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Apocynum leaf extract inhibits the progress of atherosclerosis in rats via the AMPK/mTOR pathway

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Apocynum leaf extract is an extract of the dried leaves of *Apocynum venetum* (a member of the Apocynaceae family) that has many effects on the cardiovascular system. The aim of the present study was to evaluate the protective effects of apocynum leaf extract on the atherosclerosis in rats induced by high-fat diet combined with vitamin D3 intraperitoneal injection. The atherosclerosis in rats were induced with a high-fat diet and an intraperitoneal injection of VD_3 once daily for three contiguous days at a total injection dose of 70 U/kg. At the end of the 18th week, serum total cholesterol (TC) and triglyceride (TG) contents were measured. Hydroxyproline content in the aorta were measured by the alkali hydrolysis method. The hematoxylin-eosin (HE) and immunohistochemical staining were applied to evaluate the morphological changes and the collagen I and α-smooth muscle actin expression. The protein expression and the mRNA level of AMPK and mTOR were detected by western blot analysis and reverse transcript PCR. After treatment with apocynum leaf extract, the serum total cholesterol and triglyceride concentration of the atherosclerotic rats were significantly decreased, both the Collagen I expression and the hydroxyproline content in the aorta were significantly reduced, and the α-SMA, a smooth muscle-specific marker, expression were also lower than the untreated atherosclerotic rats. Western blot analyses showed that the apocynum can marked increase the p-AMPK but decrease the mTOR protein expression. The apocynum leaf extract also exhibited higher AMPK and lower mTOR mRNA expression of the aorta in the atherosclerotic rats. We believe that the apocynum leaf extract can effectively reduce blood lipid levels in rats with atherosclerosis, delay atherosclerotic progression by inhibiting excessive collagen synthesis and inhibiting smooth muscle cell over-proliferation. The underlying mechanism may be related to the AMPK/mTOR signaling pathway activity. Our results contribute towards validation of the traditional use of apocynum leaf extract in the treatment of atherosclerosis.

1. Introduction

Atherosclerosis refers to several diseases that are associated with artery wall thickening and a loss of elasticity. Its lesions are mainly characterized by subintimal lipid accumulation in certain areas of the arteries, which is accompanied by the proliferation of smooth muscle cells and fibrous matrix components, which gradually leads to atherosclerotic plaque formation. Atherosclerosis is commonly found in men over the age of 40 and in women after menopause; is often accompanied by high blood pressure, hypercholesterolemia or diabetes; and is one of the main causes of death in the elderly. Actively investigating the pathogenesis of atherosclerosis and developing effective drugs for the prevention and treatment of atherosclerosis remain important goals of pharmacology. *Apocynum venetum* has been referred to as "the magic herb" by the Chinese, and this plant mainly grows in desert saline soil or on the sandy ground of riverbanks, ravine sand hill sides (Xie et al. 2012). Apocynum leaf extract is prepared from the dried leaves of *Apocynum venetum* (Apocynaceae), which contain many flavonoids, triterpenoids, organic acids, amino acids and other constituents; quercetin and quercetin glycosides are the main flavonoids (Kamata et al. 2008; Zhang et al. 2010; Song and Zhou 2015). Apocynum leaf extract has demonstrated many therapeutic effects, such also regulating blood pressure, reducing blood lipid concentrations and increasing coronary blood flow, and are effective in treating hypertension and hyperlipidemia (Kim et al. 2000; Wang et al. 2015). This extract also reportedly enhances immunity, prevents colds, reduces asthma and cough, eliminates depression, improves sleep quality, increases blood flow and beauty, antagonizes alcohol and protects the liver (Xie et al. 2015; Yamatsu et al. 2015). This study used a high-fat diet combined with vitamin D3 intraperitoneal injection to establish a rat aortic atherosclerosis model with the aim of studying the effect and mechanism of apocynum leaf extract in delaying atherosclerotic progression.

2. Investigations and results

2.1. Comparison of the general condition and blood lipid levels of the rats

As shown in Fig. 1 A, after feeding with high-fat diets, the rats showed slow weight gain. Starting from the 3rd week, the weight of rats on the high-fat diet was significantly (P<0.05) lower than that of the normal control group fed with the normal diet. After the 12th week of modeling, rats in the low-, medium- and high-dose apocynum leaf extract treated groups and the fluvastatin treated group received gavage treatment of the appropriate drugs for six weeks. After drug treatment, the rate of weight gain increased in all drug administration groups. From the 13th experimental week until the 17thweek, rats in the apocynum medium- and high-dose treated groups and in the fluvastatin treated group demonstrated rapid weight gain; compared with atherosclerotic rats, the differences were statistically significant (P<0.05).

As shown in Fig. 1B and C, when the experiment ended at the 18th week, the serum TG concentration of the normal control group was 0.84±0.34 mmol/L, and that of rats in the untreated athero-

sclerosis group was 1.38±0.23 mmol/L, which was 1.64±0.27 times that of the normal control group. The difference between the two groups was statistically significant (P<0.05). The serum TC concentration of rats in the normal control group was 1.54±0.24 mmol/L, and the serum TC content of the untreated atherosclerosis group was 2.80±0.21 mmol/L, which was 1.82±0.44 times that of the normal control group. The difference between the two groups was statistically significant (P<0.05). Compared with the untreated atherosclerosis group, the serum TG and TC levels in the apocynum medium- and high-dose treated groups were significantly lower (P<0.05), and the serum TC content of the fluvastatin treated group was also significantly lower than that of the untreated atherosclerosis group (P<0.05).

