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Piperine prevents cholesterol gallstones formation in mice

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ABSTRACT

Biliary cholesterol may contribute to the formation of cholesterol gallstones, and regulation of these levels could be a useful therapeutic strategy for gallstones disease. Piperine (PA) is a potential cholesterol lowering agent. In this study, we assessed the effect and mechanism of PA in preventing cholesterol gallstones formation induced by feeding lithogenic diet containing high cholesterol levels to mice. C57BL/6 inbred mice were fed lithogenic or chow diets for 10 weeks, with or without PA (15, 30 and 60 mg/kg) or ursodeoxycholic acid (UDCA, 60 mg/kg) administration. Cholesterol, phospholipids and crystals in bile, the lipid in serum, pathological changes and proteins expression in liver were analyzed. The results showed that PA could decrease the cholesterol potency and crystals in bile, reduce total cholesterol (TC), triglycerides (TG) and increase high-density lipoprotein/low-density lipoprotein (HDL/LDL) levels in serum. Furthermore, PA treatment reduced liver lipid peroxidation and protected hepatobiliary cells from liver injury by decreasing malondialdehyde (MDA) and increasing superoxide dismutase (SOD). In addition, PA inhibited the expression of ATP-binding cassette transporters G5/8 (ABCG5/8) and liver X receptor (LXR) in liver, and reduced cholesterol transport from the hepatocytes to the gallbladder. It may be the mechanism of PA in preventing cholesterol gallstones formation. PA as a potential drug for prevention cholesterol gallstones merits further investigation.

1. Introduction

Gallstones disease is a common problem caused by the presence of stones in the gallbladder. According to the chemical composition of the stones, gallstones can be divided into three categories: cholesterol gallstones, pigment gallstones and mixed gallstones. With the change of dietary habits in the populations, the incidence of gallstones is also increasing, and most gallstones are formed from cholesterol. The incidence of cholesterol gallstones is from 5.9% to 21.9% in western countries and from 3.1% to 10.7% in Asia (Chuang et al., 2012). Furthermore, cholesterol gallstones can cause biliary colic, acute cholecystitis, pancreatitis and other complications. So it is important to prevent the occurrence of cholesterol gallstones.

Although there are many reasons for the formation of cholesterol gallstones, it is mainly due to the imbalance of bile components of cholesterol, bile acids and lecithin. When levels of cholesterol are too high in bile, the bile becomes saturated and cholesterol crystallization occurs. Then the crystals precipitate, and aggregate in the gallbladder which become gallstones (Purushotham et al., 2012; Cariati and Piromalli, 2012; Ogiyama et al., 2010). Protein in the bile can also promote the cholesterol crystal nucleate. An additional factor is the gallbladder motility dysfunction. Under the combined effect of these factors and bile stasis, gallstones formation is triggered (Yang et al., 2011).

Most patients with cholesterol gallstones are willing to accept a conservative treatment, including ursodeoxycholic acid (UDCA) (vanBerge-Henegouwen et al., 2005). Although UDCA provides some benefit to patients, this approach suffers from low efficacy, a long period for the onset of action and a common recurrence of stone formation. Therefore, a better drug in treating cholesterol gallstones was needful.

The long pepper (*Piper longum* L.) is a member of the piperaceae family. Piperine (PA), extracted from the unsaponifiable oil, is found to have a variety of biological effects, such as anti-oxidant, anti-inflammatory, anti-asthmatic, anti-tumour, anti-amoebic, anti-depressant, and against CCl_4 induced hepatotoxicity (Srinivasan, 2007; Meghwal and Goswami, 2013; Mao et al., 2014; Koul and Kapil, 1993). Furthermore, it was found that PA can depress the cholesterol (Tu et al., 2014; Duanqiai et al., 2013). Moreover, it was not known whether

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PA can inhibit the cholesterol gallstones formation. In this study, the effect and the probable mechanism of PA in preventing cholesterol gallstones formation from a lithogenic diet is investigated in mice.

2. Materials and methods

2.1. Extraction method of PA

The process for PA extraction was as follows: grains of the long pepper (30.0 g) were extracted by boiling it for 30 min with acidic water (pH=3.5) three times. The acid water extract was concentrated, filtered and recrystallized to yield piperine crystals. The extract accounted for 9.57% of the dry herbs. The filtrate was saponified with an equal volume of 20% NaOH aqueous solution by heating and stirring. The mixture was extracted several times with an equal volume of chloroform until the water layer was clear and colorless. The organic layer was concentrated under pressure, and again filtered and recrystallized to form piperine crystals (Wang et al., 2005). The purity of PA is 95%. The chemical structure of PA is shown in Fig. 1.

