

The Hepatoprotective Effect and Its Possible Mechanism of Picroside II in ApoE^{-/-} Mice of Atherosclerosis

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Abstract Objective: To investigate the hepatoprotective effect and possible mechanism of picroside II in ApoE^{-/-} mice of atherosclerosis. **Methods:** ApoE^{-/-} mice fed with high-fat diet for 12 weeks were randomly divided into model group, low-, medium- and high-dose of picroside II groups and positive-drug (praluent) group, C57BL/6J mice fed with ordinary feed were the control group. After modeling, Picroside II (5, 20, 40 mg/kg/d) was injected intraperitoneally in treatment group, and praluent (10 mg/kg/w) was given intraperitoneally in the positive-drug group for 12 weeks. The remaining groups were given equal volume saline. Serum total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) were detected by biochemical assay. The aortic wall lipid deposition and collagen fibers in liver tissue were respectively observed by oil red O staining and Masson staining, the liver function was observed by measuring the content of Glutamic-oxal (o)acetic transaminase (GOT) and Glutamic-pyruvic transaminase (GPT) and Western blot was used to detect PI3K/Akt pathway and levels of the inflammatory factor interleukin-6 (IL-6) protein. **Results:** In the control, mice had better mental state and bright fur, while the mental state of mice fed with high-fat diet was slightly worse and more obese. After the end of the administration cycle, compared with the model group, the body weight of mice in the medium and high dose groups and the praluent group was significantly decreased ($P < 0.01$), the serum levels of TG, TC and LDL-C were significantly decreased ($P < 0.05$) while the HDL-C levels were significantly increased ($P < 0.05$), the lipid deposition area and the collagen volume fraction in liver tissue were significantly decreased ($P < 0.05$), the expressions of PI3K, Akt and IL-6 were significantly decreased ($P < 0.05$). **Conclusion:** Picroside II could improve lipid metabolism, improve liver fibrosis, which might related to inhibiting the activation of PI3K/Akt signaling pathway.

Keywords: Picroside II, atherosclerosis, hepatic fibrosis, inflammation, PI3K/Akt signaling pathway

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1. Introduction

Atherosclerosis (AS) is an arterial disease characterized by the formation of fibrous plaques in the intima of blood vessels. It is a common chronic inflammatory disease in clinic and the main pathological basis of vascular diseases [1,2,3]. Both non-alcoholic fatty liver disease (NAFLD) and AS are chronic metabolic diseases have similar risk factors, pathological basis and development incentives, and are often accompanied by disease [4,5,6]. Hepatic AS is an important component of hyperlipidemia and systemic AS, and an important cause of fatty cirrhosis and hepatic fibrosis [7,8]. Liver fibrosis (LF) refers to the pathophysiological process of abnormal hyperplasia of connective tissue in the liver caused by various pathogenic factors. Fibrosis occurs in the process of liver repair and healing [9]. The phosphatidylinositol 3 kinase

(PI3K)/Protein kinase B (PKB or Akt) signaling pathway plays an important role in the occurrence and development of AS [10]. High expression of transforming growth factor- β (TGF- β) is one of typical characteristics during fibrosis process [11]. Akt can be activated by TGF- β , and PI3K/Akt signaling pathway can be regulated by gene expression, which plays an important role in various patho-physiological processes such as cell survival, differentiation, growth and apoptosis [12,13]. More and more studies have shown that PI3K/Akt signaling pathway plays a key role in various liver injuries and is an effective way to treat liver fibrosis [14,15,16]. *Rhizoma picrorhizae* is a traditional Chinese herb recorded in the Classic of the Materia Medica as being able to treat "typhoid fever, labor and body heat, and redness of urine and stools like blood". Picroside II is one of its main active ingredients with neuroprotective, hepatoprotective, anticholestatic, anti-inflammatory, antioxidant and immunomodulatory activities [17,18,19,20]. Lee et al. [21] proved firstly that

picroside II can inhibit the expression and secretion of IL-33 in human monocytes and human tracheal epithelial cells by inhibit MAPK and NF- κ B pathway, which indicating that picroside II can improve lung inflammation. However, it is unclear whether picroside II can reduce inflammatory reaction by regulating PI3K/Akt signaling pathway, thus inhibiting the formation of AS. This study was based on the PI3K/Akt signaling pathway to explore the hepatoprotective effect and possible mechanism of picroside II on AS ApoE^{-/-} mice.

