Effect of nicotine on cholesterol gallstone formation in C57BL/6J mice fed on a lithogenic diet

QIAN GAO[1*], PINDUAN BI[2*], QILI MI[1], YING GUAN[1], JIARUI JIANG[1], XUEMEI LI[1] and BIN YANG[2]

[1]Yunnan Key Laboratory of Tobacco Chemistry, R&D Center of China Tobacco Yunnan Industrial Co. Ltd., Kunming, Yunnan 650106; [2]Department of Hepatobiliary Surgery, The First Affiliated Hospital of Kunming Medical University, Kunming, Yunnan 650032, P.R. China

Abstract. Gallstones are diseases of the biliary system caused by cholesterol supersaturation and/or deficiency in bile salts in bile. Early studies have shown that symptomatic gallstones are primarily a disease of non-smokers, raising the possibility that nicotine can prevent gallstone formation. The present study investigated the effect of nicotine on the formation of cholesterol gallstone in C57BL/6J mice. C57BL/6J mice (eight-weeks-old) were fed a normal or lithogenic diet (basic feed 82.45%, fat 15.8%, cholesterol 1.25% and sodium cholate 0.5%) and divided into five groups: normal diet (ND); ND + high dose nicotine (H); lithogenic diet (LD); LD + low dose nicotine (L) and LD + nicotine (H). They were treated with or without nicotine injection for 10 weeks. Nicotine treatment did not change the rate of cholesterol gallstone formation. There was no difference in TNFα, IL-1β and IL-6 among the five groups. The LD group showed the highest cholesterol levels and there was significant suppression of the total cholesterol, low-density lipoprotein-cholesterol and total bile acid levels in the serum of the nicotine-treated mice. Quantitative PCR showed nicotine altered few bile acid metabolism-related genes expression in liver tissue and significantly altered cholesterol-metabolism genes in gallbladder tissue. Hematoxylin and eosin staining and western blotting showed that protein levels of farnesoid X receptor (FXR) and megalin in the gallbladder increased in the lithogenic-diet mice, which was significantly suppressed in the nicotine-treated mice. In vitro studies using gallbladder epithelial cells showed that chenodeoxycholic acids increased megalin expression, which could be attenuated by nicotine. Nicotine could regulate bile acid metabolism via the FXR-megalin/cubilin pathways, which potentially contribute to cholesterol nucleation and subsequent gallstone formation.

Correspondence to: Dr Bin Yang, Department of Hepatobiliary Surgery, The First Affiliated Hospital of Kunming Medical University, 295 Xichang Road, Wuhua, Kunming, Yunnan 650032, P.R. China
E-mail: yangbin@kmmu.edu.cn

*Contributed equally

Introduction

Cholesterol gallstones are a major public health problem in all developed countries. In China, ~10-15% of the adult population suffers from cholesterol gallstones (1,2), which constitute one of the commonest and most costly digestive diseases (3). The development of gallstone is influenced by a number of factors, including smoking (4,5). However, several studies suggest that smoking is not a risk factor for gallstones and even has the opposite effect (6,7). In these studies, older smokers had a relatively lower prevalence of gallstones, whereas younger smokers had an increased risk of gallstones (8). Compared with non-smokers, older smokers who smoke for most of their lives have a lower risk of gallstone disease (9). In view of this, it is necessary to understand the possible mechanisms by which smoking may affect gallstone formation. Smoking may change the levels of gallstone-related protein in the gallbladder or bile composition, which could influence gallstone formation (10,11).

Bile acids (BAs), synthesized from cholesterol molecules, serve a critical role in eliminating excess cholesterol from the body and process dietary fat by facilitating the formation of micelles. The formation of gallstones is related to the metabolic disturbance of BAs. BAs, the final product of cholesterol metabolism, are the main component of bile, accounting for 50-70% of the total bile. Almost all patients with gallstone have abnormal BA metabolism (12). When the proportions of different BAs are imbalanced, cholesterol cannot maintain the state of micelles and gradually forms crystals and precipitates, eventually resulting in cholesterol stones (13,14). Thus, it is important to tightly regulate BA synthesis.

Megalin and cubilin proteins are expressed in gallbladder epithelial cells but not in hepatocytes. There are a number of megalin/cubilin ligands in bile, to mediate endocytosis of numerous ligands including high-density lipoprotein (HDL)/apolipoprotein A-I (apoA-I) (15). Dysregulation of megalin and cubilin at the mRNA and protein levels has been found in either humans or mice with gallstones (15,16). A study demonstrates that bile acids can regulate the expression

of megalin and cubilin, an effect that appeared to be mediated by the bile acid nuclear hormone receptor farnesoid X receptor (FXR) (17). It is reported that a synthetic FXR agonist could prevent gallstone formation in susceptible wild-type mice that recapitulate human cholesterol gallstone disease (5). Together, they catalyze the synthesis of two major BAs, cholic acid (CA) and chenodeoxycholic acid (CDCA) (18). Therefore, FXR/megalin/cubilin pathway serves an important role in maintaining BA homeostasis (19).

The present study attempted to discover whether nicotine, as a major component of tobacco smoke, could have a preventive effect on gallstone-susceptible C57BL6 mice. In addition, it explored if FXR/megalin/cubilin pathways had participated in this process.

Materials and methods

Animals and diets. Male C57BL6 mice (8 weeks old, 18-20 g, n=50) were purchased from the Central Laboratory of Kunming medical university. Mice were fed on normal rodent feedstuff (cholesterol <0.02%) or a lithogenic diet (1.25% cholesterol plus 0.5% cholic acid and 15.8% buffer) for 4 weeks and divided into five groups (10 mice/group): i) ND (mice fed with normal diet), ii) ND + nicotine (H) (mice fed with normal diet and treated with 6.6 mg/kg/2 days nicotine), ii) LD (mice fed with lithogenic diet), iii) LD + nicotine (L) (mice fed with lithogenic diet and treated with 1.1 mg/kg/2 days nicotine), and v) LD + nicotine (H) (mice fed with lithogenic diet and treated with 6.6 mg/kg/2 days nicotine). Mice of all groups (except group ND) were treated with nicotine (1.1 mg and 6.6 mg) for 10 weeks after being weighed every two days (20-23). All mice in groups received free access to water and were kept under the controlled condition at room temperature (22±3°C), with a relative humidity of 60-70% and a 12-h light/dark cycle. The mice were anesthetized with 4% chloral hydrate (300 mg/kg) by intraperitoneal injection. Mice were sacrificed with 150 mg/kg pentobarbital sodium by intraperitoneal (i.p.) injection. The animal experiments were approved by the Institutional Animal Care and Use Committee of Kunming medical university (approval no. kmmu2021058).

