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Original Article

Effects of capsaicin on the cholesterol lithogenesis in the gallbladder of C57BL/6 mice

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Abstract: Objective: High-fat diet was used to induce cholesterol lithogenesis in the gallbladder of C57BL/6 mice in which the effects of capsaicin on the cholesterol lithogenesis were investigated and the potential mechanism was explored. Methods: A total of 30 C57BL/6 mice were randomly didivded intocontrol group (mice were feed with normal food), gallbladder stone group (GS group; mice received high fat diet) and capsaicin group (CA group; mice received high fat diet containing 0.015% capsaicin). Results: Results showed 0%, 100% and 20% of mice had gallbladder stone in control group, GS group and CA group, respectively. All the parameters became abnormal in GS group as compared to control group, but improved to different extents after simultaneous intake of capsaicin (such as total cholesterol in bile acid, and total cholesterol, triglycerides, low-density lipoprotein and high-density lipoprotein in the serum). In GS group, cholesterol saturated index (CSI) increased significantly (1.32 vs. 0.46) as compared to control group suggesting the cholesterol supersaturated bile. The CSI was reduced to 0.8 in CA group. In addition, simultaneous intake of capsaicin significantly improved the abnormal expressions of COX-2, MUC5AC and TRPV1 in the gallbladder and abnormal expressions of CYP7A1, HMG-CoA reductase and TRPV1 in the liver. Conclusion: Capsaicin may prevent the high-fat diet induced cholesterol lithogenesis in C57BL/6 mainly via activating TRPV1 and regulating CYP7A1 and HMG-CoA reductase in the liver as well as COX-2 and MUC5AC in the gallbladder, which are key genes regulating the cholesterol metabolism.

Keywords: Cholesterol lithogenesis, gallbladder, capsaicin, TRPV1, cholesterol metabolism

Introduction

A variety of factors are involved in the cholesterol lithogenesis of the glallbaldder. These factors may promote the cholesterol supersaturation in the bile, resulting in cholesterol lithogenesis. Abnormal cholesterol metabolism is a major cause of cholesterol supersaturation and has been an important cause of cholesterol lithogenesis in the gallbladder. It is shown that HMG-CoA reductase activity remains at a higher level in lithogenesis patients in whom the cholesterol synthesis increases and the accumulation of cholesterol also elevates in the liver [1]. Cholesterol 7alpha-hydroxylase (CYP7A1) is a rate limiting enzyme in the primary synthesis of bile acid [2] and specifically localizes in the liver. In hepatocytes, cholesterol is catalyzed into 7A oxycholesterol which is then subjected to the addition of a hydroxyl group on position 7 of the steroid nucleus by the enzyme CYP7A1, hydroxylation, and side-strand break, resulting in the formation of bile acid. The synthesis of bile acid is a critical step in the cholesterol metabolism in the liver. The secreted bile acid enters the biliary tract and then dissolves the cholesterol, leading to the reduction in free cholesterol. Thus, the synthesis of bile acid may increase the free cholesterol in the liver, decrease the cholesterol in the bile and promote the cholesterol lithogenesis in the gallbladder.

Capsaicin is a compound with spicy smell and the main capsaicinoid in chili peppers. Capsaicin is also known as 8-methyl-N-vanillyl-6-nonenamide (C18H27NO3) and shows monoclinic rectangular flaky colorless crystals. Transient receptor potential vanilloid 1 (TRPV1)

Table 1. Contents of total bile acid, cholesterol and phospholipids in the bile

Group	Cholesterol mmol/L	Phospholipid mmol/L	Bile acid mmol/L	Total lipid g/dL	CSI
Control	9.1	31	156	10.41	0.46
CL	24.2	28	138	9.88	1.32
CA	15.4	29	151	10.26	0.80

CSI: cholesterol saturated index; CL group: ithogenesis group; CA group: Capsaicin group.

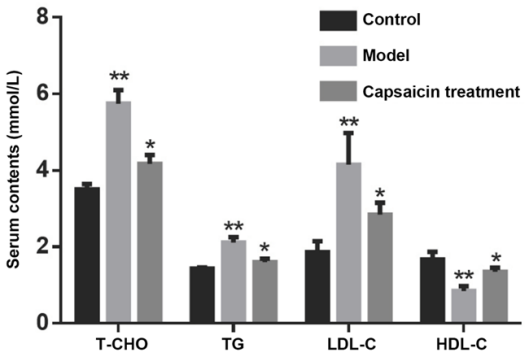

Figure 1. Serum contents of T-CHO, TG, LDL-C and HDL-C in different groups. **P < 0.01 vs. control group; *P < 0.01 vs. CL group. Normal diet group (control group): mice were fed with normal diet; Cholesterol lithogenesis group (model): mice were fed with lithogenesis inducing diet. Capsaicin group (capsaicin treatment): mice were fed with lithogenesis inducing diet mixed with capsaicin (0.015%).