2. Materials and Methods

2.1. Experimental Reagents

Praluent (Aliciumab injection), S20190042, Sanofi, France; Picroside II, C₂₃H₂₈O₁₃, molecular weight: 492.47, HPLC $\geq$ 98%, batch No. HS18118B1, Baoji Chenguang Biotechnology Co. Ltd; Improved oil red O staining kit (G1261), Masson staining kit (G1340), BCA protein quantitative kit (PC0020), Beijing Soraibao Technology Co. Ltd; HE staining kit (G1120), GOT/AST test kit (BC1565), GPT/ALT activity test kit (BC1555), Solarbio, China; RIPA cracking solution (P0013B), PMSF (ST506), Beyotime Biotechnology Co. Ltd., China. Rabbit anti mouse PI3K (4292S), Akt (4691S), IL-6 (12912S) antibodies, HRP-goat anti-rabbit IgG secondary antibody (7074P2), Cell Signaling Technology, USA; β -actin (AF7018), IL-6 (DF6087) antibodies, Affinity Biosciences, USA.

2.2. Laboratory Animals

2.2.1. Animal source: 6 C57BL/6J mice, 30 ApoE^{-/-} mice, male, 6 weeks old, SPF grade, body mass (20 $\pm$ 2) g, purchased from Hangzhou Ziyuan Experimental Animal Science and Technology Co. Ltd.: SCXK (Zhejiang) 2019-0004. It is raised in SPF environmental animal room, with relative humidity of 50%-70%, temperature of (22 $\pm$ 2) °C, 12h/12h day and night environment, and free drinking water. This experiment was approved by the Experimental Animal Welfare Ethics Committee of Qingdao University (No. 20220917C573620230517080), and the experimental steps strictly comply with the ethical requirements of animal welfare.

2.2.2 Establishment of model: ApoE^{-/-} atherosclerosis mouse model [22] was established by high-fat diet. High fat feed (D12108C) was purchased from Jinan Pengyue Experimental Animal Breeding Co. Ltd. Formula: corn starch 23.51%, casein 22.18%, cocoa butter 17.19%, sucrose 12.53%, maltodextrin 7.87%, cellulose 5.54%, soybean oil 2.77%, potassium citrate 1.83%, calcium phosphate 1.44%, cholesterol 1.25%, mineral premix 1.11%, vitamin premix 1.11%, calcium carbonate 0.61%, sodium cholate 0.50%, cystine 0.33%, choline 0.22%. The model was successfully established after 12 weeks of continuous feeding [23].

2.2.3 Success signs: ApoE^{-/-} male mice have dark, dry fur and shedding, of which the hair shedding rate of ApoE^{-/-} mice is 23.95%, but the hair shedding rate of C57BL/6J mice is 0%. Three mice were randomly selected from C57BL/6J mice and ApoE^{-/-} mice fed with high-fat diet for

dissection. The thoracic aorta was stained with oil red O, and the aortic lumen showed obvious lipid deposition. A large number of atherosclerotic plaques were formed, which indicates that the AS model is successful.

2.3. Grouping and Intervention

Seven C57BL/6J mice were randomly assigned to the control group. Thirty seven ApoE^{-/-} mice successfully modeled were randomly divided into model group, positive-drug (praluent) group (10 mg/kg/w, converted according to the dose used in clinical human body) [24], picroside II low-dose (PII-L), medium-dose (PII-M) and high-dose (PII-H) groups (5, 20, 40 mg/kg/d). The mice in the control group and the model group were given equal volume of normal saline (10mg/kg/d) by intraperitoneal injection. At the 12th week, all mice were dissected for blood sampling. The experiment lasted for 24 weeks.