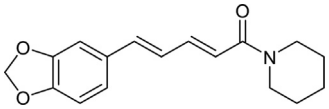

Fig. 1. The chemical structure of PA.

2.2. Animals and treatment

Male C57BL/6 mice (20 g ± 2 g; SPF obtained from Vital River Laboratories of Beijing, China) were used in all experiments. Mice were housed in a temperature-and-light control room (23 °C, 12 h light cycle) and had free access to water. The control group mice were fed a chow diet [0.02% (wt/wt) cholesterol], other groups mice were fed to a high-cholesterol lithogenic diet [15% total fat, 1.25% cholesterol and 0.5% cholic acid] (Wang et al., 2008). All animals were handled in accordance with the standards established in the Guide for the Care and Use of Laboratory Animals published by the Institute of Laboratory Animal Resources of the National Research Council (United States) and approved by the Animal Care Committee of the Peking Union Medical College and the Chinese Academy of Medical Sciences.

Mice were divided into six groups. The control and model groups were given carboxymethyl cellulose (CMC) aqueous solution without PA, and the 15, 30 and 60 mg/kg PA and 60 mg/kg UDCA groups were given same volume of CMC aqueous solution with the respective concentrations of PA (Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing, China) and UDCA (Changzhou Pharmaceutical Factory, Jiangsu, China). Every day the mice were administered drugs once by gavage, and the experiment lasted for 10 weeks. Surgery was performed on mice rapidly. Blood was collected by eye bleeding, and serum was separated. Gallbladder was removed, and the bile was obtained. The livers were collected and stored at −70 °C.