Collection of gallbladder biles and gallstone and microscopic studies. At week 10 of the diet feeding, non-fasted animals were weighed and anesthetized with an i.p. injection of 35 mg/kg pentobarbital. After cholecystectomy, gallbladder volume was measured by weighing the whole gallbladder and equating gallbladder weight (including stones) with gallbladder volume. Gallbladders were then opened and 5 μl of fresh gallbladder bile was examined for solid and liquid crystals and gallstones. The pooled gallbladder biles were centrifuged at 100,000 x g for 30 min at 37°C and filtered through a preheated (37°C) Swinnex-GS filter (0.22 μm) assembly (MilliporeSigma), samples were frozen and stored at -20°C for further lipid analyses. Mouse blood was collected from the vena cava and separated serum samples were stored in a -80°C biofreezer. The liver and gallbladder were isolated and frozen in liquid N_2 until required for analysis.

Blood chemical analysis. The serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol, HDL-cholesterol, phospholipids and triglycerides were determined using an automated Hitachi Clinical Analyzer (model 7020; Hitachi Ltd.).

Lipid analysis. Levels of bile cholesterol, phospholipid, bile salts as well as cholesterol and triglyceride in bile were determined respectively following the manufacturer's instructions. Bile from 10 mins' drainage was sampled. Bile cholesterol and phospholipid concentrations were determined by Cholesterol E-Test Wako kit (cat. no. 999-02601; FUJIFILM Wako Pure Chemical Corporation) and Phospholipid C-Test Wako kit (cat. no. 433-36201; FUJIFILM Wako Pure Chemical Corporation), respectively. The bile salts concentrations were measured by a Nexera X2 Ultra High-Performance Liquid Chromatography system (Shimadzu, Kyoto, Japan) according to the method of Paulusma *et al* (24). Briefly, 5 μl of diluted bile (1:100) were spiked with 20 μl of internal standard solution in methanol:water (1:1 v/v). Protein precipitation was performed with 30 μl of methanol, followed by 10,000 x g centrifugation for 10 min at 4°C. The supernatant was used for LC-MS/MS analysis.

Histopathological stain and Immunohistochemistry analysis. Paraffin-embedded gallbladder and liver sections (5 μm in thickness) were dewaxed twice in xylene solutions for 10 min each and rehydrated in descending alcohol series. Then paraffin sections were stained with hematoxylin for 5 min at room temperature and differentiated with 0.1% hydrochloric acid ethanol for 1 min, followed with eosin for 5 min at room temperature, dehydrated in ascending alcohol series, followed by being cleared twice in xylene solutions for 10 min each. They were observed at x100 magnification under a light microscope.

To confirm the expression of megalin and FXR, 10% formalin (48 h at 20-22°C)-fixed tissues were embedded in paraffin. The 5 μm paraffin sections were subjected to anti-FXR (1:500, Novus Biologicals) or anti-megalin antibody (1:200, Abcam) overnight at 4°C and stained with a DAB (3,3'Diaminobenzidine) kit (MXB Biotechnologies) for 15 min at 20-22°C. The positive staining was taken photograph under a light microscope (magnification, x40).

All samples were blindly inspected by two independent pathologists. Positive immunostaining was visualized as brown granules contained in the cytoplasm. The immunostaining was scored by evaluating the intensity and percentage of positively stained cells. The intensity of FXR or megalin staining was scored as follows: 0, none; 1, weak; 2, moderate; and 3, strong. The percentage scores were assigned, as follows: 1, ≤25; 2, 26-50; 3, 51-75; and 4, >75%. These scores were multiplied to arrive at a final score ranging between 0 and 12.

Cell culture and treatments. The human gallbladder epithelial cell line GBEC were purchased from iCell Bioscience Inc. and were cultured in low-glucose DMEM supplemented with 2 mM glutamine, 1% MEM non-essential amino acids solution, 1% MEM vitamin solution, 7.5% FBS and P/S (all from Gibco; Thermo Fisher Scientific, Inc.). The cells were seeded at ~70-80% confluence in complete media for 24 h and maintained at 37°C in 5% CO_2. For CDCA and nicotine treatments, GBECs were plated at 3x10^4 cells/cm^2 for 48 h and subsequently cultured overnight at 37°C in the low-serum

medium. 10 μM CDCA was added medium for 6 h, then 2 μM nicotine was added. Cells were cultured at 37°C in 5% CO_2 for another 18 h for reverse transcription-quantitative (RT-q) PCR and 48 h for western blot analysis.

RT-qPCR. Total RNA was isolated from $1x10^5$ cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's instructions and the concentration of RNA was calculated by spectrophotometry. cDNA was prepared using the PrimeScript RT reagent kit with gDNA Eraser (Takara Biotechnology Co., Ltd.). Reverse transcription PCR was performed u sing SYBR Green Premix Ex Taq (Takara) according to the manufacturer's protocols Sequences for the primers used are in Table I. Primer sequences were synthesized by TsingKe Biological Technology.

For reverse transcription PCR, the PCR mixture was denatured at 95°C for 10 sec, annealed at 60°C for 20 sec and then extended at 72°C for 30 sec. This process was repeated for a total of 40 cycles. The relation of NPC1 like intracellular cholesterol transporter 1 (NPC1L1) and sterol regulatory element-binding protein 2 (SREBP-2) mRNA expression with β-actin was calculated based on the threshold cycle (Ct) values. The relative mRNA expression of β-actin was calculated by the inverse log of $\Delta\Delta$Cq (25). All experiments were performed in triplicate.