2.4. Sample Collection

2.4.1. Serum: The mice fasted 12 hours and 10% chloral hydrate solution was intraperitoneally injected (300 mg/kg) for anesthesia. 1 mL of blood was taken from the heart. Serum was separated by a low speed centrifuge (centrifugation at 4000 rpm for 10 min) and stored in a -20°C.

2.4.2. Slicing: After blood collection, open the chest and abdominal cavity, peel off the liver and partially freeze save and fix part of the liver tissue in 4% paraformaldehyde, dehydrated with conventional gradient ethanol, transparent with xylene, embed in paraffin, and cut continuously with a thickness of 5 μ m. Some liver tissues were immersed in appropriate amount of OCT embedding agent, and put into a small cup containing liquid nitrogen. After 20 sec, the tissues were quickly frozen into blocks. The frozen microtome (Leica CM1950, Germany) was cut with a thickness of 10 μ m and stored at -20°C.

2.4.3. Total protein extraction: Take 100 mg of the above cryopreserved liver tissue into a 1.5 mL EP tube with a high-speed cryogenic tissue grinder (KZ-III-F, Wuhan Xavier Biotechnology Co. Ltd.), add RIPA lysate and PMSF to lyse and centrifugate at 12000 rpm for 10 min with a high-speed centrifuge (Eppendorf 5430R, Germany) to remove the sediment and leave the supernant. Draw BCA standard curve to measure protein concentration, add 1/4 $\times$ loading buffer, store at -80°C.

2.5. Detection of Indicators

2.5.1. General condition: The body mass of mice was measured once two weeks, and the mental state, fur gloss, activity and body mass of mice in each group were observed.

2.5.2. Blood lipid detection: The level of serum total cholesterol (TC, mmol/L), triglyceride (TG, mmol/L), high-density lipoprotein (HDL-C, mmol/L) and low-density lipoprotein (LDL-C, mmol/L) was detected by the automatic biochemical analysis system (Modularp 800, Roche, Switzerland).

2.5.3. Liver function: Measure the standard curve according to the instructions of the test kit, add reagents in the 96 well plate according to the instructions, mix well,

place at room temperature for 10 min, measure the absorbance of each tube at the wavelength of 505 nm with an automatic microplate reader (Bio rad 680, USA), and calculate the GOT/GPT activity (U/g quality).

2.5.4. HE staining: Take the paraffin sections of the above liver tissue, and put the sections in the 56 °C oven 1 hour in advance. Xylene dewaxing and hydration, and distilled water for 2 min. Then hematoxylin for 2 min and eosin for 1 min, conventional dehydration and sealing film. The histopathological changes were observed under microscope (CX31, Olympus, Japan) in three different visual fields.

2.5.5. Oil red O staining: The freeze section was rewarm, dried and immersed in oil red dye solution for 10 min; 60% isopropanol differentiated twice, washed for 10 sec × 2 times in water; re-staining with hematoxylin and washing with water twice; sealing films with glycerol gelatin to observe lipid deposition under microscope (MF43, Guangzhou Mingmei Technology Co. Ltd.). Three different visual fields were selected from the slices, and the percentage of fat deposition area (%) each group was quantitatively analyzed by Image J software = the red area of fat deposition/the total area of tissue in the visual field.

2.5.6. Masson staining: Paraffin sections are dewaxed and hydrated, and Masson composite staining solution is added dropwise for 5 min. After washing with distilled water, add phosphomolybdic acid for 5 min. After washing with distilled water, add aniline blue is directly for 5 min. After slightly washing with distilled water, differentiation solution is added for 45 sec. After slightly washing with distilled water, differentiation solution is added for 45 sec. Conventional dehydration, transparency, the elastic fibers of abdominal aorta were observed under the microscope. Three different hepatic tissue visual fields were selected, and the collagen volume fraction (CVF) = collagen positive blue area/total tissue area in the visual field in each group was quantitatively analyzed by Image J software.