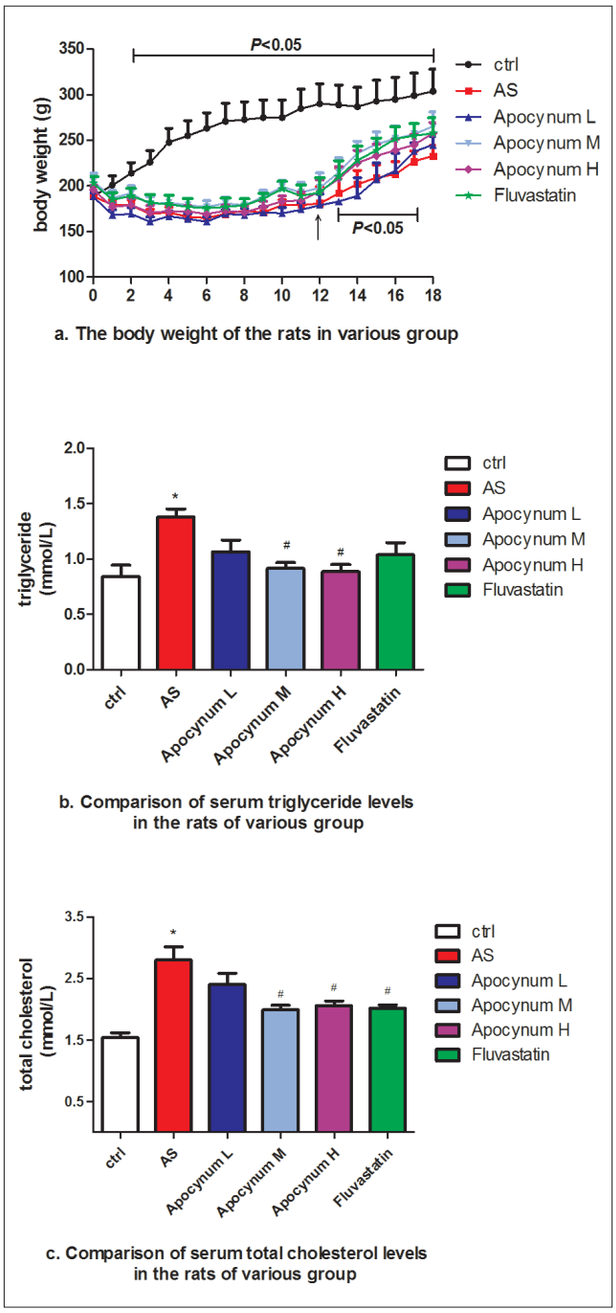


Fig. 1: Comparison of the body weight and the blood lipid of the rats in various group. Ctrl: control group; AS: untreated atherosclerosis group; apocynum L, M, H: apocynum leaf extract low-, medium- and high-dose treated group; fluvastatin: fluvastatin treated group. * compare with the control group P<0.05; # compared with the AS group P<0.05.

2.2. Histological examination of the rats' aorta tissue

As Fig. 2 shows, the tunica intimae of aortas in the normal groups was smooth without lipid deposition and contained a small amount of collagen fibers and smooth muscle fibers, as well as uniformly distributed elastic fibers. The rats in the untreated atherosclerosis group showed obvious atherosclerosis. Those with mild symptoms showed increased lipids and various numbers of visible foam cells. Those with severe symptoms presented arterial wall calcification. Rats in the apocynum groups and in the simvastatin group also showed various degrees of atherosclerotic lesions in the arterial wall; the numbers of calcification lesions were significantly lower and the extent of calcification was significantly less than those in the untreated atherosclerosis group.

2.3. Comparison of arterial wall collagen component

Collagen I immunohistochemistry results are shown in Fig. 3 A and B. The aortic walls of rats in all groups were positive for collagen I. Compared with the normal control group, the arterial wall of rats in the untreated atherosclerosis group presented significantly increased collagen I expression (P<0.05), whereas the apocynum low-, medium- and high-dose treated groups and the fluvastatin treated groups all had significantly lower arterial wall collagen I expression than the untreated atherosclerosis group; the differences were significant (P<0.05). To quantitatively evaluate the collagen content in the arterial wall of the rats, we examined the hydroxyproline content; the results are shown in Fig. 3C. The arterial wall hydroxyproline content was 20.81±1.35 μg/mg tissue in the normal control group and was 48.19±2.54 μg/mg tissue in the untreated atherosclerosis group, i.e., 2.4 times that of the normal control group. The apocynum medium- and high-dose treated groups and the fluvastatin treated group all had significantly decreased hydroxyproline levels in the arterial wall, and the differences were statistically significant compared to the untreated atherosclerosis group (P<0.05).

2.4. α-SMA expression in the arterial wall

In this study, we used α-SMA as a marker to label aortic smooth muscle cells. Immunohistochemistry analysis showed that smooth muscle cells were present in the tunica media of the normal rat arterial wall and were neatly organized and evenly distributed in the tunica media of the artery. As shown in Fig. 4, compared to the normal control group, the α-SMA expression level in the arterial wall of rats with atherosclerosis was significantly higher (P<0.05). The apocynum medium- and high-dose treated groups and the fluvastatin treated group showed lower α-SMA expression to various degrees, and the differences were significant compared with the untreated atherosclerosis group (P<0.05).

2.5. Western blot results

The molecular weight of the p-AMPK protein is 64 kDa. Compared with the normal control group, aortic p-AMPK protein expression was significantly (P<0.05) lower in the atherosclerotic rats. p-AMPK protein expression in the aortic wall of rats in the apocynum high-dose treated group was significantly (P<0.05) higher than that in the untreated atherosclerosis group which was shown in Fig. 5 A and B. The molecular weight of mTOR is 289 kDa. Optical density analysis showed that, compared with the normal control group, aortic mTOR protein expression level was significantly (P<0.05) higher in the untreated atherosclerosis group. Aortic mTOR protein expression was significantly lower (P<0.05) in the apocynum high-dose group and the fluvastatin group than in the untreated atherosclerosis group as shown in Fig. 5 A and C.