Western blot analysis. Protein were lysed using RIPA lysis buffer (Beyotime Biotechnology, Inc.). Protein concentration was measured using the bicinchoninic acid (BCA) protein assay (Beyotime Biotechnology, Inc.). Protein samples (20 μg) were separated on 8% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (MilliporeSigma). Non-specific binding to the membrane was blocked for 1 h at room temperature with 5% fat-free milk in TBST (Tris-buffered saline with 0.1% Tween 20 detergent) and then the membranes were incubated with 1:2,000 FXR primary antibody (cat. no. NB300-259; Novus Biologicals, LLC), 1:1,000 megalin primary antibody (cat. no. ab56014; Abcam) and 1:1,000 cubilin primary antibody (ab251050, Abcam) respectively at 4°C overnight. Then, the membrane was washed four times with TBST and incubated with a 1:5,000 dilution of the HRP conjugated secondary antibody (cat. no. ab205718; Abcam) at room temperature for 45 mins. After the membrane was washed twice with TBST, membrane-bound antibody was visualized using an enhanced chemiluminescent kit (MilliporeSigma) according to manufacturer's instructions. The densities were quantified via photodensitometric scanning using Quantity One-4.2.3 software (Bio-Rad Laboratories, Inc.).

Statistical analysis. Values are given as the mean ± SD. Statistical differences between multiple groups were compared using Kruskal-Wallis analysis. When statistical significance was identified based on the nonparametric test, Dunn's test was used for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of nicotine on the liver weight-to-body weight ratio and liver function. The liver weight-to-body weight ratio

Table I. Sequences of primers in the present study.

Gene name	Primer sequence (5'-3')
FXR	Forward: GTGGTGGCAAATCCTCCATCAG
	Reverse: AGATTCGCTGGCGTCAGTGTCT
CYP7A1	Forward: CACCATTCCTGCAACCTTCTGG
	Reverse: ATGGCATTCCCTCCAGAGCTGA
CYP7B1	Forward: CGGAAATCTTCGATGCTCCAAAG
	Reverse: GCTTGTTCCGAGTCCAAAAGGC
CYP8B1	Forward: CATGAAGGCTGTGCGTGAGGAA
	Reverse: CATCACGCTGTCCAACACTGGA
CYP27A1	Forward: TCAGGAGACCATCGGCACCTTT
	Reverse: CCAGTCACTTCCTTGTGCAAGG
BSEP	Forward: CCTTGGTAGAGAAGAGGCGACA
	Reverse: ATGGCTACCCTTTGCTTCTGCC
NTCP	Forward: CCTGATGCCTTTCACTGGCTTC
	Reverse: GGATGGTAGAACAGAGTTGGACG
OATP1	Forward: GCTGTTCAGTCTTACGAGTGTGC
	Reverse: CAAGGCATACTGGAGGCAAGCT
OSTα	Forward: GCCTGCCATTTTCTCCATCTTGG
	Reverse: CAGCACTGTCATCAGGAAGGTC
OSTβ	Forward: CAAGCATGTTCCTCCTGAGAAGG
	Reverse: CTCTTAGGAAGACCTGGCTGTTG
MRP2	Forward: TACCAGCGAGTTATCGAAGCGTG
	Reverse: TGCTTCTGACCGCCACTGAGAT
MRP3	Forward: ACTTCCTCCGAAACTACGCACC
	Reverse: GCTGGCTCATTGTCTGTCAGGT
MRP4	Forward: CACTCAGGAAACGAACCTTCTCC
	Reverse: TTGCACTGCCTGCGTGTTCTCT
NPC1L1	Forward: ATCGCACTACCATCCAGGACCTx
	Reverse: CCCAGAGTAGCCTTGGAATCCA
ABCG5	Forward: TGCCATCCTGACTTACGGAGAG
	Reverse: CTGCTTTGGGTGTCCACTGATG
SR-BI	Forward: ACACCCGAATCCTCGCTGGAAT
	Reverse: CCGTTGGCAAACAGAGTATCGG
Megalin	Forward: CCAATGGACTCACTCTGGACCT
	Reverse: GAATGGAAGGCAGTGCTGATGAC
Cubilin	Forward: TCCGCTTCACATCAGATGGCAG
	Reverse: GGAGCAGTTGAGATTGGGAAGG
β-actin	Forward: CTGTGCCCATCTACGAGGGCTAT
	Reverse: TTTGATGTCACGCACGATTTCC

FXR, farnesoid X receptor; CYP7A1, cholesterol 7α-hydroxylase; CYP7B1, oxysterol 7α-hydroxylase; CYP7B1, oxysterol 7α-hydroxylase; CYP8B1, sterol 12-α-hydroxylase; CYP27A1, cytochrome P450 family 27 subfamily A member 1; BSEP, bile salt export pump; NTCP, Na⁺-taurocholate cotransporting polypeptide; OATP1, organic anion transporting polypeptide 1; OST, organic solute transporter; MRP, multidrug resistance-associated protein; NPC1L1, NPC1 like intracellular cholesterol transporter 1; ABC, ATP-binding cassette; SR-BI, scavenger receptor class B member 1.