is an important non-selective cation channel on cell membrane and its activation may cause increase in intracellular calcium, which plays crucial roles in the regulation of multiple pathophysiological processes. TRPV1 is a specific receptor of capsaicin [3]. In recent years, capsaicin has been widely used in clinical practice. In cardiovascular studies, capsaicin with analgesic and anti-inflammatory activities is found to prevent obesity, induce the apoptosis of cancer cells, lower blood pressure and reduce blood lipids. However, its influence on the lithogenesis in the gallbladder has never been reported. There is evidence showing that capsaicin may reduce the HMG-CoA reductase expression in the liver [4]. HMG-CoA reductase is a key enzyme in the cholesterol metabolism. Thus, to reduce HMG-CoA reductase expression may have the potential to influence the cholesterol lithogenesis [5]. In addition, capsaicin is able to effective up-regulate the CYP7A1 expression in the liver and promote the transformation of cholesterol into bile acid [6]. Mice with reduced CYP7A1 expression are more susceptible to gallbladder lithogenesis [7]. Capsaicin may exert anti-inflammatory effect via regulating the expression of some pro-inflammatory cytokines (such as COX-2) [8]. Nilsson et al found high COX-2 expression in the mucosa of inflammatory gallbladder and lithogenesis was closely related to inflammation [9]. Lithogenesis is frequently accompanied by the chronic inflammatory process, and to inhibit gallbladder inflammation may inhibit the lithogenesis and its recurrence [10].

In this study, high fat diet was used to induce the cholesterol lithogenesis in the gallbladder of C57BL/6 and then: 1) the total bile acid, total cholesterol and phospholipids in the bile were measured, and cholesterol saturated index (CSI) was calculated; 2) the total cholesterol (T-CHO), triglyceride (TG), low density lipoprotein (LDL-C) and high density lipoprotein (HDL-C) in the serum were measured; 3) the gallbladder was collected and processed for HE staining and immunohistochemistry for COX-2, MUC5AC and TRPV1; 4) the liver was collected and processed for immunohistochemistry for HMG-CoA reductase, TRPV1 and CYP7A1, and Western blot assay was performed to detect the CYP7A1, HMG-CoA reductase and TRPV1 expressions in the liver. This study aimed to investigate the pathogenesis of cholesterol lithogenesis in the gallbladder which may provide scientific evidence for the prevention and therapy of cholesterol lithogenesis in the gallbladder.

Materials and methods

Preparation of high fat diet

The normal diet for rodents was prepared according to the AIN-93 standard developed by the American Nutrition Society and contained 0.02% cholesterol (wt/wt). The diet was produced in the Experimental Animal Center of Daping Hospital, the Third Military Medical University. Then 2% cholesterol, 0.5% cholic acid and 15% lard were added to the normal diet to induce cholesterol lithogenesis.

Grouping and feeding

A total of 30 C57BL/6 mice were purchased from the Experimental Animal Center of Daping Hospital, the Third Military Medical University

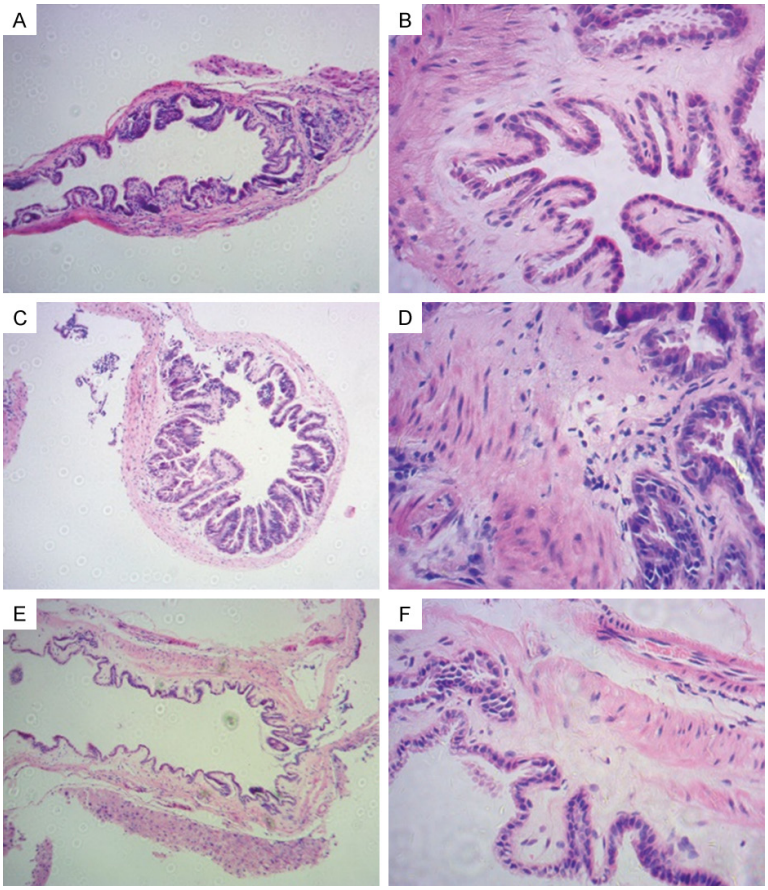

Figure 2. HE staining of the gallbladder in different groups. A: Control group (×100); B: Control group (×400); C: CL group (×100); D: CL group (×400); E: CA group (×100); F: CA group (×400). Normal diet group (control group): micewere fed with normal diet; Cholesterol lithogenesis group (CL group): micewere fed with lithogenesis inducing diet. Capsaicin group (CA group): mice were fed with lithogenesis inducing diet mixed with capsaicin (0.015%).