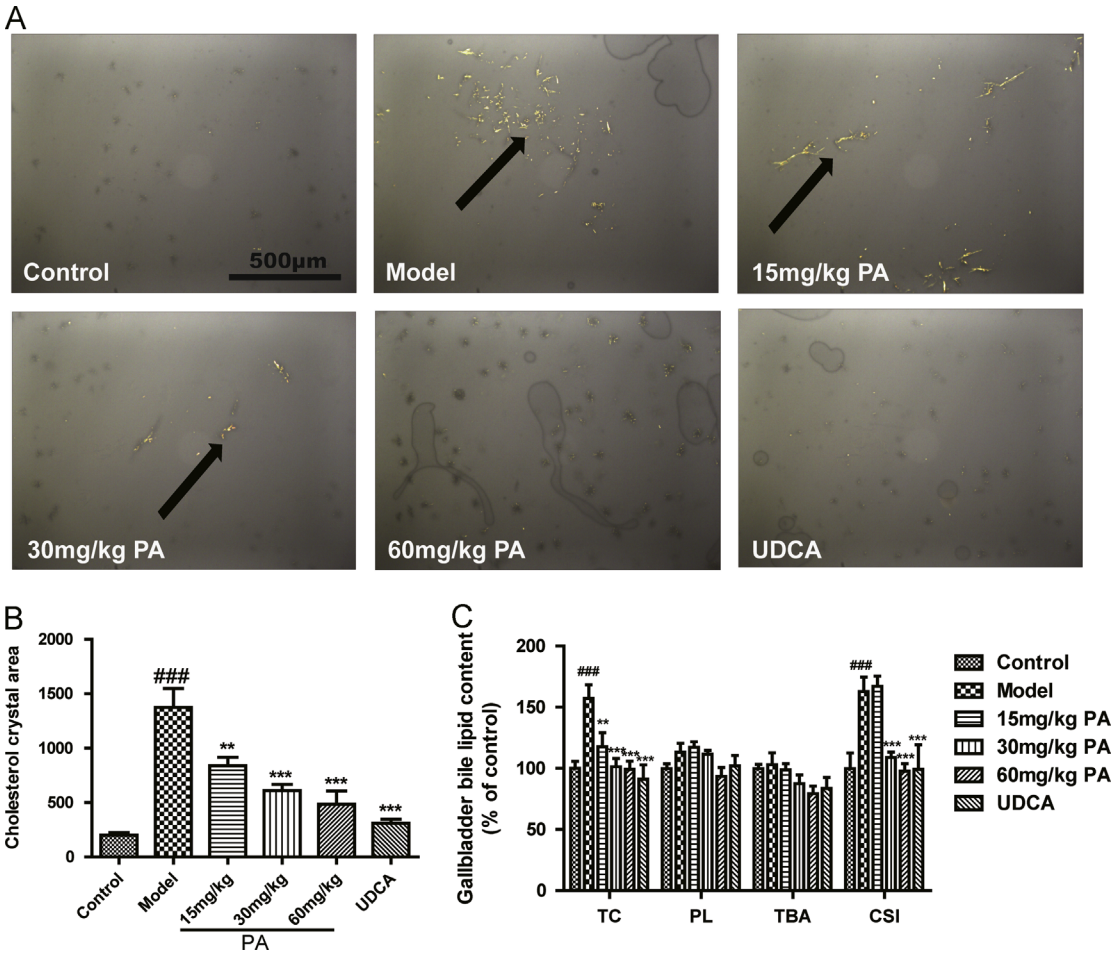

Fig. 2. Effect of PA in cholesterol crystal induced by a lithogenic diet in mice. (A) Images of biliary sludge detected by polarizing light microscopy. (B) The quantization result of (A). (C) The percentage content of TC, PL, TBA and CSI compared with control group in gallbladder bile ($n=6$). Results are mean ± S.E.M., $P<0.001$ vs. control group, $^{**}P<0.01$ and $^{***}P<0.001$ vs. model group.

2.3. Serum lipid analysis

Blood serum total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL) and low-density lipoprotein (LDL) concentrations were measured by a 40FR automatic biochemical analyzer (BioSino Biotechnology and Science, Inc., Beijing, China).

2.4. Biliary cholesterol crystals and lipid analysis

Gallbladder bile was examined with polarizing light microscopy for the presence of cholesterol crystals (Abela et al., 2012; Wang et al., 2010). Biliary total bile acid (TBA), phospholipids (PL) and TC levels were measured by the 40FR automatic biochemical analyzer as before (PL kit purchased from Beijing Jinhao Pharmaceutical Co., Ltd., Beijing, China; The TBA and TC analysis kits were purchased from BioSino Biotechnology and Science, Inc., Beijing, China). Results from these methods were used to calculate the cholesterol saturation index (CSI) (Carey, 1978).

2.5. Hepatic oxidative stress marker and antioxidant enzyme analysis

Hepatic malondialdehyde (MDA) and superoxide dismutase (SOD) content were determined by the Lipid Peroxidation MDA Assay Kit and Total Superoxide Dismutase Assay Kit (Beyotime Institute of Biotechnology, Jiangsu, China).

2.6. Histological analysis

Liver samples were soaked in 4% paraformaldehyde, embedded in paraffin, sectioned and stained using haematoxylin and eosin (H&E) for routine microscopic examination (Feng et al., 2011). Sections of snap-frozen liver were cut at 10 μm and then stained with Oil Red O (CI 26125) to look for the presence of lipids (Vidyashankar et al., 2010).

2.7. Expression of cholesterol transporters and nuclear receptors in liver

Proteins of the cholesterol transporters (ABCG5, ABCG8) and the nuclear receptor (LXR) which regulate cellular cholesterol homeostasis were measured as described previously (van Straten et al., 2008). Briefly, liver samples (250 μg) from mice were fractionated by a Nucl-Cyto-Mem Preparation kit (Applygen Technologies Inc., Jiangsu, China). Separated proteins were used for western blot analysis. Briefly, samples were separated by 10% SDS–PAGE and then transferred to PVDF membrane (Millipore, Temrcula, CA), and immunoblotted with polyclonal antibodies against the murine ABC cholesterol transporters (ABCG5, ABCG8) and the nuclear receptor (LXR) (Grefhorst et al., 2012). Polyclonal anti-GAPDH and anti-PCNA antibodies were used as the protein-loading control of the membrane protein and nuclear protein. Bands were visualized by an enhanced chemiluminescence procedure (Applygen Technologies Inc., Beijing, China) and quantified with a Microplate Reader (Thermo Fisher Scientific Inc., USA) and an ImageQuant Las 4000 mini (GE Healthcare Life Sciences, Shanghai, China).

2.8. Statistical analysis

All values are expressed as the mean ± S.E.M. Data were analyzed by two-tailed, unpaired Student's one-way ANOVA using SPSS 13.0. It was considered statistically significant when P values <0.05 were found.

