulation of myocardial extracellular matrix and interstitial fibrosis [11-14]. In our opinions, another mechanism of APS therapeutic effects on DC is to reduce Akt phosphorylation, inhibit NF-κb activation, reduce FN and Col III expression, and inhibit accumulation of myocardial extracellular matrix and interstitial fibrosis, leading to the restoration of myocardial function.

In summary, APS can effectively treat DC in rats, which provides an experimental basis for its clinical application. However, given the numerous biological activities of APS, further in-depth studies are warranted to elucidate its mechanisms of action on DC.

Disclosure of conflict of interest

None.

Address correspondence to: Jingyu Yang, Department of Cardiology, Tianjin Chest Hospital, Tianjin, China. E-mail: yangjingyu2001@126.com

References

[1] Kandula V, Kosuru R, Li H, Yan D, Zhu Q, Lian Q, Ge RS, Xia Z, Irwin MG. Forkhead box transcription factor 1: role in the pathogenesis of diabetic cardiomyopathy. Cardiovasc Diabetol 2016; 1: 44.

[2] Joubert M, Bellevre D, Legallois D, Elie N, Coulbault L, Allouche S, Manrique A. Hyperglycemia-Induced Hypovolemia Is Involved in Early Cardiac Magnetic Resonance Alterations in Streptozotocin-Induced Diabetic Mice: A Comparison with Furosemide-Induced Hypovolemia. PLoS One 2016; 2: e0149808.

[3] Jani Y, Kamberi A, Xhunga S, Pocesta B, Ferati F, Lala D, Zeqiri A, Rexhepi A. The influence of type 2 diabetes and gender on ventricular repolarization dispersion in patients with subclinic left ventricular diastolic dysfunction. Am J Cardiovasc Dis 2015; 4: 155-66.

[4] Frustaci A, Ciccosanti F, Chimenti C, Nardacci R, Corazzari M, Verardo R, Ippolito G, Petrosillo N, Fimia GM, Piacentini M. Histological and proteomic profile of diabetic versus non-diabetic dilated cardiomyopathy. Int J Cardiol 2016; 203: 282-9.

[5] Zhang M, Gu H, Chen J, Zhou X. Involvement of long noncoding RNA MALAT1 in the pathogenesis of diabetic cardiomyopathy. Int J Cardiol 2016; 202: 753-5.

[6] Hou J, Zheng D, Fung G, Deng H, Chen L, Liang J, Jiang Y, Hu Y. Mangiferin suppressed advanced glycation end products (AGEs) through NF-κB deactivation and displayed anti-inflammatory effects in streptozotocin and high fat diet-diabetic cardiomyopathy rats. Can J Physiol Pharmacol 2016; 3: 332-40.

[7] Wu H, Li GN, Xie J, Li R, Chen QH, Chen JZ, Wei ZH, Kang LN, Xu B.Resveratrol ameliorates

myocardial fibrosis by inhibiting ROS/ERK/TGF-β/periostin pathway in STZ-induced diabetic mice. BMC Cardiovasc Disord 2016; 16: 5.

[8] Ni R, Cao T, Xiong S, Ma J, Fan GC, Lacefield JC, Lu Y, Tissier SL, Peng T. Therapeutic inhibition of mitochondrial reactive oxygen species with mito-TEMPO reduces diabetic cardiomyopathy. Free Radic Biol Med 2016; 90: 12-23.

[9] Frustaci A, Ciccosanti F, Chimenti C, Nardacci R, Corazzari M, Verardo R, Ippolito G, Petrosillo N, Fimia GM, Piacentini M. Histological and proteomic profile of diabetic versus non-diabetic dilated cardiomyopathy. Int J Cardiol 2016; 203: 282-9.

[10] Hou J, Zheng D, Fung G, Deng H, Chen L, Liang J, Jiang Y, Hu Y. Mangiferin suppressed advanced glycation end products (AGEs) through NF-κB deactivation and displayed anti-inflammatory effects in streptozotocin and high fat diet-diabetic cardiomyopathy rats. Can J Physiol Pharmacol 2016; 3: 332-40.

[11] Hung YC, Yang HT, Yin MC. Asiatic acid and maslinic acid protected heart via anti-glycative and anti-coagulatory activities in diabetic mice. Food Funct 2015; 9: 2967-74.

[12] Suzuki H, Kayama Y, Sakamoto M, Iuchi H, Shimizu I, Yoshino T, Katoh D, Nagoshi T, Tojo K, Minamino T, Yoshimura M, Utsunomiya K. Arachidonate 12/15-lipoxygenase-induced inflammation and oxidative stress are involved in the development of diabetic cardiomyopathy. Diabetes 2015; 2: 618-30

[13] Varga ZV, Giricz Z, Liaudet L, Haskó G, Ferdinandy P, Pacher P. Interplay of oxidative, nitrosative/nitrative stress, inflammation, cell death and autophagy in diabetic cardiomyopathy. Biochim Biophys Acta 2015; 2: 232-42.

[14] Wen HL, Liang ZS, Zhang R, Yang K. Anti-inflammatory effects of triptolide improve left ventricular function in a rat model of diabetic cardiomyopathy. Cardiovasc Diabetol 2013; 12: 50.