2.5.7. Western blotting to detect the expressions of PI3K, Akt and IL-6 in mouse liver tissue: Take protein samples 20 µg. Add SDS protein loading buffer in proportion, preheat with a dry thermostat, heat at 100 °C for 5 min, cool at room temperature, centrifugate at 10000 rpm for 30 min, take the supernatant for standby. After preparation of PAGE gel, take sample for electrophoresis, transferring membrane, seal 5% skimmed milk powder at room temperature for 1 h, dilute PI3K (1:2000), Akt (1:4000) IL-6 (1:2000) and internal reference protein β-actin (1:3000) primary antibody, incubated overnight; Dilute the secondary antibody at 1:4000 and incubate it at 37 °C for 1h. ECL luminous solution emits light and fixes after development to β-actin protein, scanned by Bio Rad 2000 gel imaging system, Image J analyzes the gray value of each band, calculates the relative gray value (RVP)= the gray value of the target protein/the internal reference gray value, repeats the test three times, and takes the average value.

2.6. Statistical Analysis

SPSS26.0 statistical software was used for analysis. Image J was used to read image information, and

GraphPad Prism 6.0 software was used for drawing. Mean ± standard deviation of data between groups ($\bar{x} \pm s$) means that the difference between groups is significant ($P < 0.05$), and the difference is extremely significant ($P < 0.01$).

3. Results

3.1. Effect of Picroside II on Body Mass of Mice

During the experiment, the control group mice had good mental status, bright fur, flexible activities, and no significant changes in body mass ($P > 0.05$). Before modeling, the mice had good mental state, bright fur and flexible activities. After modelling, the mental state of the mice was slightly worse, the body was relatively obese, and the body mass was significantly higher than before modelling ($P < 0.05$). After treatment, the body mass of mice in the PII-M, PII-H groups and the praluent group decreased than that in the model group ($P < 0.05$). See Table 1.

Table 1 Effect of picroside II on body mass of mice ($\bar{x} \pm s$, n=6)

Groups	Before modelling (g)	After modelling (g)	After treatment (g)
control	19.37±1.13	29.95±0.80	29.90±0.94
model	20.15±0.82	30.85±1.88	31.23±3.02
praluent	20.45±1.19	32.23±1.28	30.32±1.99*
PII-L	19.83±0.96	31.92±0.53	31.25±1.89
PII-M	20.03±0.73	31.30±1.20	30.60±2.01*
PII-H	19.55±0.73	32.26±2.31	30.58±2.10*

* $P < 0.05$ vs model group

3.2. Effect of Picroside II on Serum Level of Lipid in Mice

Compared with the control group, the serum levels of TC, TG and LDL-C in the model group were significantly increased ($P < 0.01$), while HDL-C were significantly decreased ($P < 0.05$). Compared with the model group, the serum level of TG in the PII-H group was significantly lower ($P < 0.05$), while the TC, TG, LDL-C in the PII-M, PII-H groups and the praluent group were significantly lower ($P < 0.05$), and the level of HDL-C was significantly higher ($P < 0.05$). There was no significant difference between the PII-L group and the model group ($P > 0.05$). There was no significant difference among the PII-M, PII-H and the praluent groups ($P > 0.05$). See Table 2.