and weighed, followed by numbering. Then, they were housed separately with 5 mice in each cage and given ad libitum access to water and food. Mice were fed with normal diet for 1 week and then with either normal diet, lithogenesis inducing diet or lithogenesis inducing diet containing capsaicin (0.015%) for 8 weeks. All the animal procedures were in accordance with the Guideline for Animal Use and Care of AAALAC.

Normal diet group (control group): mice (n=10) were fed with normal diet; Cholesterol lithogenesis group (CL group): mice (n=10) were fed with lithogenesis inducing diet. Capsaicin group (CA group): mice (n=10) were fed with lithogenesis inducing diet mixed with capsaicin (0.015%).

Observations

During the study, the general conditions of C57BL/6 mice in different groups were observed and recorded: the body weight, food intake, hair, activity and spirit.

Sample collection

After the mice were housed for 8 weeks, they received food deprivation overnight and anesthetized with ether on the second day. The animals were fixed on an operation table and the hair in the abdomen was removed. After sterilization with ethanol, a midline incision was made at the abdomen, followed by laparotomy. The liver, gallbladder and gallbladder lithogenesis were observed and photographed. The gallbladder was carefully peeled off the gallbladder bed with tweezers. The cystic duct was clipped, and the gallbladder was removed into a PCR tube. The gallbladder was cut by anophthalmic scissors. The bile was collected into a PCR tube, marked and stored at -20°C. Then, a micro-syringe (2 ml) was used to collect the blood from left ventricle. The collected blood was slowly injected into a biochemistry tube, followed by centrifugation at 3000 rpm for 30 min at 4°C. Then, the supernatant (serum) was collected and stored at 4°C. The liver and gallbladder were fixedin 4% paraformaldehyde and stored at 4°C.

Observation of gallbladder bile under a polarizing microscope

A drop of bile was randomly collected from two mice in each group smeared on a slide which was dried in air. The slide was numbered and observed for cholesterol crystals under a polarizing microscope.

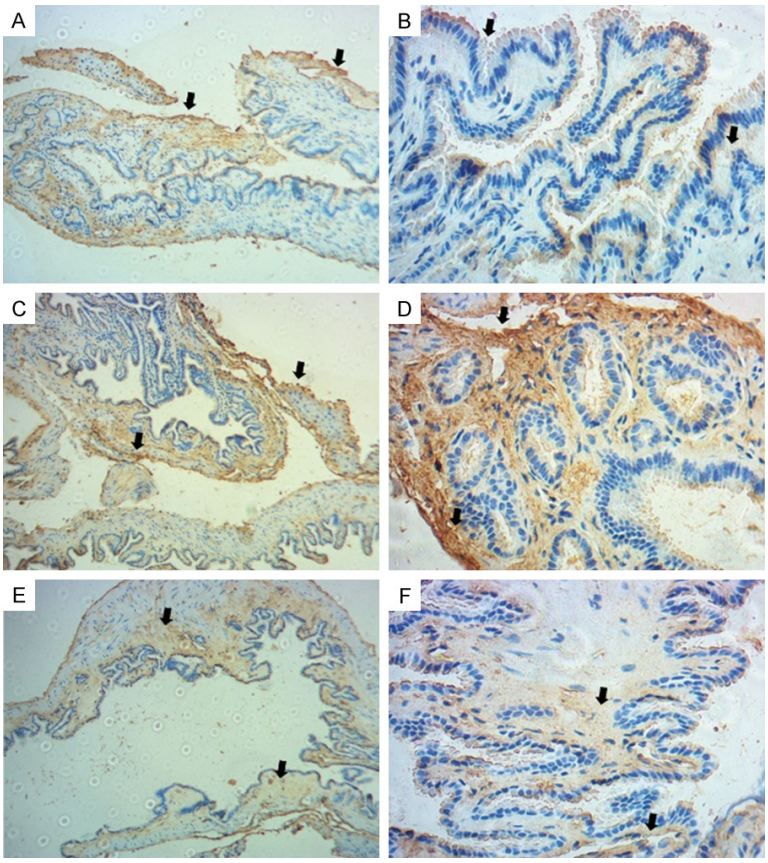

Figure 3. Immunohistochemistry for COX expression in the gallbladder. A: Control group (×100); B: Control group (×400); C: CL group (×100); D: CL group (×400); E: CA group (×100); F: CA group (×400). Arrow: COX expression. Normal diet group (control group): mice were fed with normal diet; Cholesterol lithogenesis group (CL group): mice were fed with lithogenesis inducing diet. Capsaicin group (CA group): mice were fed with lithogenesis inducing diet mixed with capsaicin (0.015%).