was not significantly different among the different treatment groups. H&E staining of the liver in Fig. 1 did not reveal obvious histomorphological differences among different
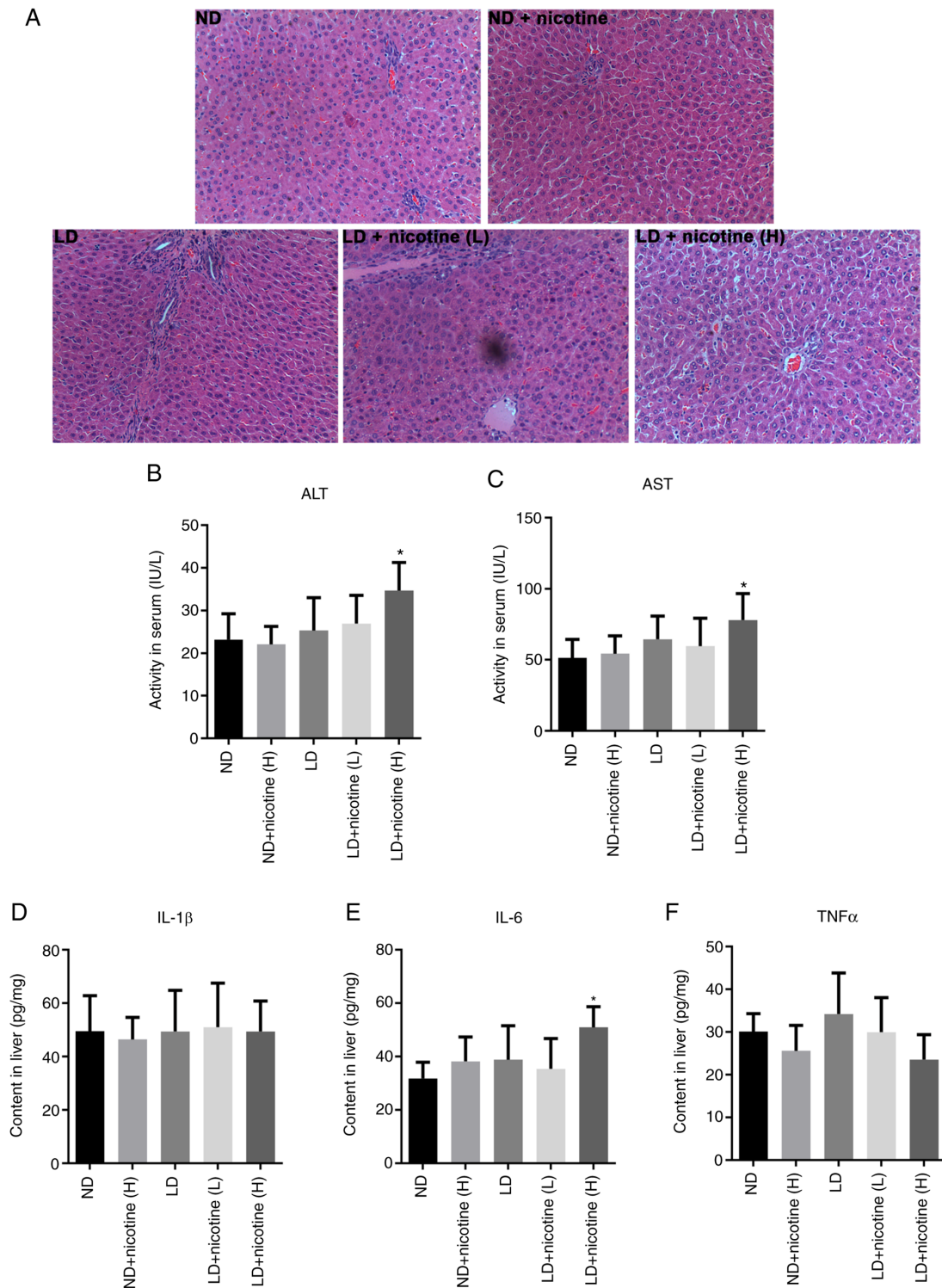
Figure 1. Nicotine does not affect the liver function in mice fed with ND or LD. (A) No difference observed in pathological morphology of liver tissue by hematoxylin and eosin staining (magnification, x100). (B) ALT and (C) AST serum levels were normal among the five groups. (D) TNFα, (E) IL-1β and (F) IL-6 levels did not change after nicotine injection in mice fed with normal or lithogenic diet. Data are expressed as the mean ± standard deviation of the mean with three independent experiments. *P<0.05 vs. the ND group. ND, normal diet; LD, lithogenic diet; ALT, alanine aminotransferase; AST, aspartate aminotransferase; H, 6.6 mg/kg/2 days nicotine; L, 1.1 mg/kg/2 days nicotine.

groups, except the high-dose nicotine treatment which caused liver injury (Fig. 1A).

Serum ALT and AST levels did not change after low-dose nicotine treatment, but increased in the high-dose nicotine treatment group (Fig. 1B and C). In addition, no significant differences were observed in the levels of inflammatory factors following nicotine treatment (Fig. 1D-F). This suggested that low-dose nicotine treatment showed no significant toxic side effects on liver function after 10 weeks.

Effects of nicotine on gallstone formation. Mice fed with a normal diet treated with high-dose nicotine showed no abnormal behavior

Table II. Gallstone formation rate, gallbladder size and volume in different groups.

Groups	Formation rate (%)	Gallbladder size		
		Length (mm)	Width (mm)	Volume (μl)
ND	0	6.3±0.6	6.0±0.50	30.02±4.93
ND + nicotine (H)	0	6.2±0.4	5.9±0.7	28.95±4.09
LD	100	7.8±1.1	3.4±0.4	67.00±12.84
LD + nicotine (L)	90	7.7±0.9	5.5±0.9	61.50±9.52
LD + nicotine (H)	90	7.6±1.0	6.8±1.8	53.82±7.25