Table 2 Effect of picroside II on serum levels of lipids in mice ($\bar{x} \pm s$, n=6)

Groups	TG (mmol/L)	TC (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)
control	0.36±0.11	2.39±0.27	1.32±0.20	0.15±0.05
model	1.30±0.67**	19.07±2.12**	1.04±0.17*	2.15±0.29**
praluent	0.54±0.24 [#]	11.01±3.56 [#]	1.39±0.40 [#]	1.15±0.50 [#]
PII-L	1.20±0.46	18.69±3.08	1.07±0.13	2.02±0.66
PII-M	0.71±0.33 [#]	13.33±3.38 [#]	1.18±0.22 [#]	1.52±0.51 [#]
PII-H	0.61±0.56 [#]	12.22±2.02 [#]	1.40±0.35 [#]	1.44±0.72 [#]

** $P < 0.01$ vs control group, * $P < 0.01$ vs control group, [#] $P < 0.05$ vs model group

3.3. Effect of Picroside II on Liver Function in Mice

Compared with the control group, the activity of GOT and GPT in the model group increased significantly ($P < 0.05$), indicating that there was liver injury in the model group; Compared with the model group, the activity levels of GOT and GPT in the PII-M, PII-H and praluent groups were significantly reduced ($P < 0.05$); There was no significant difference between the PII-L and the model groups ($P > 0.05$); There was no significant difference ($P > 0.05$) among the PII-M, PII-H and praluent groups. See Table 3.

Table 3 Effect of picroside II on liver function in mice ($\bar{x} \pm s$, $n=6$)

Groups	GOT (U/g)	GPT (U/g)
control	36.94±0.93	88.54±5.60
model	49.74±2.72*	118.22±3.92*
praluent	38.15±2.40 [#]	94.23±3.90 [#]
PII-L	49.99±3.28	114.33±4.68
PII-M	41.23±3.15 [#]	109.20±6.50 [#]
PII-H	39.70±3.08 [#]	99.37±6.32 [#]

* $P < 0.01$ vs control group, [#] $P < 0.05$ vs model group

3.4. Effect of Picroside II on Pathological Changes of Liver Tissue in Mice

In the control group, there was no obvious monocyte infiltration between liver cells, and the cell morphology was normal, while in the model group, there was a large number of monocyte infiltration between liver cells, with irregular cell arrangement, obvious cell swelling, obvious lipid vacuoles, and typical AS changes. Inflammatory infiltration and lipid vacuoles in mice of picroside II (low, medium and high dose groups) and praluent groups decreased to varying degrees. See Figure 1.

3.5. Effect of Picroside II on Lipid Deposition in Liver Tissue of Mice

No lipid deposition was found in the liver tissue cells of the control group, and obvious lipid deposition color blocks were found in the liver tissue of the model group. The lipid staining area of the PII-M and praluent groups was significantly lower than that of the model group ($P < 0.05$), but there was no significant difference between the PII-L and model group ($P > 0.05$). There was no significant difference ($P > 0.05$) between the PII-M, PII-H and praluent groups. See Figure 2).