Biochemistry of the bile

Bile stored at 20°C was thawed on crushed ice, and then samples were absorbed according to the instruction of Kit. The cholesterol content was measured with the 3000 semi-automatic biochemical analyzer (Beckman Coulter, USA), while the total bile acid and phospholipids were measured with correspondent kits (ELISA). According to the contents of total cholesterol, bile acid and phospholipids, their mole percentage were calculated separated, and the total lipid concentration as well as phospholipid/(phospholipids + TBA) was calculated on the basis of which the corresponding CSI was searched in the Carey table [11].

Detection of lipids in the serum

The serum was collected, and the lipids (triglycerides [TG], total cholesterol [TC], high-density lipoprotein cholesterol [HDL-C] and low-density lipoprotein cholesterol [LDL-C]) were measured with the 3000 semi-automatic biochemical analyzer by enzyme colorimetric method, while the serum total bile acid [TBA] and glutamic-pyruvic transaminase were measured with correspondent kits (ELISA). Serum was stored at -20°C for further use.

Pathological examination of the gallbladder

The gallbladder was fixed, washed, dehydrated, transparentized, embedded in paraffin, and sectioned, followed by HE staining. Sections were observed under a light microscope. Cell nucleus was blue, cartilage matrix and calcium particles were dark blue, and the mucus was grey blue. The cytoplasm was pink, and the eosinophilic granules in the cytoplasm were bright red. The collagens were light red, elastic fibers were light pink, red blood cells were orange and protein containing liquid was pink.

Immunohistochemistry for COX-2, MUC5AC and TRPV1 in the gallbladder and CYP7A1, HMG-CoA reductase and TRPV1 in the liver

After deparaffinization and hydration, sections were incubated with 3% H_2O_2 for 5-10 min, followed by antigen retrieval in 0.01 M citric acid. After blocking in 5% BSA for 20 min, sections were treated with phospho-MAPK polyclonal antibody (1:1000) at 4°C overnight. Following incubation with HRP conjugated goat anti-rabbit IgG (1:1000) at 37°C for 20 min, visualization was performed with DAB, and counterstaining was performed with hematoxylin, followed by dehydration, transparentization and mounting. In blank control group, the primary antibody was replaced with PBS. Positive cells had brown or yellow granules in the nucleus and/or cytoplasm. The staining intensity was classified as no staining (negative), light yellow (weakly positive), and brown-yellow (positive).

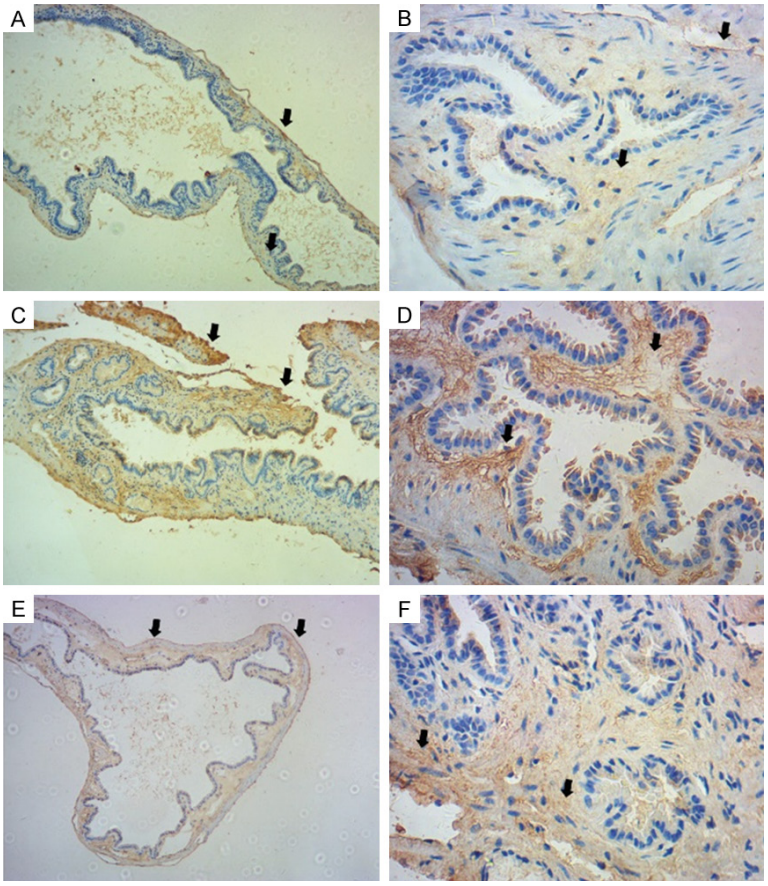

Figure 4. Immunohistochemistry for MUC5AC expression in the gallbladder. A: Control group (×100); B: Control group (×400); C: CL group (×100); D: CL group (×400); E: CA group (×100); F: CA group (×400). Arrow: MUC5AC expression. Normal diet group (control group): mice were fed with normal diet; Cholesterol lithogenesis group (CL group): mice were fed with lithogenesis inducing diet. Capsaicin group (CA group): mice were fed with lithogenesis inducing diet mixed with capsaicin (0.015%).