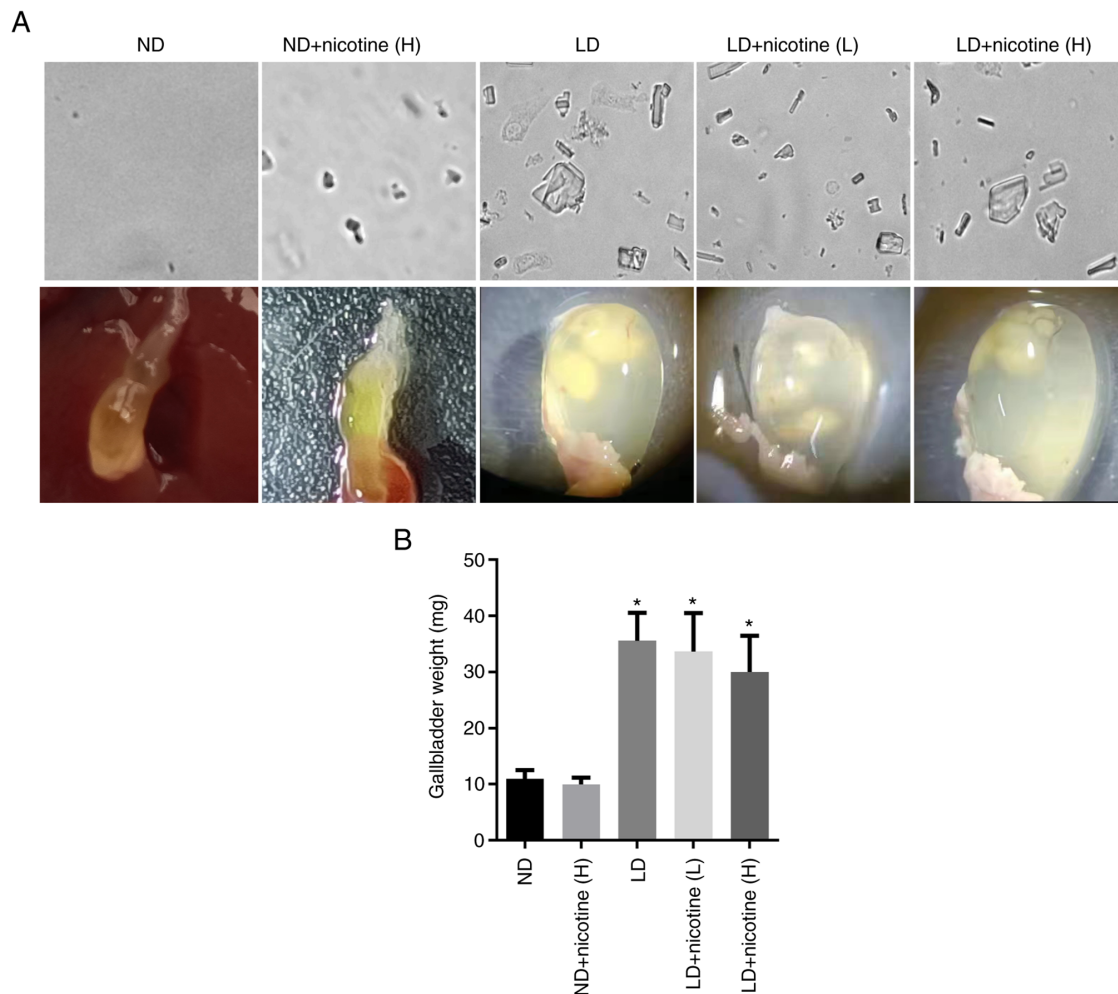ND, normal diet; LD, lithogenic diet; H, 6.6 mg/kg/2 days nicotine; L, 1.1 mg/kg/2 days nicotine.

Figure 2. Nicotine does not reduce gallstone formation induced by LD. C57BL/6J mice were fed LD for 10 weeks and were administered intraperitoneal injections of nicotine. (A) Image of gallstone formation (magnification, x10) and (B) weight of gallbladder were measured (n=10). Data are expressed as the mean ± standard deviation of the mean with three independent experiments. *P<0.05 vs. the ND group. ND, normal diet; LD, lithogenic diet; H, 6.6 mg/kg/2 days nicotine; L, 1.1 mg/kg/2 days nicotine.

and no gallbladder stone formation. Crystals were observed in the lithogenic diet groups. Gross analysis of the gallbladder showed that LD + nicotine groups did not exhibit significant lower stone formation compared to the LD group (Fig. 2A). The gallbladder volume of mice in the LD + nicotine (H) group was lower than that in the LD group, while low concentrations of nicotine did not change gallbladder volume (Fig. 2B; Table II).

The effect of nicotine on lipid levels in bile and serum. Compared with the LD group, the cholesterol level in the gallbladder bile from the LD + nicotine (H) group was significantly reduced, (Fig. 3A). The cholesterol level did not significantly decrease in LD + nicotine (L) group compared with the LD group. Compared with the LD group, there were no significant differences in the
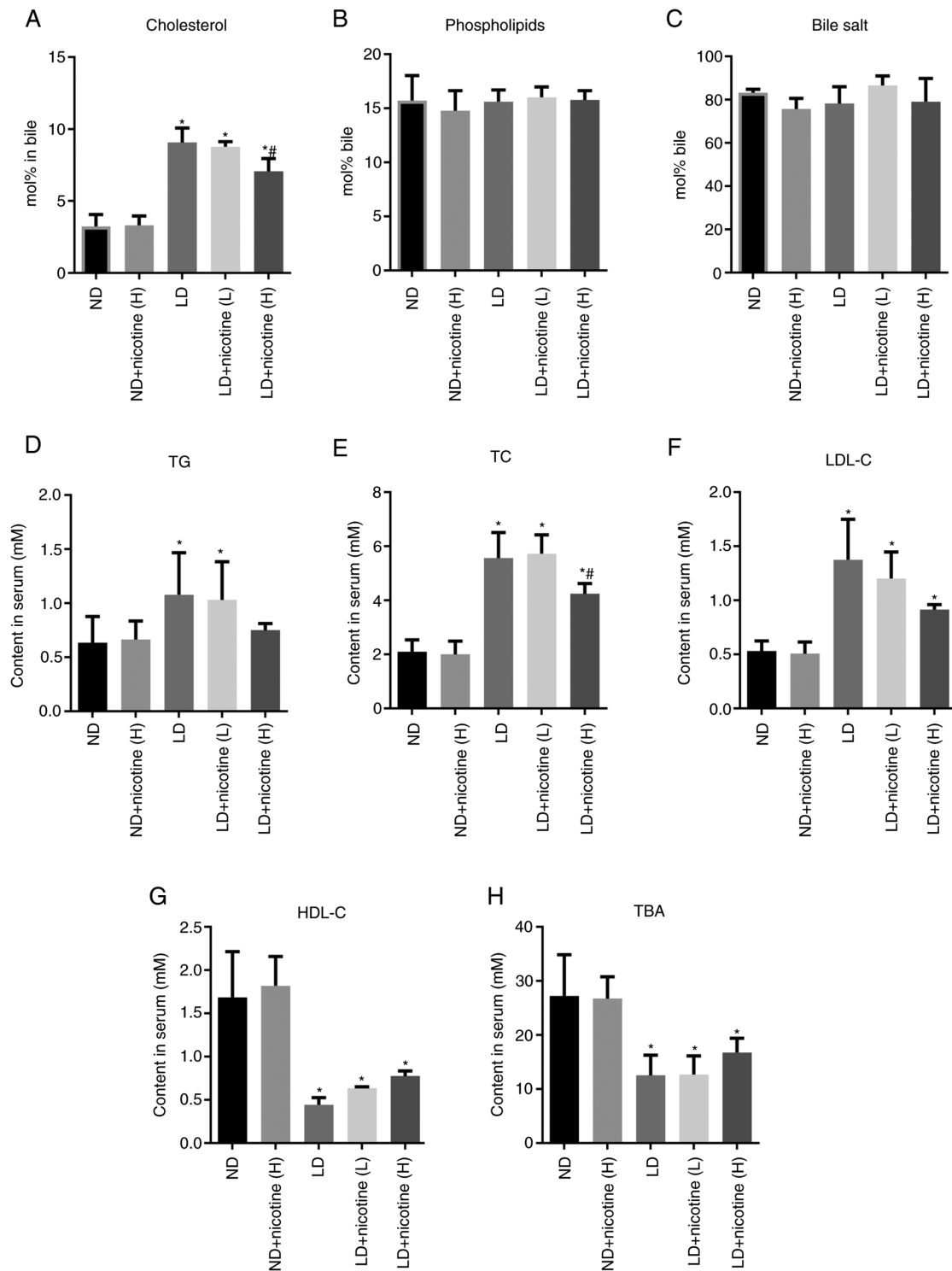
Figure 3. Effects of nicotine on the levels of cholesterol and lipids in the serum and bile. The bile level of (A) cholesterol, not (B) phospholipid or (C) bile salts was significantly elevated in LD groups and reduced in the nicotine treatment group. Compared with the ND group, the serum levels of (D) TG, (E) TC and (F) LDL-C increased, while (G) HDL-C and (H) TBA decreased. Notably, nicotine could attenuate LD-increased TC levels in LD + nicotine (H) group. Data are presented as the mean ± standard deviation (n=10 mice per group). *P<0.05 vs. the ND group; $^\#$P<0.05 vs. the LD group. LD, lithogenic diet; ND, normal diet; H, 6.6 mg/kg/2 days nicotine; L, 1.1 mg/kg/2 days nicotine; TG, triglycerides; TC, total cholesterol; LDL-C, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein cholesterol; TBA, total bile acids.