2.6. Real-time PCR results

AMPK mRNA expression in the aortic wall of atherosclerotic rats was significantly (P<0.05) lower than that in the normal control group (63±6%). AMPK mRNA expression levels were significantly

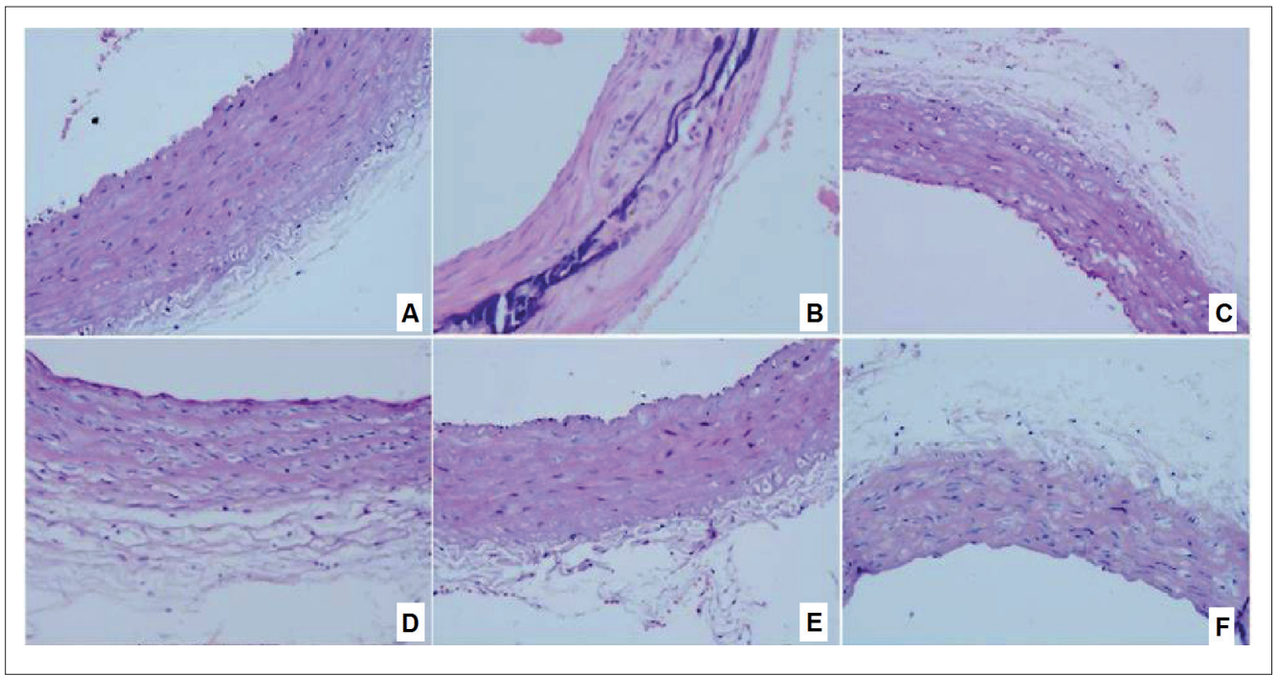


Fig. 2: Hematoxylin-eosin (HE) staining results in the rats of various group (X200). A. the normal control groups; B. the untreated atherosclerosis group; C. the apocynum leaf extract low-dose treated group; D. the apocynum leaf extract medium-dose treated group; E. the apocynum leaf extract high-dose treated group; F. the fluvastatin treated group.

higher (P<0.05) in the apocynum low-, medium- and high-dose treated groups and the fluvastatin treated group than in the untreated atherosclerosis group as shown in Fig. 6 A. In contrast with AMPK, aortic mTOR mRNA expression was more than twice as high in rats with atherosclerosis than in the normal control group (2.26±0.54); this difference was significant (P<0.05). Compared with the untreated atherosclerosis group, the apocynum low-, medium- and high-dose groups and the fluvastatin group showed significantly lower (P<0.05) mTOR mRNA expression which was shown in Fig. 6 B.

3. Discussion

Hyperlipidemia is a major risk factor for atherosclerosis. Therefore, most atherosclerosis models are based on high-fat diets. However, because rats do not have a gallbladder and ingest low levels of cholesterol and other lipids, pure high-fat diets lead only to lipid deposition and rarely induce atherosclerosis in rats. Pang et al. (2010) fed Sprague-Dawley (SD) rats with basic diet plus VD3 for 4 days, followed by the intragastric injection of a high-fat emulsion for three months, ultimately leading to the formation of atherosclerosis. Some researchers believe that vitamin D3 elevates serum calcium (thereby damaging the endothelial integrity of the arterial wall), can accelerate elastic lamina fracture (thus greatly accelerating lipid and calcium salt deposition in the vascular wall and the invasion of inflammatory cells in the bloodstream) and promotes the formation of atherosclerosis (Almofti et al. 2006; Tukaj et al., 2012). Therefore, atherosclerosis models induced by VD3 coupled with high-fat diets in rats have received increasing attention from researchers. However, no consensus has been reached on the specific VD3 dose, the route of application or the experimental cycle. In this study, we applied a dose of 70 U/kg VD3, which was delivered in three separate intraperitoneal injections. At the end of the experiment in the 18th week, rats in the model group showed significantly increased serum cholesterol and triglyceride levels, and histological examination revealed that the walls of the rat thoracic aorta had increased lipid components and collagen fibers, smooth muscle cell (SMC) proliferation and foam cell formation, as well as significant calcification in severe cases. This approach avoided the toxic effects caused by a single large-dose injection and was not hampered by the poor absorption rate see for oral administration. The animals were generally in a good state, with low mortality and a high rate of successful model establishment. Thus, this method is suitable for application.

Apocynum leaf is recorded in the "Chinese Pharmacopoeia" as a commonly used Chinese medicine. This product is the dried leaf of *Apocynum venetum* L., a member ofthe Apocynaceae family. The most recent research shows that the total tannins and total flavonoids in apocynum leaf extract exhibit a good lipid-lowering effect (Ma and Chen 1989). In this study, after 6 weeks of apocynum leaf extract gavage in rats with atherosclerosis, the rat serum TC and TG levels were significantly reduced.