Table1
Effect of lithogenic diet feeding and PA on serum TC, TG, and HDL/LDL levels in mice.

Group	TC (mmol/l)	TG (mmol/l)	HDL/LDL
Control	3.15 ± 0.22	1.18 ± 0.27	17.00 ± 1.65
Model	3.59 ± 0.41[a]	2.16 ± 0.33[b]	13.43 ± 2.10[c]
15 mg/kg PA	2.85 ± 0.18[d]	1.58 ± 0.31[e]	17.35 ± 1.47[e]
30 mg/kg PA	2.65 ± 0.53[f]	1.23 ± 0.25[d]	22.79 ± 5.27[e]
60 mg/kg PA	2.61 ± 0.29[d]	1.16 ± 0.28[d]	19.21 ± 4.43[e]
UDCA	3.17 ± 0.36[e]	1.50 ± 0.32[e]	18.19 ± 2.13[e]

Data represents mean ± S.E.M., $n=12$.
[a] $P<0.05$.
[c] $P<0.01$.
[b] $P<0.001$ vs. control group.
[f] $P<0.05$.
[e] $P<0.01$.
[d] $P<0.001$ vs. model group.

3. Results

3.1. Effect of PA in cholesterol crystals in bile

Cholesterol crystals of lithogenic diet mice were significantly increased compared with the chow diet. The crystal area was increased from 201.3 to 1374.0 $\mu m^2/mm^2$. However, these lithogenic effects were fully blocked by mice with PA and UDCA administration (Fig. 2(A) and (B)). And PA treatment groups showed a dose dependent effect. Among the four treatment groups, 60 mg/kg PA and UDCA groups showed the best effect in inhibiting the formation of cholesterol crystals. The crystal area was 483.3 and 311.3 $\mu m^2/mm^2$, correspondingly. Fig. 2(B) is the quantization result of Fig. 2(A).

We next investigated the concentrations of bile salts and lipids as well as the CSI in gallbladder bile (Fig. 2(C)). Mice fed a lithogenic diet

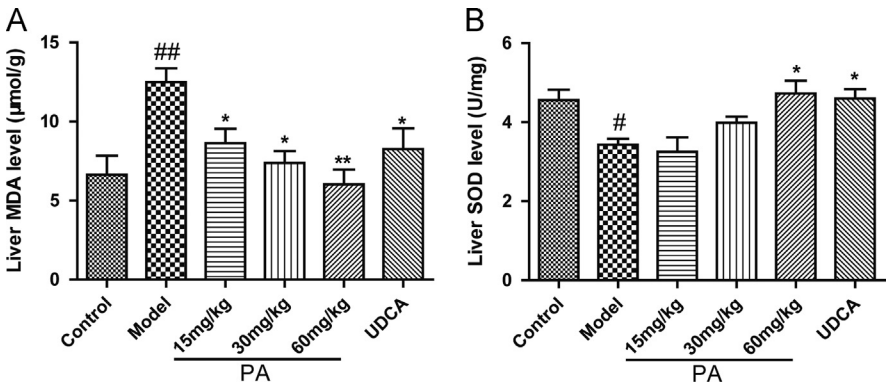

Fig. 3. Effect of PA in hepatic lipid peroxidation induced by a lithogenic diet in mice. (A) Liver MDA levels. (B) Liver SOD levels. Data represents mean ± S.E.M. $n=3$, $P<0.05$, $P<0.01$ vs. control group, $^*P<0.05$ and $^{**}P<0.01$ vs. model group.

showed high levels of cholesterol and CSI (CSI > 100%) which were positively correlated with the formation of gallstones. However, lithogenic diet mice administrated PA or UDCA displayed lower levels of cholesterol and CSI. These results showed that PA can inhibit the formation of cholesterol crystals in mice fed a lithogenic diet.

3.2. Effect of PA in reducing serum lipid

The levels of TC, TG, and HDL/LDL in serum were determined. The data presented in Table 1 shows that there was a significant increase in TC, TG and a significant decrease in the HDL/LDL ratio in serum of mice given a lithogenic diet compared with control group. And all of these were reversed in mice administrated PA and UDCA. These results indicated that PA could reduce serum lipid levels.