phospholipids and bile salts in the bile of the experimental groups (Fig. 3B and C).

Serum lipid levels between different groups were also compared, including triglycerides (TG), total cholesterol (TC), low-density lipoprotein-cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) and total bile acid (TBA). The results showed that compared with the ND group, the serum levels of TG, TC and LDL-C in the LD group significantly increased, while HDL-C and TBA significantly decreased (Fig. 3D-H). After low-concentration
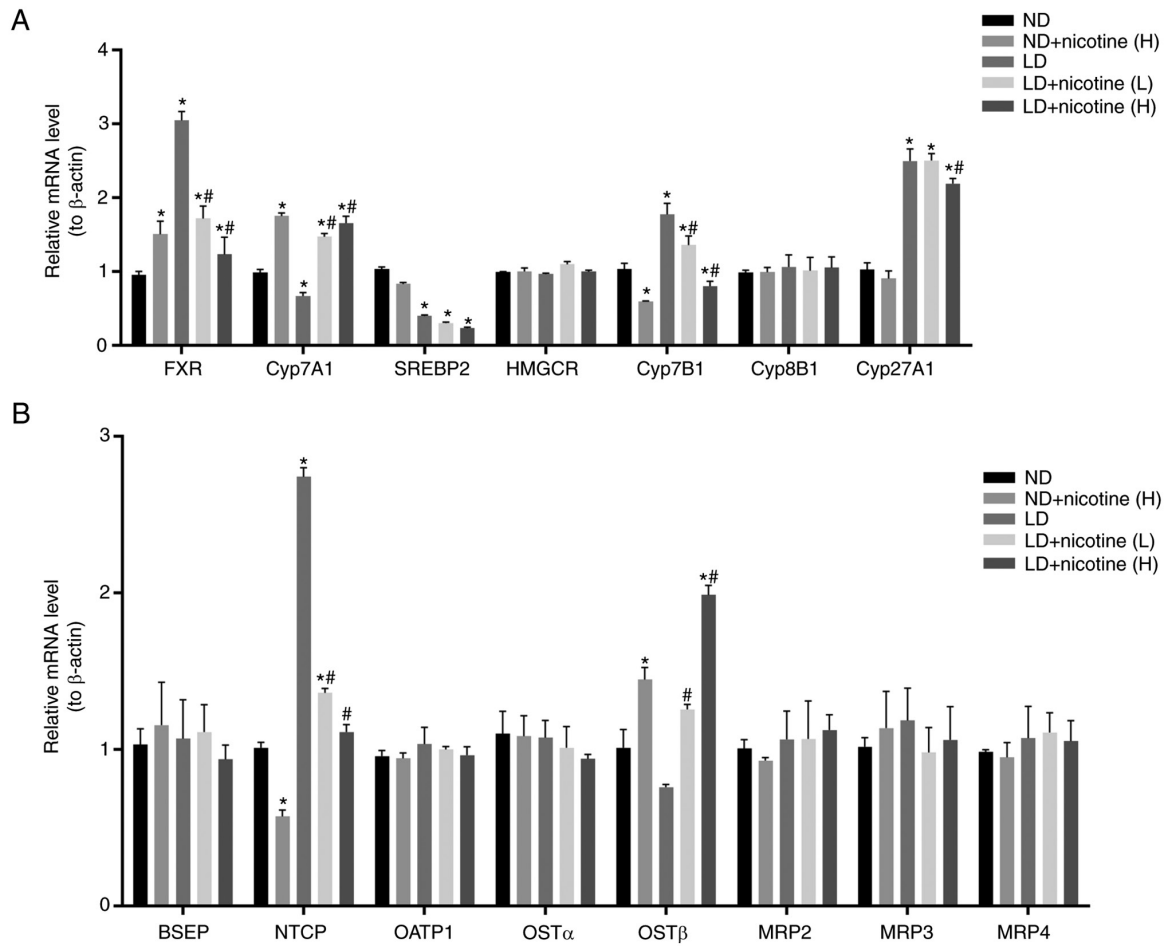
Figure 4. Effect of nicotine on mRNA expression of bile acid metabolism-related gene in liver tissues. mRNA levels of (A) bile acid synthesis-related genes and (B) bile acid transport-related genes in the livers of mice injected with nicotine were measured by quantitative polymerase chain reaction (n=3). Data are expressed as the mean ± standard deviation of the mean. $^{*}P<0.05$ vs. the ND group; $^{\#}P<0.05$ vs. the LD group. ND, normal diet; LD, lithogenic diet. FXR, farnesoid X receptor; CYP7A1, cholesterol 7α-hydroxylase; SREBP2. sterol regulatory element-binding protein 2; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; CYP7B1, oxysterol 7α-hydroxylase; CYP27A1, cytochrome P450 family 27 subfamily A member 1; BSEP, bile salt export pump; NTCP, Na^{+}-taurocholate cotransporting polypeptide; OATP1, organic anion transporting polypeptide 1; OST, organic solute transporter; MRP, multidrug resistance-associated protein.