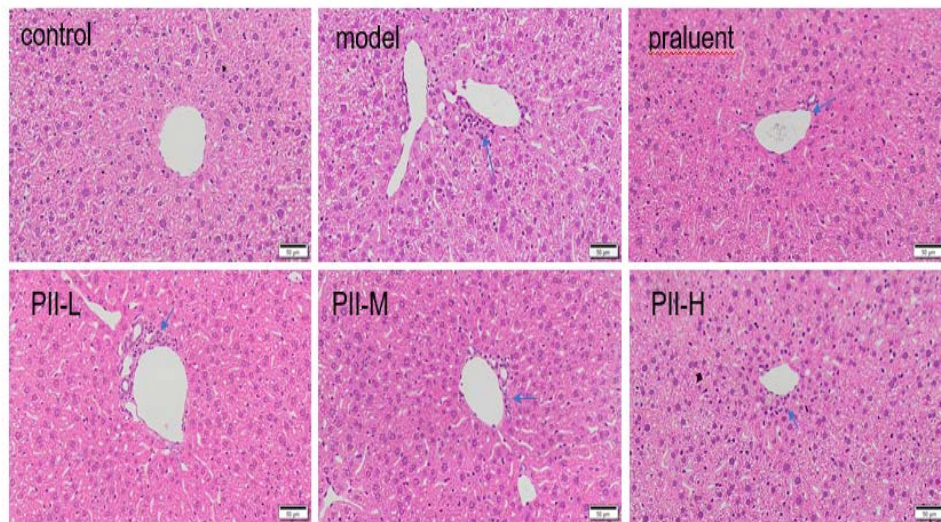
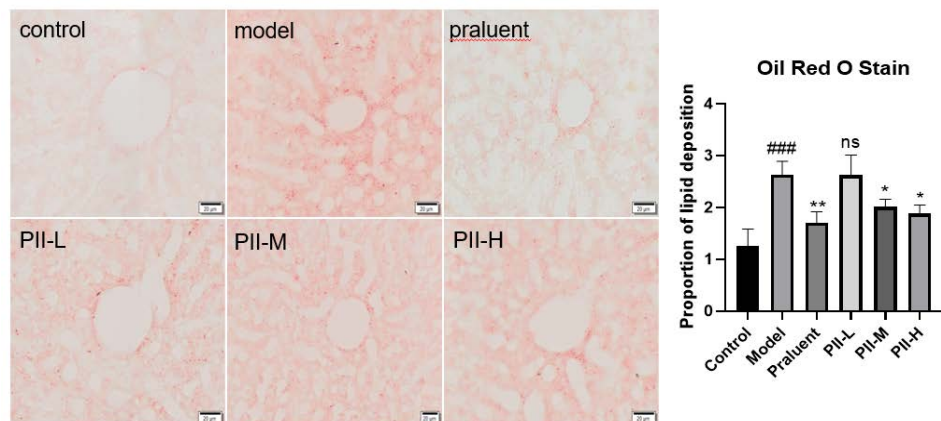


Figure 1. ApoE^{-/-} mouse liver tissue HE staining 10×



$P < 0.001$ vs Control; ** $P < 0.01$ vs Model; * $P < 0.05$ vs Model

Figure 2. Oil Red O staining of ApoE^{-/-} mouse liver tissue 20×

3.6. Effect of Picroside II on Fibrosis in Liver Tissue of Mice

In the control group, the distribution of collagen in the liver tissue was uniform, the adjacent collagen fiber network was intact, and no fiber proliferation was found. In the model group, the collagen fibers in the liver tissue was significantly increased, the collagen fiber network was broken, and the collagen fibers were connected with each other. The CVF of liver tissue in model group was significantly increased than that in control group ($P < 0.01$), while the CVF of liver tissue in praluent and PII-H groups significantly decreased than that in model group ($P < 0.05$). See Figure 3.

3.7. Effect of Picroside II on Expressions of PI3K, Akt and IL-6 in Liver Tissue of Mice

The expressions of PI3K, Akt, IL-6 in liver issue of model group were significantly enhanced than those of control group, while in praluent group were decreased than those of model group ($P < 0.01$). The expression of PI3K in PII-H group was lower than that in model group ($P < 0.01$), and the expressions of Akt and IL-6 in PII-M and PII-L groups were lower than those in model group ($P < 0.05$), but no difference existed among the other groups ($P > 0.05$). See Figure 4.