Statistical analysis

All the data are expressed as mean ± standard deviation (SD) and statistical analysis was performed with SPSS version 13.0. Comparisons were performed with t test and a value of $P < 0.05$ was considered statistically significant.

Results

General condition and incidence of lithogenesis

In control group, mice were fed with normal diet, in CL group and CA group, mice were fed with lithogenesis inducing diet for 8 weeks, but mice in CA group were simultaneously fed with 0.015% capsaicin. In three groups, none died during the study, and the spirit status, hair condition, food intake, body weight and activity were comparable among them. The bile was clear and light yellow and had no stones in control group (0/10). In CL group, the bile was thick and yellow, granular stones were found in the gallbladder and the incidence of lithogenesis was 100% (10/10). After capsaicin treatment, only a small amount of sand-like stones was found in the gallbladder and the incidence of lithogenesis was only 20% (2/10), the bile was light yellow. Under a polarized light microscope, no cholesterol crystals were observed in the bile of mice in control group. In CL group, a large amount of characteristic cholesterol-hydrate crystals were found in the bile. In CA group, the cholesterol crystals were only occasionally observed, and the size and amount of crystals were smaller than those in CL group.

Contents of total bile acid, cholesterol and phospholipids in the bile

When compared with control group (9.1 mmol/L), the cholesterol content in CL group increased significantly ($P < 0.01$), but it reduced after capsaicin treatment in CA group (15.4 mmol/L). The CSI in CL group was significantly higher than in control group (1.32 VS. 0.46), and the CSI in CL group was higher than 1, suggesting the cholesterol supersaturated bile. In CA group, the CSI reduced significantly (0.8) when compared with CL group (**Table 1**).

Serum lipids and liver function in different groups

In CL group, the contents of serum total cholesterol (T-CHO), triglyceride (TG) and low density lipoprotein (LDL-C) increased significantly, but

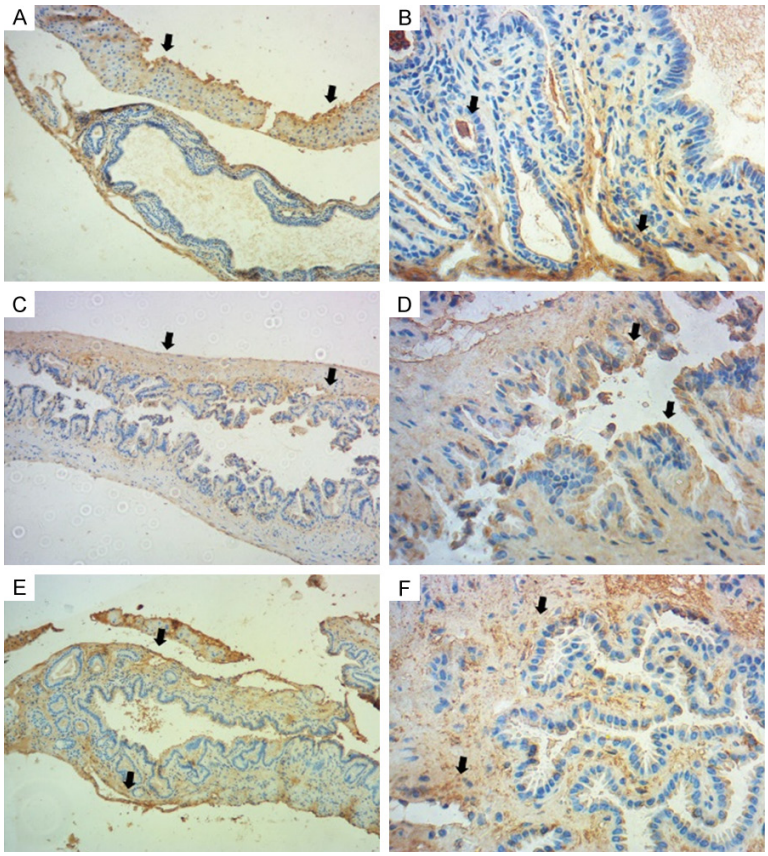

Figure 5. Immunohistochemistry for TRPV1 expression in the gallbladder. A: Control group (×100); B: Control group (×400); C: CL group (×100); D: CL group (×400); E: CA group (×100); F: CA group (×400). Arrow: TRPV1 expression. Normal diet group (control group): mice were fed with normal diet; Cholesterol lithogenesis group (CL group): mice were fed with lithogenesis inducing diet. Capsaicin group (CA group): mice were fed with lithogenesis inducing diet mixed with capsaicin (0.015%).

the content of high density lipoprotein (HDL-C) reduced markedly when compared with control group (**Figure 1**). However, the serum contents of T-CHO, TG and LDL-C reduced markedly and the serum content of HDL-C increased dramatically after capsaicin treatment in CA group when compared with CL group ($P < 0.05$; **Figure 1**).