3.3. Effect of PA in hepatic lipid peroxidation

The liver of lithogenic diet feeding mice showed an increase in MDA and a decrease in SOD compared with the control group ($P < 0.05$). 60 mg/kg PA and UDCA groups showed decreased MDA and significantly increased SOD levels (Fig. 3), which showed that PA could reduce lipid peroxidation.

3.4. Effect of PA in hepatic cells injury

Histological examination of liver tissue was carried out to assess the liver injury and the effect of PA. It is showed that the lithogenic diet induced the accumulation of cholesterol esters and hepatocyte damage in the liver. PA treatment markedly reduced the accumulation of cholesterol and prevented liver injury (Fig. 4).

3.5. Effect of PA in modulating the expression of ABCG5/8 and LXR in liver

We next evaluated whether PA inhibited cholesterol accumulation in the liver through regulating the expression of transporters. As expected, we found that the amount of LXR, ABCG5 and ABCG8 was significantly increased in mice fed a lithogenic diet compared with control. However, PA and UDCA treatment significantly decreased these levels compared with those of mice in model group (Fig. 5).

4. Discussion

In this experiment, C57BL/6 mice were fed a lithogenic diet for 10 weeks by the same method as reported in the literatures (Zheng et al., 2008; Li et al., 2011) to make biliary cholesterol supersaturation and cholesterol gallstones. It was reported that the oil extracted from *P. longum* unsaponifiable matter plays an important role in the prevention of cholesterol gallstones formation (Xu et al., 2013). PA is the active ingredient of this extract and our results indicate the effect of PA in preventing the formation of cholesterol crystals.

When levels of cholesterol are too high in bile, the bile becomes saturated and forms cholesterol crystals. Then the crystals are precipitated, and formed aggregates in the gallbladder which become gallstones (Purushotham et al., 2012; Cariati and Piromalli, 2012; Yang et al., 2011). It is found in our research that biliary TC and CSI were significantly elevated and cholesterol crystals appeared in the gallbladder bile in model group. However, PA or UDCA administration decreased the level of TC and CSI, and also reduced the crystals area in gallbladder bile (Fig. 2).

High cholesterol diet can lead to high blood lipid and increased cholesterol gallstones formation (Di Ciaula et al., 2013; Raghavendra and Srinivasan, 2014). So the levels of TC, TG, and HDL/LDL in serum were detected. Our results indicated that the content of TC and TG was elevated, while HDL/LDL was decreased in serum of model group (Table 1). However, all of these changes could be relieved by administrating PA or UDCA, which showed that PA could improve lipid metabolic.

The formation of cholesterol gallstones was associated with the disorder of liver metabolism and cholesterol transport from the hepatocytes to the gallbladder. So the healthy liver could ensure normal secretion of bile and transport (Buko et al., 2011; Song et al., 2011). In this study, PA decreased MDA, and increased the level of SOD induced by lithogenic diet, which improved the liver lipid peroxidation (Fig. 3). From the histology, both in H&E and Oil Red O staining (Fig. 4), we can see liver in model group damaged seriously, hepatic cord arrangement disorder and lipid droplets vacuoles apparent. However, PA or UDCA treatment group could reduce liver damage in a dose-dependent manner. In other words, PA can protect live cells from lithogenic diet injury.

The major lipid components of bile—cholesterol, bile acids and phospholipids—are transported from the liver to the bile duct by distinct transporters (Goralski and Sinal, 2004). ABCG5 and ABCG8 are major transporters involved in cholesterol reverse transport in