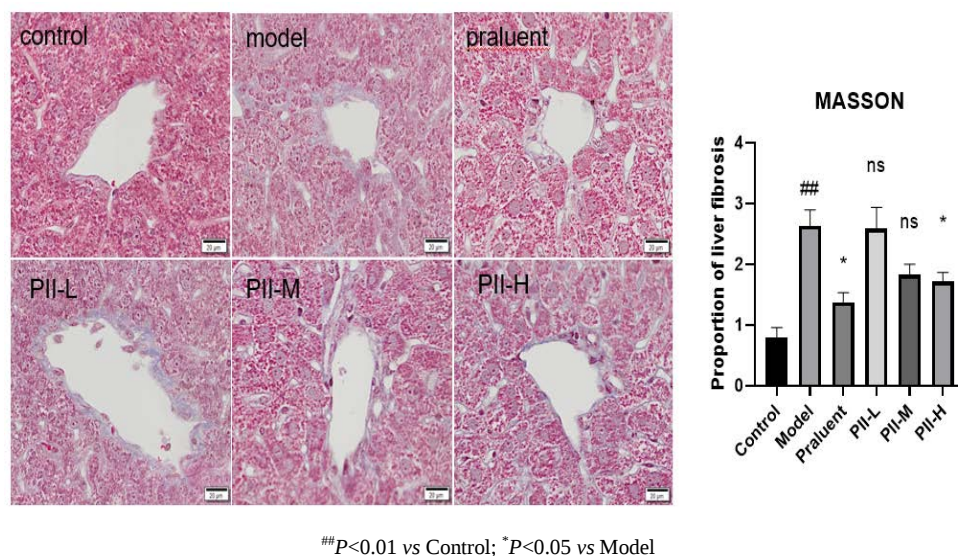


Figure 3. Masson staining of ApoE^{-/-} mouse liver tissue 20 ×

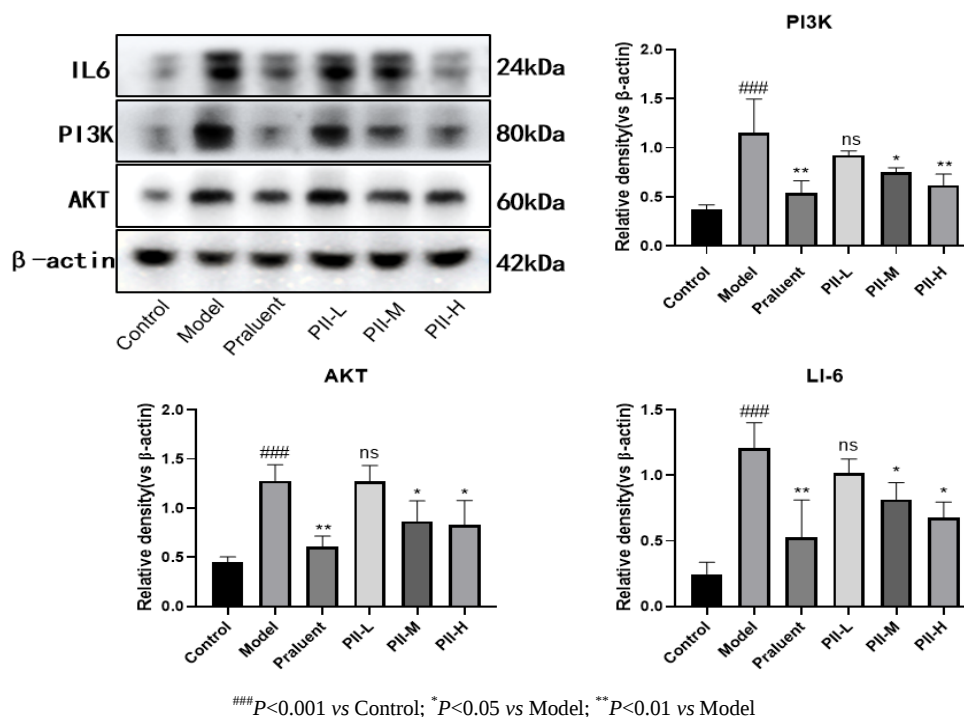


Figure 4. Western blotting to detect expressions of PI3K/AKT/IL-6

4. Discussion

AS is a chronic inflammatory vascular disease caused by various factors. It is the main reason that directly or indirectly leads to myocardial infarction, myocardial ischemia, stroke and other cardiovascular and cerebrovascular diseases [1]. AS has a long course of disease and various symptoms, but its causes and molecular mechanisms have not been fully explored now. Epidemiological investigation has confirmed AS is the results of the interaction of hypertension, smoking, aging, obesity, irregular work and other factors [25]. Excessive adipose tissue deposition is closely related to the formation of AS plaque, and has become one of the independent risk factors for the occurrence and development of AS [26]. Studies have shown that the prevalence of atherosclerotic dyslipidemia in patients with metabolic fatty liver disease (MAFLD) in the middle to late stages of liver fibrosis is higher than that in patients without MAFLD. The oxidation of free fatty acids in the liver of patients with hyperlipidemia is increased, and the utilization of liver glycogen is inhibited, which shows that lipid components are deposited in hepatocytes, further aggravating liver fibrosis [27]. The liver is exposed to high concentration of free fatty acids in the whole portal vein circulation, and visceral adipose tissue is proinflammatory, which may be positively correlated with the development of AS [28].