nicotine treatment, the levels of these indicators are unchanged. Compared with the LD group, the serum TG, TC and LDL-C of the mice treated with the high-dose nicotine group decreased significantly, while the levels of HDL-C and TBA increased (Fig. 3D-H).

The effect of nicotine on mRNA expression of BA-related gene in liver tissues. The present study examined major genes involved in bile acid metabolism in liver tissue from mice with or without nicotine treatment. As shown in Fig. 4, compared to the ND group, FXR, cholesterol 7α-hydroxylase (CYP7A1) and oxysterol 7α-hydroxylase (CYP7B1) and Na^{+}-taurocholate cotransporting polypeptide (NTCP) mRNA levels increased, while CYP7A1 and sterol regulatory element-binding protein 2 (SREBP-2) decreased in the LD group. These effects were reversed by high-dose nicotine treatment (Fig. 4). Notably, no changes in some genes expression were observed following nicotine treatment, including 3-hydroxy-3-methylglutaryl-CoA reductase, sterol 12-α-hydroxylase (CYP8B1), organic solute transporter α (OSTα), bile salt export pump (BSEP), organic anion transporting polypeptide 1 (OATP1), multidrug resistance-associated protein (MRP)2, MRP3,

MRP4. This suggests that nicotine may induce changes in cholesterol in other ways in addition to partially affecting bile acids.

Effect of nicotine on bile acid metabolism in the gallbladder epithelial tissues. The present study investigated the cholesterol metabolism in the gallbladder epithelial cells. It detected the mRNA levels of NPC1L1, ABCG5/G8, SR-BI and megalin/cubilin expression by RT-qPCR. The results showed that compared with the ND group, the expression level of NPC1L1 in the LD group significantly decreased, while megalin, ABCG5/G8 and SR-BI increased. The administration of low and high concentrations of nicotine could attenuate their expression levels (Fig. 5A).

Generally, the FXR-megalin/cubilin signaling pathway regulates cholesterol balance and participates in the formation of stones (17). Therefore, the present study also detected the levels of FXR-megalin/cubilin expression by western blotting and immunohistochemistry. Similar to mRNA expression, nicotine did not alter cubilin protein levels, but restored LD-downregulated FXR and megalin levels (Fig. 5B and C). These results suggest that nicotine can affect the formation
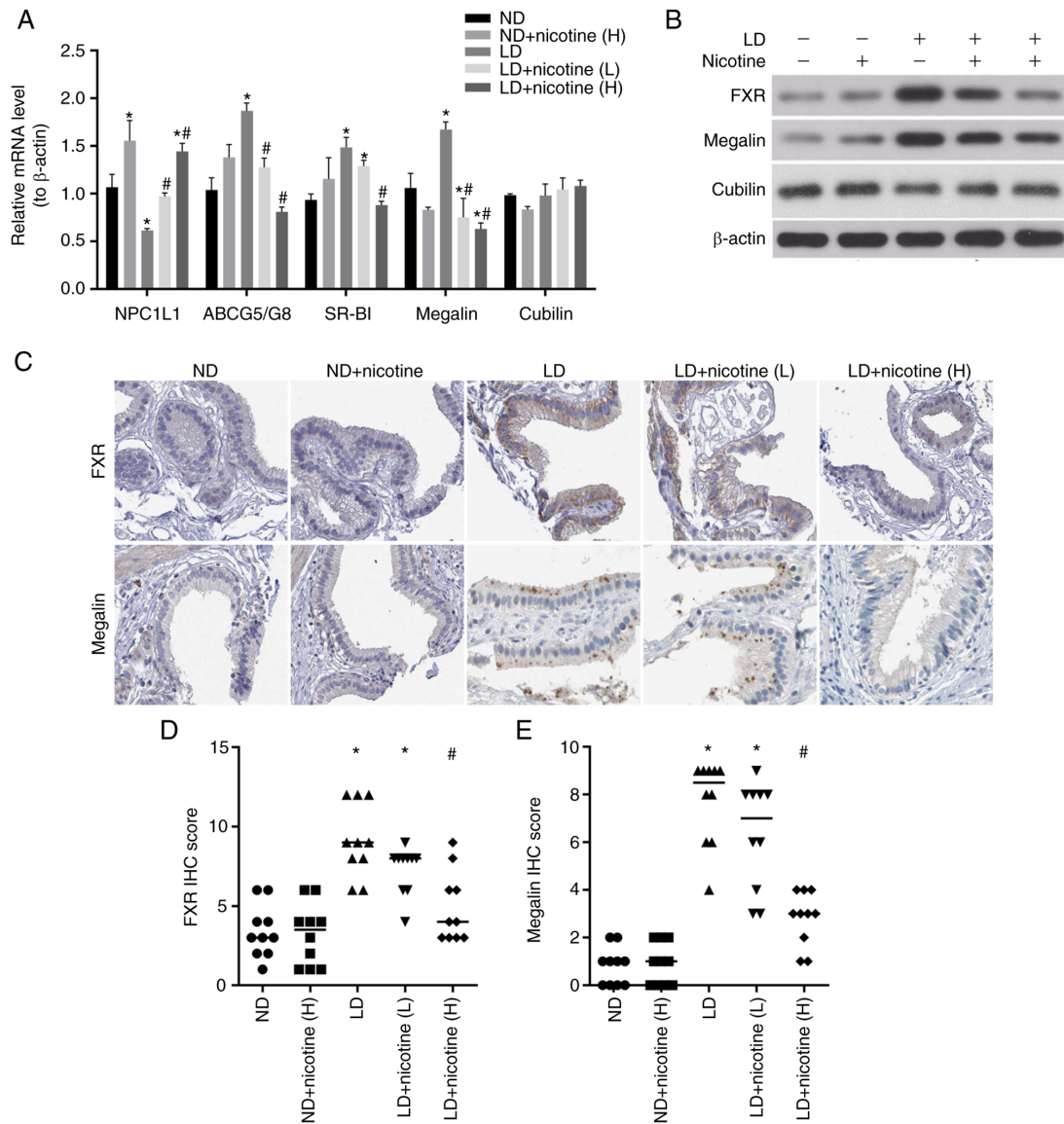
Figure 5. Effect of nicotine on cholesterol metabolism by regulating in the gallbladder epithelial tissues. (A) mRNA levels of bile acid metabolism in the gallbladder epithelial tissues of mice injected with nicotine was measured by quantitative polymerase chain reaction (n=3). (B) Representative western blot analyses and (C-E) immunohistochemical staining of FXR and megalin (magnification, x100). Data are expressed as the mean ± standard deviation of the mean (n=10 mice per group). *P<0.05 vs. the ND group; #P<0.05 vs. the LD group. ND, normal diet; LD, lithogenic diet; H, 6.6 mg/kg/2 days nicotine; L, 1.1 mg/kg/2 days nicotine; NPC1L1, NPC1 like intracellular cholesterol transporter 1; ABC, ATP-binding cassette; SR-BI, scavenger receptor class B member 1; FXR, farnesoid X receptor.