Collagen is an important non-cell component of the normal arterial wall and can form networks together with proteoglycans, thereby supporting vascular wall cells, maintaining blood vessel elasticity and affecting blood vessel wall cell morphology and agglomeration (Silver et al. 2001). Collagen content can be used as an important indicator for the evaluation of organ fibrosis. We used immunohistochemistry to detect collagen I expression in the arterial wall and measured the hydroxyproline content to qualitatively and quantitatively evaluate the extent of atherosclerosis in rats. The hydroxyproline content in the arterial wall in rats with atherosclerosis was significantly higher than that in the normal control group (more than twice as high), indicating that in atherosclerosis, an increase in the collagen component of the arterial wall is involved in the pathogenesis of atherosclerosis. We also used immunohistochemistry to detect collagen I expression levels in the aortic wall in the rat groups. The arterial wall of normal rats expressed collagen I at basal levels, and collagen I expression was significantly higher in the arterial wall of rats in the untreated atherosclerosis group than in the normal control group. This result suggested that collagen I, an important collagen component of the arterial wall, is involved in atherosclerosis; thus, inhibiting collagen I expression might slow the progression of atherosclerosis. Our results demonstrated that after atherosclerotic rats were treated with apocynum leaf extract for 6 weeks, the hydroxyproline content and collagen I expression in the aortic wall were significantly decreased, and histological examination showed that the extent of arterial wall thickening in treated rats was ameliorated, indicating that the oral administration of apocynum leaf extract can delay atherosclerotic progression.

In the tunica media of the normal arterial wall, variable amounts of collagen, elastic fibers and glycoproteins encircle the smooth

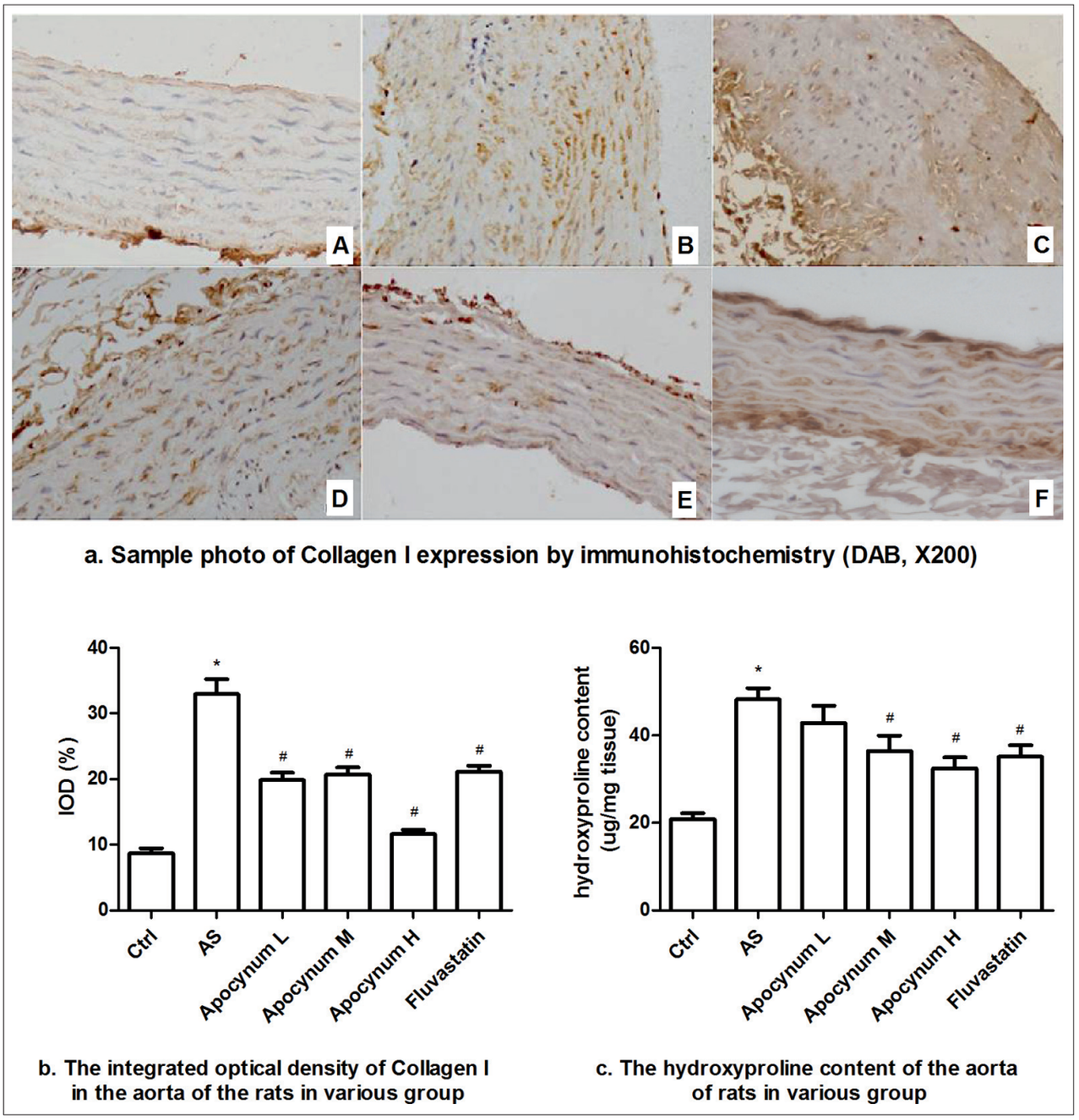
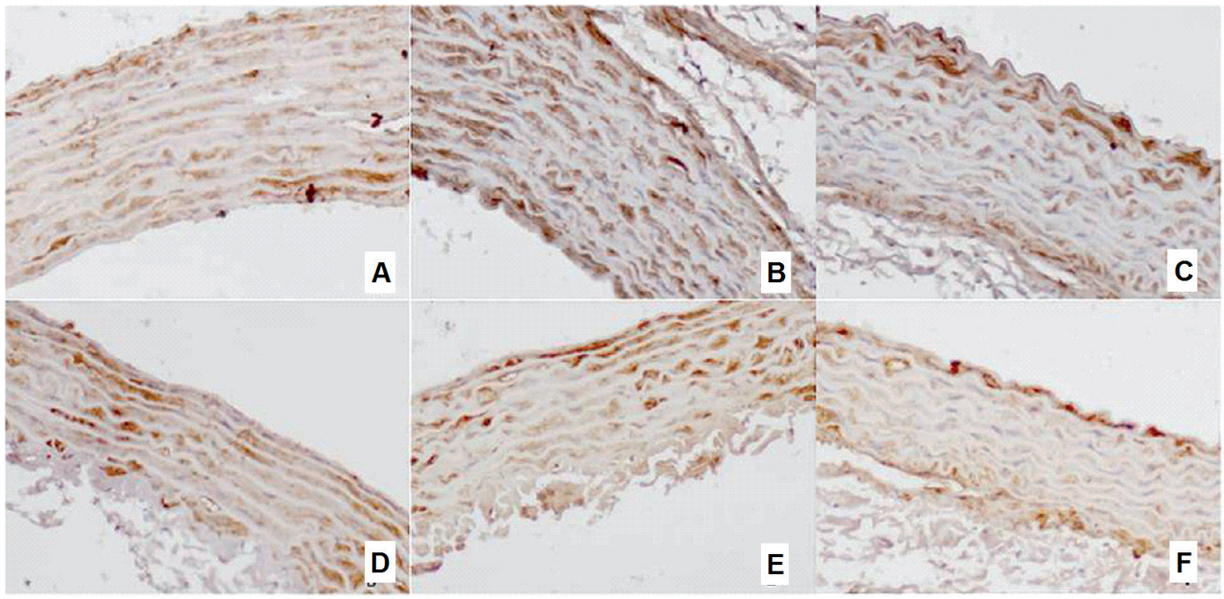
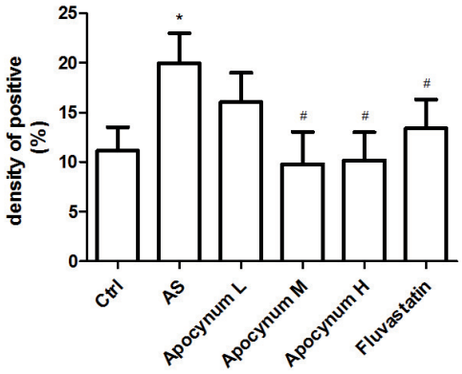