HE staining of the gallbladder

Under a light microscope, the mucus in the gallbladder increased significantly, the connective tissues in the lamina propria increased, and the small vessels and collagens elevated in CL group when compared with control group, but these pathological changes were attenuated after capsaicin treatment in CA group (**Figure 2**).

Immunohistochemistry of the gallbladder

The gallbladder was collected and processed for immunohistochemistry. Results showed the expressions of COX-2 and MUC5AC in the gallbladder increased, but TRPV1 expression reduced significantly in CL group as compared to control group. However, after capsaicin treatment, the expressions of COX-2 and MUC5AC in the gallbladder reduced significantly, and the TRPV1 expression increased markedly as compared to CL group (**Figures 3**-**5**).

Immunohistochemistry of the liver

The liver was harvested and processed for immunohistochemistry. Results showed the expressions of CYP7A1 and TRPV1 in the liver reduced significantly, but the HMG-CoA reductase increased markedly in CL group when compared with control group. However, after capsaicin treatment, the expressions of CYP7A1 and TRPV1 in the liver increased significantly, but the HMG-CoA reductase reduced markedly when compared with CL group (**Figures 6**-**8**).

Expression of CYP7A1, HMGCoA reductase and TRPV1

Expression of CYP7A1, HMGCoA reductase and TRPV1 was detected with western blot to investigate the effects of expression regulation of hepatobiliary transporters in cholesterol gallstone on accumulation of cholesterolin liver. Results of western blot showed that when compared with control group, expression of CYP7A1 and TRPV1 decreased significantly while HMGCoA reductase increased significantly in CL group; and expression of CYP7A1 and TRPV1 increased significantly while

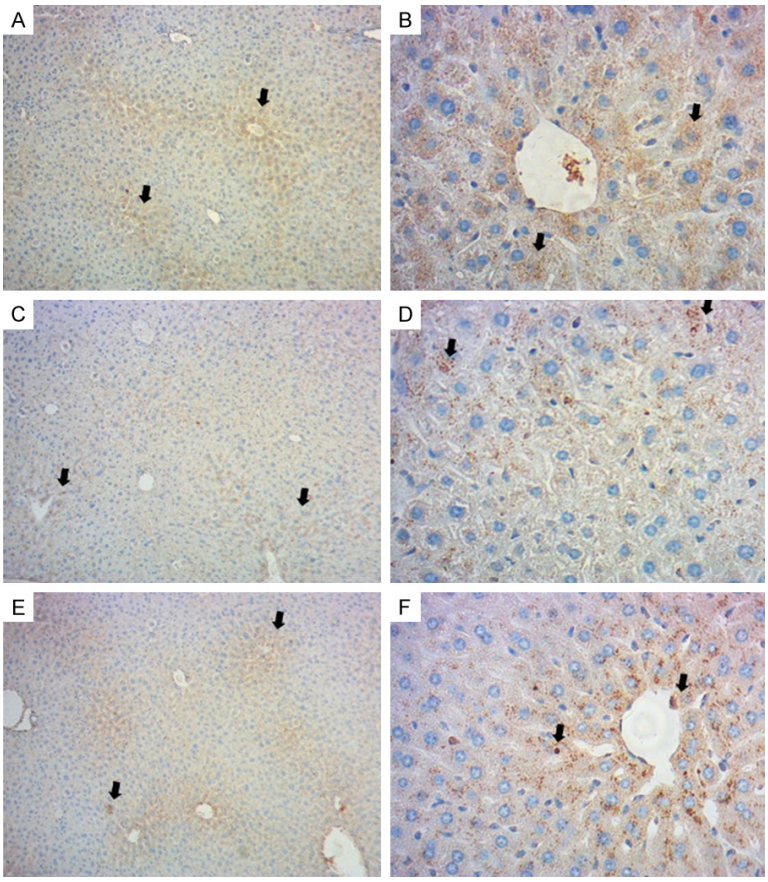

Figure 6. Immunohistochemistry for CYP7A1 expression in the liver. A: Control group (×100); B: Control group (×400); C: CL group (×100); D: CL group (×400); E: CA group (×100); F: CA group (×400). Arrow: CYP7A1 expression. Normal diet group (control group): mice were fed with normal diet; Cholesterol lithogenesis group (CL group): mice were fed with lithogenesis inducing diet. Capsaicin group (CA group): mice were fed with lithogenesis inducing diet mixed with capsaicin (0.015%).

HMGCoA reductase decreased significantly in CA group.