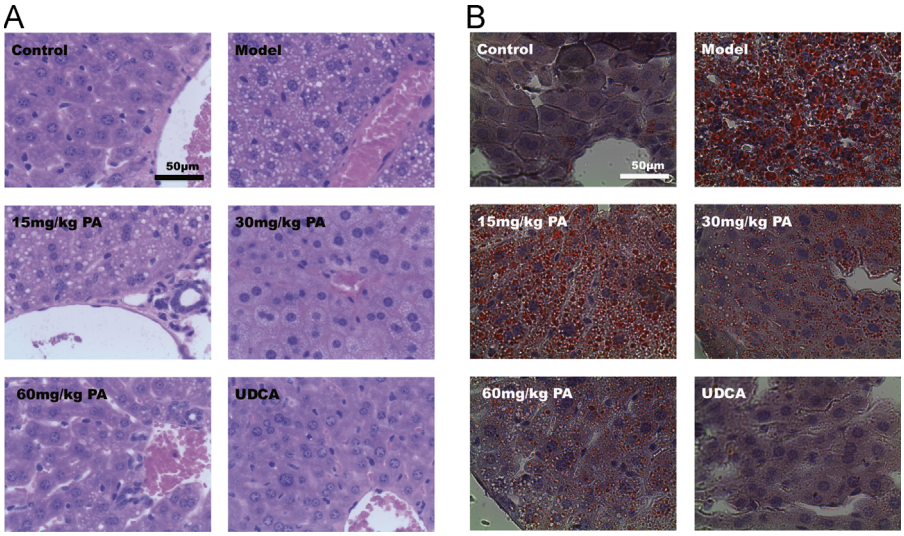

Fig. 4. Effect of PA in hepatic cells injury by histology analysis induced by a lithogenic diet in mice ($n=6$). (A) H&E staining. (B) Oil Red O staining.

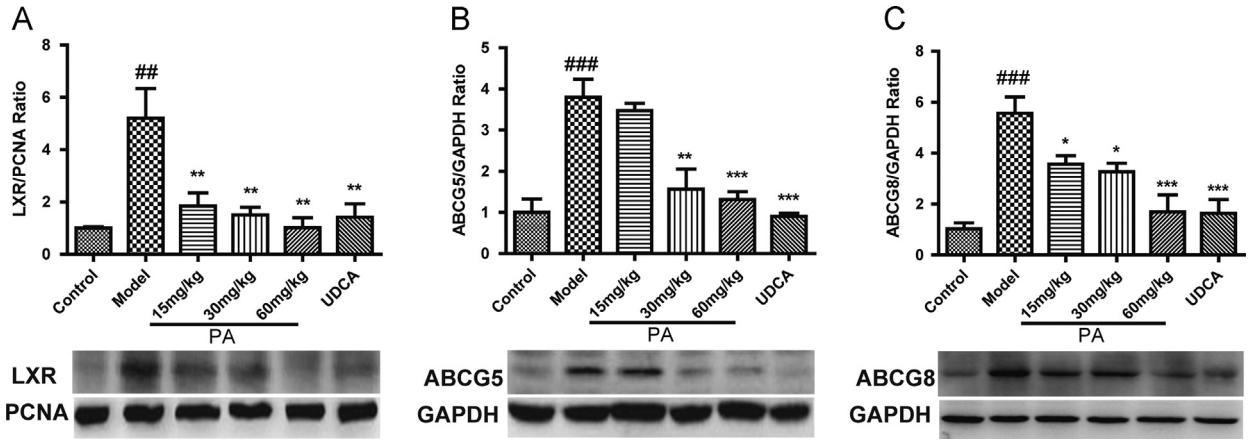

Fig. 5. Effect of PA in the expression of ABCG5/8 and LXR by western blot induced by a lithogenic diet in mice. The protein expression data for LXR (A), ABCG5 (B) and ABCG8 (C) is represented as mean ± S.E.M., $n=3$, $P<0.01$, $P<0.001$ vs. control group, $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ vs. model group.

hepatocytes (Marschall et al., 2010; Calpe-Berdiel et al., 2008). In addition, LXR plays an important role in regulating cholesterol metabolism. The lithogenic diet could induce high expression of LXR, which regulated the transcription of both ABCG5 and ABCG8, and was found to be consistent with the expression of ABCG5/8 (Vázquez et al., 2012; Halilbasic et al., 2013; Jiang et al., 2008). Therefore, our data suggest that the expression of ABCG5, ABCG8 and LXR proteins increased significantly in lithogenic diet group, which were significantly lower by PA or UDCA administration (Fig. 5).

In conclusion, PA prevented cholesterol gallstones formation and reduced biliary cholesterol secretion induced by lithogenic diet in C57BL/6 mice. The possible action mechanism of PA is by suppression in the expression of the hepatic proteins ABCG5, ABCG8 and LXR. The cholesterol from bile re-absorption and transport from hepatocytes to the gallbladder is reduced. Then the level of cholesterol and CSI in gallbladder bile is reduced. And cholesterol crystal formation in the gallbladder is decreased. Moreover, PA alleviated the serum lipid and hepatic peroxidation may also protect the liver and decrease the cholesterol crystal formation. However, there may be other mechanisms of PA in preventing gallstone formation which need further confirmation. PA as a potential drug for preventing cholesterol gallstones merits further investigation.

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