Fig. 3: Collagen content comparison in the various group. A. the normal control groups; B. the untreated atherosclerosis group; C. the apocynum leaf extract low-dose treated group; D. the apocynum leaf extract medium-dose treated group; E. the apocynum leaf extract high-dose treated group; F. the fluvastatin treated group. Ctrl: control group; AS: untreated atherosclerosis group; apocynum L, M, H: apocynum leaf extract low-, medium- and high-dose treated group; fluvastatin: fluvastatin treated group. * compared with the control group $P<0.05$; # compared with the AS group $P<0.05$.

muscle cells. As the main cell component of the tunica media of the arterial wall, smooth muscle cells play an important role in maintaining the flexibility and function of arterial walls (Maiolino et al. 2013; Qiu et al. 2014; Chistiakov et al. 2015; Ramji and Davies 2015): (1) smooth muscle cells secrete a variety of cytokines, such as PG12 and PGE, and secrete chemokines to stimulate phagocytic cell and smooth muscle cell proliferation. (2) The surface of smooth muscle cells contains lipoprotein receptors. For example, LDL-R binds to LDL, followed by endocytosis, and is involved in lipoprotein metabolism. (3) The surface of smooth muscle cells displays a variety of cytokine receptors and growth factor receptors. (4) Smooth muscle cells can synthesize and secrete stromal components of vascular wall connective tissue. It has been shown that atherosclerosis is due to SMC proliferation in atherosclerotic plaques; furthermore, proliferated SMCs exhibit internal structure changes, and the phenotype of SMC changes accordingly, switching from the main function of contraction to that of proliferation. At this time, it is known that SMC exhibits an enhanced ability to synthesize collagen and other extracellular matrix components and intake lipid for their gradual transformation into foam cells. Therefore, most researchers recognize that SMC proliferation plays an important role in the occurrence and development of atherosclerosis. In this study, we used α-SMA as a vascular smooth muscle cell marker and used immunohistochemistry to detect its expression in the aortic wall of rats. α-SMA expression was found to be significantly higher in the arterial wall of atherosclerotic rats than in that of normal rats, suggesting that a high-fat diet coupled with VD3 injection can stimulate SC



a. Sample photo of α -SMA immunohistochemistry results (DAB, X200)



b. The integrated optical density of α -SMA in the aorta of the rats in various group

Fig. 4: α-SMA expression of the arterial wall comparisons in the various group. A. the normal control groups; B. the untreated atherosclerosis group; C. the apocynum leaf extract low-dose treated group; D. the apocynum leaf extract medium-dose treated group; E. the apocynum leaf extract high-dose treated group; F. the fluvastatin treated group.

proliferation in rat aortic wall. After 6 weeks of intervention with apocynum extract, α-SMA expression in the arterial wall of atherosclerotic rats was decreased, indicating that apocynum extract can inhibit SMC hyperproliferation in the arterial wall, thus delaying the progression of atherosclerosis.

AMP-Activated Protein Kinase (AMPK, 64 kDa) is the catalytic subunit of 5'-prime-AMP-activated protein kinase and is a member of the ser/thr protein kinase family (Motoshima et al. 2006). The kinase activity of AMPK is activated by stimuli that increase the cellular AMP/ATP ratio. AMPK regulates the activities of several key metabolic enzymes through phosphorylation and protects cells from stress that causes ATP depletion by switching off ATP-consuming biosynthetic pathways. Intracellular signaling protein kinases that play a signaling role in the regulation of cellular energy metabolism are involved in lipid metabolism, which in turn provides the substrates that are needed to convert AMP to ATP. AMPK negatively regulates ATP-consuming biosynthetic processes including gluconeogenesis and lipid and protein synthesis. Due to its role as a central regulator of both lipid and glucose metabolism, AMPK is considered a potential therapeutic target for the treatment of type II diabetes mellitus, obesity and cancer (Xu and Si 2010; Steinberg and Schertzer 2014). AMPK has also been implicated in a number of species as a critical modulator of aging through its interactions with mTOR. The mechanistic target of rapamycin (mTOR) is an atypical serine/threonine kinase that is present in two distinct complexes. This kinase is a master growth regulator that senses and integrates diverse nutritional and environmental cues, including growth factors, energy levels, cellular stress and amino acids. It couples these signals to the promotion of cellular growth by phosphorylating substrates that potentiate anabolic processes such as mRNA translation and lipid synthesis or that limit catabolic processes such as autophagy. Aberrant mTOR signaling is involved in many disease states, including