Discussion

The pathogenesis of cholesterol lithogenesis is very complex, and the excess secretion of cholesterol and the formation of cholesterol supersaturated crystals in the bile are the major causes. In addition, abnormal lipid metabolism in the blood may cause changes in physical and chemical characteristics of the bile, which also plays an important role in the lithogenesis of the gallbladder. The serum contents of cholesterol, TG and LDL-C increased significantly, and the contents of total bile acid, cholesterol and phospholipids in the bile increased markedly in mice with cholesterol lithogenesis. Moreover, there is evidence showing that capsaicin is effective to reduce the contents of cholesterol and triglycerides in the plasma and bile, which may be associated with the capsaicin induced oxidation and decomposition of fat [12, 13]. In a study, rats were fed with capsaicin at 50 mg/kg/d for 60 d, and results showed the contents of cholesterol, triglycerides and phospholipids in the plasma reduced significantly [14]. Rabbits were fed with a diet containing 0.5% cholesterol and capsaicin at 8 mg/kg/d for 30 d, and the contents of cholesterol and triglycerides in the plasma reduced dramatically [15]. Treatment with capsaicin at a low dose for 8 weeks is able to reduce the plasma cholesterol in rats with hyperlipidemia, the reduction in plasma cholesterol after capsaicin treatment is mainly ascribed to the decrease in LDL-C [16], and the plasma HDL-C remains stable. In the present study, mice were simultaneously fed with capsaicin in CA group, and results showed the plasma contents of cholesterol, triglycerides and LDL-C reduced significantly, accompanied by improvement of serum lipoproteins and reductions in total bile acid, cholesterol and phospholipids, leading to the inhibition of cholesterol crystal formation and the improvement of gallbladder pathology. During the study, all the mice showed a good growth status, none died, and abnormalities were also not observed. Capsaicin had no influence on the food intake, body weight and activity. These findings suggest that capsaicin is able to prevent the cholesterol lithogenesis.

Liver is a major organ where the cholesterol is synthesized and metabolized. HMG-CoA reductase is a key rate limiting enzyme in the synthesis of endogenous cholesterol, and the biochemical synthesis of a large amount of cholesterol requires the catalysis by HMG-CoA

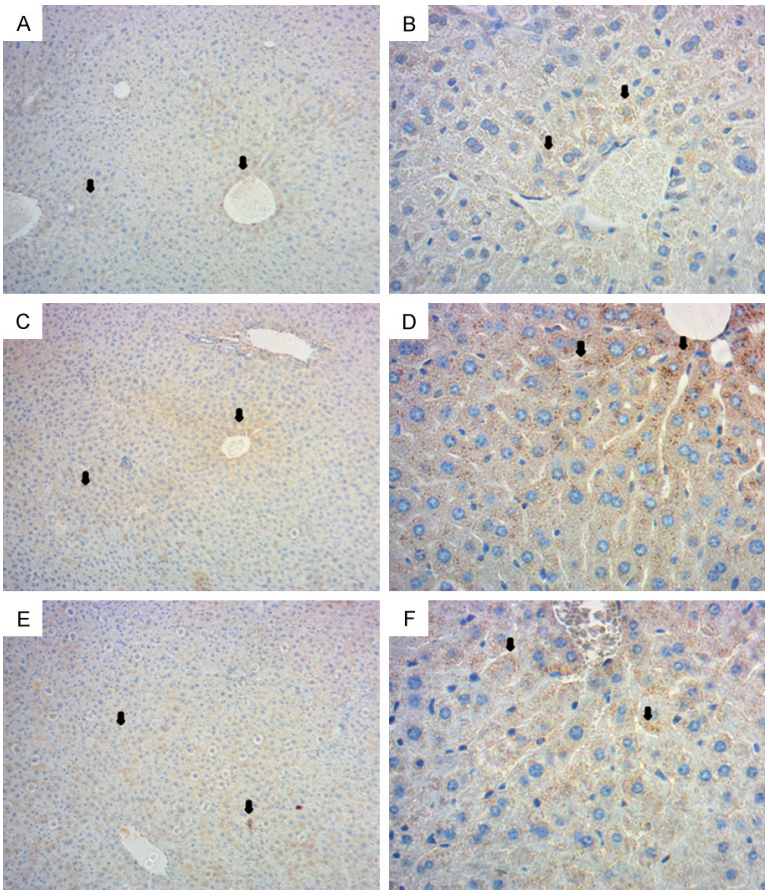

Figure 7. Immunohistochemistry for HMG-CoA reductase expression in the liver. A: Control group (×100); B: Control group (×400); C: CL group (×100); D: CL group (×400); E: CA group (×100); F: CA group (×400). Normal diet group (control group): mice were fed with normal diet; Cholesterol lithogenesis group (CL group): mice were fed with lithogenesis inducing diet. Capsaicin group (CA group): mice were fed with lithogenesis inducing diet mixed with capsaicin (0.015%).