Rhizoma picrorhizae is a commonly used traditional Chinese medicine, whose efficacy can clear away heat and dampness. Pharmacological studies show that *Rhizoma Picrorhizae* has many effects, such as protecting liver and cholagogue, anti-inflammatory, lipid regulating, and immune regulating. At present, the main active ingredients studied most are iridoids, and the most important of them is picroside II [29]. Research shows that picroside II can improve oxidative damage, inflammation and apoptosis. Wang et al. [18] reported that picroside II attenuates hyperhomocysteinemia-induced endothelial injury by reducing inflammation, oxidative stress and cell apoptosis. Picroside II inhibits TLR4/NF κ B signaling pathway, thereby reducing the oxidative stress and inflammation of renal tissue induced by ischemic reperfusion [19].

PI3K/Akt/mTOR signaling pathway plays an important role in many maturation processes such as cell growth, proliferation and differentiation. The abnormality of this pathway is usually closely related to the occurrence of cardiovascular and cerebrovascular diseases and other dangerous diseases [30]. In the classical pathway, activated PI3K phosphorylates Akt domain Thr308 through phosphoinositide dependent kinase-1 (PDK1), and mammalian target of rapamycin complex (mTORC2) phosphorylates Akt, and activated Akt directly activates mTORC1 through phosphorylation of Ser2448 site. On the other hand, Akt inhibits brain Ras homolog enriched in brain (Rheb) and activates mTORC1 by phosphorylating tuberous sclerosis complex (TSC2). Activated mTORC1 phosphorylates its effector ribosomal protein S6 kinase 70kDa (p70S6K) and eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1). The activated p70S6K can inhibit the function of mTORC2, forming a negative feedback mechanism of

PI3K signaling pathway [31]. At present, research has gradually proved that some effective ingredients of Chinese herbal medicine can enhance the autophagic activity of macrophages by inhibiting the expression of PI3K/Akt/mTOR signaling pathway, play a role in reducing lipid metabolism and inhibiting the inflammatory response of vulnerable plaque of AS [32,33,34]. It can effectively prevent or delay the progress of AS, and has significant influence in the formation of AS [10].

In this study, the lipid deposition in the liver tissue of ApoE^{-/-} mice was observed by oil red O staining. The results showed that after the treatment with picroside II, the lipid deposition in the liver tissue cells of ApoE^{-/-} mice was significantly reduced than that in the model group. Masson staining indicated that after the treatment with picroside II, the proliferation of collagen fibers in the liver tissue of ApoE^{-/-} mice was significantly reduced than that in the model group, with normal cell morphology, orderly arrangement, uniform distribution of collagen, and intact adjacent collagen fiber network. There is no significant difference between the PII-L and the model group, which indicated picroside II may improve the lipid deposition in the liver tissue of AS mice in a dose-dependent manner, alleviate the degree of liver fibrosis, and thus achieve the effect of inhibiting AS.

This experimental study confirmed that picroside II may play a role by mediating the PI3K/Akt signaling pathway to inhibit the inflammation of AS and improve the lipid metabolism of ApoE^{-/-} mice, and provide a theoretical basis for the clinical application of picroside II.

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Ethics Approval and Consent to Participate

This experiment was approved by the Ethics Committee for Laboratory Animal Welfare of Qingdao University (No. 20220917C573620230517080).

Conflicts of Interest Statement

The authors declare that there are no conflict of interest.

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