cancer, cardiovascular disease and diabetes (Maiese 2015). In this study, we used Western blot analysis to analyze the expression of p-AMPK and mTOR and used real-time PCR to analyze AMPK and mTOR mRNA expression in the arterial wall in rats. AMPK mRNA expression in the arteries of rats with atherosclerosis was reduced to approximately 60% that of normal rats, and p-AMPK protein expression was also significantly reduced; however, mTOR mRNA expression was more than twice that of the normal control group, and expression of the corresponding protein also increased significantly. Our results suggest that the AMPK/mTOR pathway is involved in atherosclerotic progression. After atherosclerotic rats were treated with apocynum leaf extract, AMPK protein and mRNA expression levels were increased accordingly, and mTOR protein and mRNA expression levels were reduced. We thus hypothesize that the AMPK/mTOR pathway may be the target by which apocynum leaf extract inhibits the progression of arterial atherosclerosis.

In summary, apocynum extract can effectively reduce blood lipid levels in rats with atherosclerosis and delay atherosclerotic progression by inhibiting excessive collagen synthesis in the aorta and the over-proliferation of smooth muscle cells. This process may be related to the ability of apocynum extract to inhibit AMPK/mTOR signaling pathway activity.

4. Experimental

4.1. Reagents

Hydroxyproline (alkali hydrolysis method, A030-2), total cholesterol (single reagent COD-PAP method, CAT #: A111-2) and triglyceride (single reagent GPO-PAP enzymatic method, CAT #: A1010-2) test kits were purchased from Nanjing Jiancheng Bioengineering Institute, China. An anti-rat α-smooth muscle actin (SMA) monoclonal

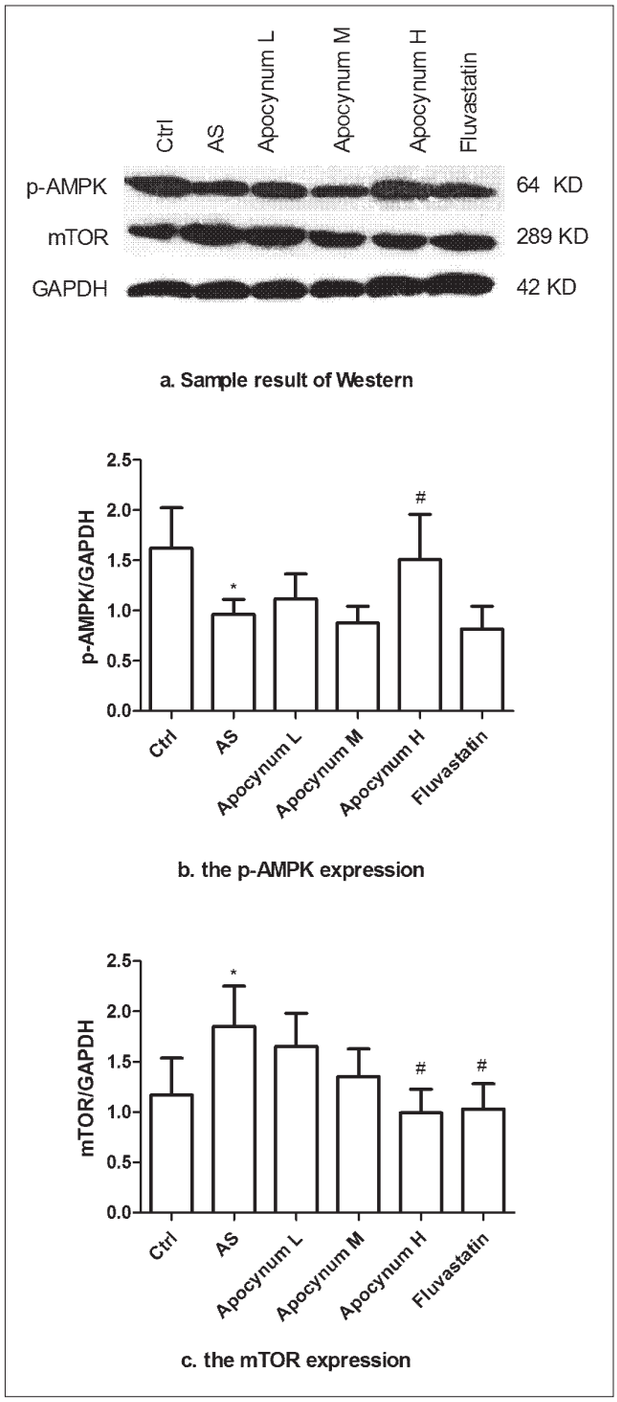


Fig. 5: Western blot results of p-AMPK and mTOR in the aortic wall. Ctrl: control group; AS: untreated atherosclerosis group; apocynum L, M, H: apocynum leaf extract low-, medium- and high-dose treated group; fluvastatin: fluvastatin treated group. * compare with the control group $P<0.05$; # compared with the AS group $P<0.05$.