reductase [17, 18]. Thus, to inhibit the HMG-CoA reductase expression has been a major strategy to lower the plasma cholesterol [19, 20]. It has been shown that plant extracts and spices are able to reduce the cholesterol synthesis by inhibiting HMG-CoA reductase expression [21]. In the present study, our results also showed the HMG-CoA reductase expression increased significantly in the liver of CL group, but supplement with capsaicin markedly reduced the HMG-CoA reductase expression (**Figure 6**). It is indicated that capsaicin is able to inhibit the endogenous cholesterol synthesis in the liver. Generally, the cholesterol may enter the bile duct along with the bile or be transformed into bile acid which is then excreted. CYP7A1 is a rate-limiting enzyme in the synthesis of bile acid, and to regulate the CYP7A1 is able to promote the transformation of cholesterol into bile acid and maintain the balance between cholesterol and bile acid [22]. Thus, the capsaicin induced inhibition of cholesterol lithogenesis may be related to its suppression on HMG-CoA reductase expression and its induction of CYP7A1 expression in the liver. Studies have revealed that capsaicin is an agonist of TRPV1 and may activate TRPV1 to exert the body weight-lowering effect, analgesic effect, cardioprotective effect, cerebroprotective effect and gastrointestinal effect [23-25]. However, these effects reduce significantly after blocking of TRPV1. In our study, results showed capsaicin significantly increased the TRPV1 protein expression in the liver and gallbladder of mice with cholesterol lithogenesis (**Figures 4** and **7**).

In addition, the MUC5AC expression rate was as high as 61% in the gallbladder with cholesterol lithogenesis and 45.4% in the gallbladder with bilirubin lithogenesis, suggesting that MUC5AC over-expression is closely related to the gallbladder lithogenesis. COX-2 is a key factor in the gallbladder lithogenesis and is closely associated with the activity of cholesterol synthesis enzyme and gallbladder inflammation. In our study, results showed the protein expressions of MUC5AC and COX-2 increased in the liver and gallbladder of mice with cholesterol lithogenesis, which, however, were inhibited by capsaicin (**Figures 2** and **3**).

In our study, lithogenesis inducing diet was used to establish the animal model of cholesterol lithogenesis of the gallbladder in C57BL/6 mice, and the protective effects of capsaicin on the cholesterol lithogenesis were investigated. In addition, the expressions of key enzymes in the synthesis of cholesterol were detected in

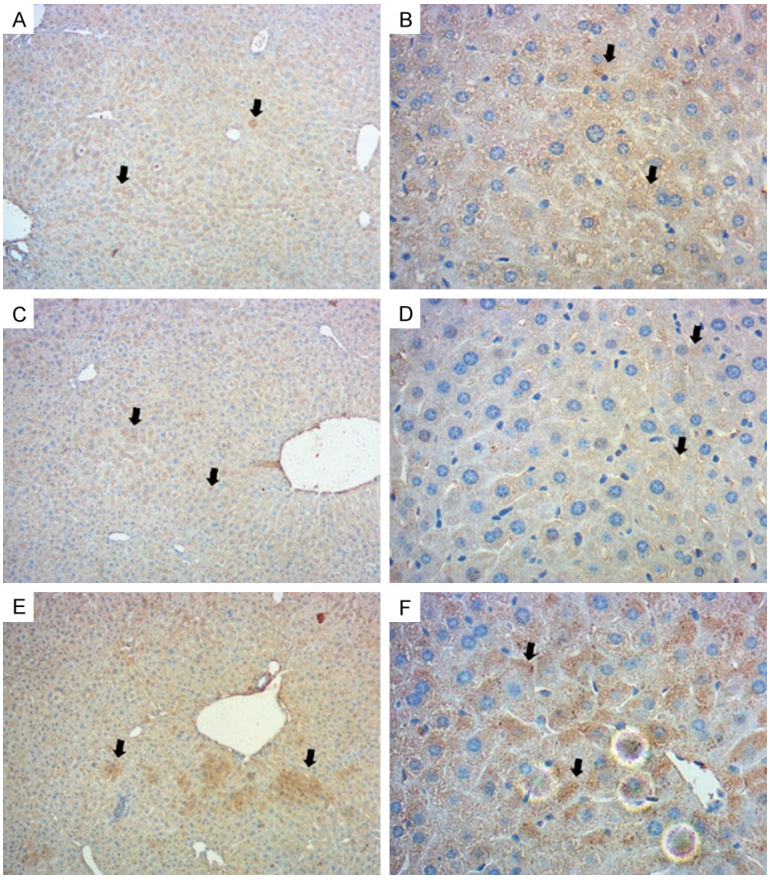

Figure 8. Immunohistochemistry for TRPV1 expression in the liver. A: Control group (×100); B: Control group (×400); C: CL group (×100); D: CL group (×400); E: CA group (×100); F: CA group (×400). Arrow: TRPV1 expression. Normal diet group (control group): mice were fed with normal diet; Cholesterol lithogenesis group (CL group): mice were fed with lithogenesis inducing diet. Capsaicin group (CA group): mice were fed with lithogenesis inducing diet mixed with capsaicin (0.015%).

the liver and gallbladder, aiming to elucidate the mechanism underlying the protective effects of capsaicin on the cholesterol lithogenesis. Our findings provide experimental evidence on the anti-lithogenic effect of capsaicin.

Disclosure of conflict of interest

None.

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