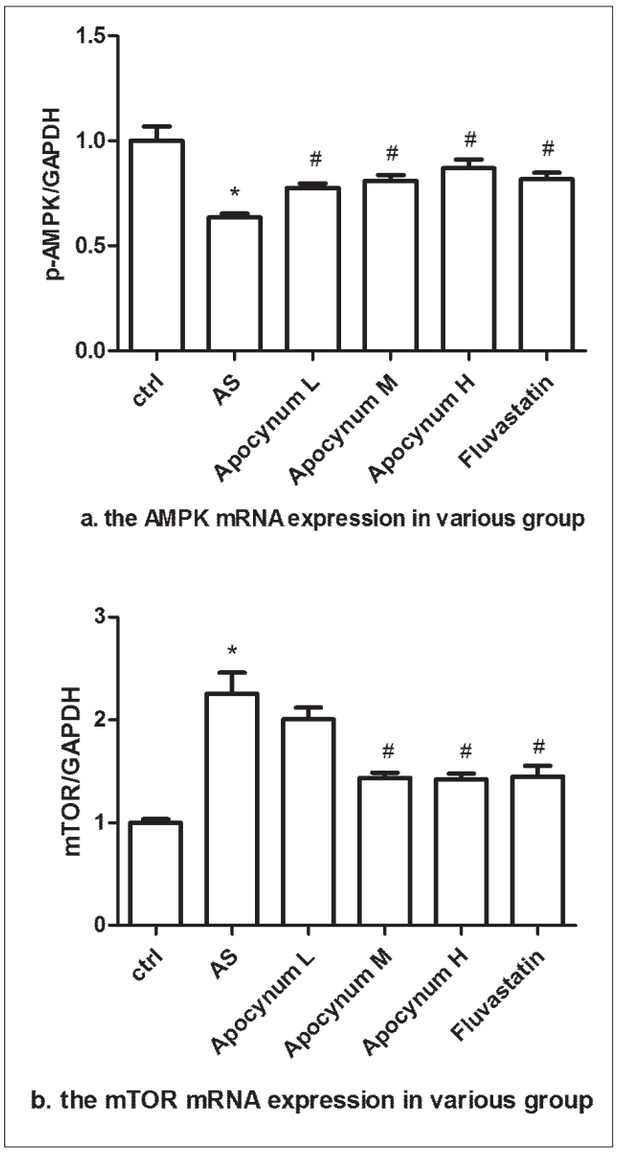


Fig. 6: Real-time PCR results of AMPK and mTOR in the aortic wall. Ctrl: control group; AS: untreated atherosclerosis group; apocynum L, M, H: apocynum leaf extract low-, medium- and high-dose treated group; fluvastatin: fluvastatin treated group. * compare with the control group $P<0.05$; # compare with the AS group $P<0.05$.

antibody (A2625), a rabbit anti-rat phospho-adenosine monophosphate-activated kinase (p-AMPK) polyclonal antibody (AP0116, ABclonal) and a rabbit anti-rat mammalian target of rapamycin (mTOR) polyclonal antibody (A2445, ABclonal) were purchased from ABclonal Biotech Co., Ltd., College Park, MD, USA. A rabbit anti-collagen I monoclonal antibody (ZA-0616), a mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (TA-08), a one-step polymer detection system for mouse, rabbit and tat antibodies (PV-6001) and a concentrated DAB kit (ZLI-9017) were purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd., China. ProteinExt™ Mammalian Total Protein Extraction Kit (DE101), Easy Protein Quantitative Kit (Bradford method) (DQ101-01) and TransScript Green Two-Step qRT-PCR SuperMix (AQ201-01) were from Beijing TransGen Biotech, China. TRIzol® reagent (15596026) was purchased from Life Technologies, Carlsbad, CA, USA.

4.2. Apocynum leaf total flavonoid extraction, separation, purification and quantification

Apocynum herb (raw material) was produced and collected in Baicheng City, Jilin Province, China. The dried apocynum leaves were crushed, sifted through a 20-50 mesh and pulverized. The powder was heated under reflux with 10 volumes of 75% ethanol for 2 h. The extraction was performed twice, and the extracts were combined and filtered, followed by ethanol recovery and concentration, yielding the concentrated extract. The concentrate was then purified by passing through a D101 macroporous resin, which was washed first with water and subsequently with 50% ethanol. The filtrates were combined and concentrated to obtain the total flavonoids. Based on spectrophotometry, the total flavonoid content (measured as rutin) of the extract was at least 72%.The flavonoid content of the extract was then analyzed using HPLC; the extract contained 4.59 % hyperosides, 9.86 % rutin and 0.12 % quercetin.

4.3. Establishment of an atherosclerosis model and experimental groups

Sixty SPF-grade healthy male Wistar rats weighing 180-200 g were purchased from the Experimental Animal Center of Jilin University. All animals were individually caged, had free access to drinking water and were weighed weekly. After one week of adaptive feeding, the rats were randomly divided into the following six groups: normal control group (Ctrl), untreated atherosclerosis group (AS), fluvastatin treated group (fluvastatin) and apocynum low-, medium- and high-dose treated groups (apocynum L, apocynum M and apocynum H) (10 rats per group). With the exception of the normal control group, the animals received an intraperitoneal injection of VD_3 once daily for three contiguous days at a total injection dose of 70 U/kg and were fed with a high-fat diet until the end of the experiment. The control group of the 10 animals was fed with a normal diet and water. During the 12th week of dieting, animals in the apocynum low-, medium- and high-dose groups received daily gavage of apocynum total flavonoids at doses of 25, 50 and 100 mg/kg • d, respectively, and animals in the fluvastatin group received fluvastatin at 5 μg/kg (gavage). The high-fat diet comprised 80.8% basic diet, 3.5% cholesterol, 10% lard, 0.2% propylthiouracil, 0.5% sodium cholate and 5% white granulated sugar.

4.4. Blood lipid examination

When the experiment was terminated at the end of the 18th week, serum was collected from each group (2000 rpm, 15 min, 4 °C). Serum total cholesterol (TC) and triglyceride (TG) contents were measured according to the kit instructions.

4.5. Hydroxyproline content determination

Accurately weighed rat thoracic aorta (30 mg wet weight) were placed in a test tube, and 1 ml of lysis buffer was added. The sample was allowed to hydrolyze for 20 min in aboiling water bath. After cooling, the lysate was adjusted to pH 6.0-6.8. All procedures were performed according to the manufacturer's instructions, and the absorbance was measured at 550 nm after subtracting a blank (doubly distilled water). The hydroxyproline content of each sample was calculated as follows:

$$\text{Hydroxproline content (μg/mg wet weight)} = \frac{\text{Measured OD value – blank OD value}}{\text{Standard OD value – blank OD value}} \text{ X standard content (5 μgml) X } \frac{\text{lysate total volume (10 ml)}}{\text{Tissue wet weight (mg)}}$$

4.6. Morphological examination

The full length of the rat thoracic aorta is approximately 2 cm. The proximal end (0.5 cm) was fixed with 10% formalin, embedded in paraffin and prepared as 5-μm-thick sections. The sections were subjected to serial alcohol deparaffinization and stained in hematoxylin-eosin (HE). Differences in histology between the rat aortic tissues of the various groups were observed under a light microscope.

4.7. Immunohistochemical staining

Immunohistochemical staining was conducted according to the manual provided with the one-step polymer detection system. The main steps are as follows: paraffin sections were subjected to conventional deparaffinization and hydration, followed by antigen retrieval using microwave heating. After cooling to room temperature, the slides were incubated with 3% H_2O_2/deionized water for 5 min to block endogenous peroxidase. Appropriately diluted primary antibodies (α-SMA antibody diluted by 1:500, collagen I antibody diluted by 1:200) were added for overnight incubation at 4 °C. Goat anti-rabbit IgG/HRP conjugate was then added, followed by 15-20 min of incubation at room temperature and 3,3'-diaminobenzidine (DAB) chromogenesis. At the end of chromogenesis, the sections were washed with distilled water, double-stained with hematoxylin, dehydrated, clarified and mounted for microscopic examination. Immunohistochemically stained slides were observed under a microscope. Five non-overlapping fields of vision were randomly selected for each observation area (× 400 magnification). Images were recorded using the Nikon 80i imaging system, and Image-Pro Plus 5.1 image processing software was used to measure the integrated optical density (IOD) of positive signals.

4.8. Western blot analysis

The distal end of rat thoracic aorta was analyzed for p-AMPK and mTOR expression in the arterial wall (Western blot). A rat aortic sample (100 mg) was diced, placed into 1 ml RIPA lysis buffer (containing 1 μmol/L PMSF) and fully homogenized, followed by centrifugation at 12,000 g for 20 min. The total protein concentration in the supernatant was determined using the Bradford method. Fifty micrograms of total protein was loaded into each well of a 10% SDS-PAGE gel and resolved at 120 V for 1.5 h, followed by membrane transfer at 4 °C and 100 V for 1 h. The PVDF membrane was fully washed in TBST (20 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.05% Tween 20) and blocked in 5% fat-free milk for 1 h. The membrane was then incubated with appropriately diluted primary antibodies (antibodies for p-AMPK and mTOR were diluted 1:500, and the anti-GAPDH antibody was diluted 1:2000) at 4 °C overnight, followed by a 1-h incubation at room temperature with the corresponding HRP-conjugated secondary antibodies (goat anti-rabbit IgG was used for p-AMPK and mTOR, andrabbit anti-mouse IgG was used for GAPDH; the dilution ratios were both 1:3000). Finally, signals were detected using the enhanced chemiluminescence (ECL) chromogenic reaction. The optical density of the bands was analyzed using ImageJ 1.50g (NIH, USA). The relative expression of the target protein was calculated as the ratio of the optical densities of the target protein band and the GAPDH band.

4.9. Aortic total RNA extraction and reverse transcript PCR

4.9.1. Aortic total RNA extraction

The total RNA was extracted according to the TRIzol® reagent manual. A total of 100 mg of rat aorta was weighed and cut, followed by thorough homogenization in1 ml TRIzol® reagent. The homogenate was mixed with 200 μl of chloroform, shaken vigorously for 15 s and then placed on ice for 2 min, followed by centrifugation at 4 °C and 12,000 × g for 15 min. The supernatant was transferred to a new tube; 500 μl isopropanol was then added, mixing well, and allowed to stand for 20 min. The mixture was then centrifuged at 12,000 × g for 15 min. The RNA precipitate was washed with 70% ethanol and dissolved in RNase-free water. OD260 and OD280 were measured. When OD260/OD280> 1.8, 1 μg of total RNA was used in reverse transcription (RT) PCR experiments.

4.9.2. Real-time RT-PCR detection of gene expression

According to the manual supplied with the TransScript® Green Two-step qRT-PCR Supermix, we used two-step qPCR to measure AMPK and mTOR mRNA expression levels in different groups of rats. The following procedure was adopted for first-strand cDNA synthesis and gDNA removal: 1 μg of total tissue RNA, 4 μl of 5X TransScript®All-in-one Supermix for qPCR, 1 μl of gDNA remover and RNase-free water were mixed in a total reaction volume of 20 μl. For reverse transcription, 15 min of incubation at 42 °C was followed by heating at 85 °C for 5 s to inactivate the reverse transcriptase and gDNA remover. The qPCR system comprised 1 μl of cDNA template, 0.4 μl each of forward and reverse primers (10 μmol/L), 10 μl of 2XTransStart®Tip Green qPCR Supermix and sufficient H_2O to reach a total volume of 20 μl. The PCR conditions were as follows: denaturation at 94 °C for 30 s, followed by 45 cycles of 94 °C for 5 s, 60 °C for 15 s and 72 °C for10 s; the CT value of each sample was recorded. Based on the CT values for the target gene and reference genes, we used $2^{-\Delta\Delta CT}$ to represent the relative expression levels of the target gene, according to $\Delta\Delta CT = (CT_{target}\text{-}CT_{reference})_{experiment} - (CT_{target}\text{-}CT_{reference})_{control}$ (Livak and Schmittgen 2001).

The AMPK (NM_023991) primer sequences were as follows: forward primer 5'-AGCATCGATGATGAGGTGGT-3' and reverse primer 5'-GAGACCGGTGGTGGGTTATC-3', leading to a product length of 267 bp. The mTOR (NM_019906.1) primer sequences were as follows: forward primer 5'-ATGCCACACAGTGATGGAGG-3' and reverse primer 5'-AGAAATCCCGACCAGTGAGC-3', leading to a product length of 344 bp. GAPDH (NM_017008.4) was used as an internal reference; the primer sequences were as follows: forward primer 5'-GTTACCAGGGCTGCCTTCTC-3' and reverse primer 5'-GATGGTGATGGGTTTCCCGT-3', leading to a product length of 177 bp.

4.10. Data analysis

The data were analyzed using GraphPad Prism 5.0 software. The data are expressed as means±standard deviation and were compared using one-way analysis of variance (ANOVA); intragroup pairwise comparisons were conducted using the Newman-Keuls method, the significance was set at $P< 0.05$.

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