process of gallstones by regulating cholesterol metabolism in the gallbladder.

Effect of nicotine on the regulation of FXR/megalin/cubilin protein expression in the gallbladder epithelial cells. As shown in Fig. 6A, the FXR agonists CDCA increased the expression of megalin in GBEC. CDCA clearly increased the expression of FXR and megalin in GBEC, while not changing the expression of cubilin. The nicotine inhibited CDCA-induced expression of FXR and megalin in GBEC. Immunofluorescence analyses of megalin expression in GBEC showed that megalin is expressed in perinuclear and vesicular, decreased by nicotine (Fig. 6B). It suggested that nicotine could regulate FXR and megalin, which might be involved in the regulation of the expression of the genes related to bile acid metabolism.

Discussion

Nicotine has a little beneficial effect on human diseases, including colitis and oral ulcers (26-28). The present study investigated the effects of nicotine on cholesterol metabolism and gallstone prevention in gallstone-susceptible C57L mice. It found that nicotine did not prevent cholesterol gallstone formation, but decreased biliary cholesterol secretion, retarding phase transition of cholesterol and that this is likely due to nicotine changing the expression of FXR/megalin pathway.

The present study first investigated the effects of nicotine on liver function. H&E staining and liver function assay showed that nicotine did not induce liver damage. Currently, research on the effects of nicotine on the liver is inconsistent. A few studies suggest that nicotine has toxicity in the liver (29-31). A longer study period would have allowed for
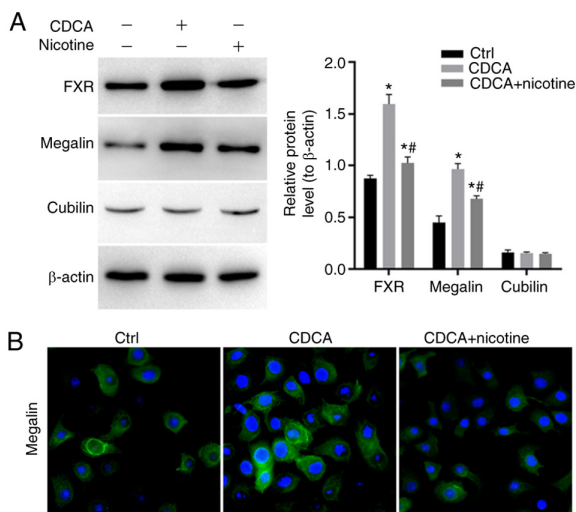
Figure 6. Effect of nicotine on the regulation of FXR/megalin/cubilin in the gallbladder epithelial cells. (A) GBEC cells were treated with 2 μM nicotine for 48 h, then total cell lysates (40 μg) from GBECs were subjected to western blot analysis. (B) Immunofluorescence for megalin in confluent GBECs treated with nicotine for 48 h in the presence of FXR agonist CDCA (10 μM; magnification, x100). *P<0.05 vs. control; #P<0.05 vs. the CDCA. FXR, farnesoid X receptor; CDCA, chenodeoxycholic acid.

potentially increased differences in liver damage levels. By contrast, several studies suggest that chronic nicotine treatment has no significant effect on the liver (32) and even alleviates poison-induced liver injury (33-35). The inconsistency of these findings may be due to differences in the concentration, duration of nicotine treatment and the types of substances that induce liver injury.

The present study next examined the effect of nicotine on gallstone formation and gallbladder bile composition. Neither inhibitory nor increased effect of nicotine on the rate of gallstone formation was observed in LD diet mice. Almost all studies suggest that smoking increases the progression of gallbladder disease, including gallstones (36,37). However, earlier research showed that nicotine can protect against the formation of gallstones (9,38). There may be two reasons for this: i) As well as nicotine, tobacco contains a variety of other substances that promote gallstone formation. ii) Smoking prolongs the maximal gallbladder emptying time and delays gallbladder contraction, then results in bile stasis in gallbladder, which causes most gallbladder disorders (39).

The effect of nicotine on bile cholesterol metabolism was investigated. There were significant changes in the composition of bile after 10 weeks of nicotine treatment with a reduction in total bile cholesterol concentrations. The data showed high concentrations of nicotine increased the TBA levels. Since gallbladder volume and bile secretion were similar before and after nicotine treatment, the increase in TBA may be caused by changes in the ratio of different types of BAs. The rise in total BA concentration in bile tends to reduce gallstone formation by increasing cholesterol solubility (40,41).

FXR can regulate the synthesis of BAs in a tissue-specific manner, regulating bile acid reabsorption, maintaining bile acid cycle homeostasis and reducing cholesterol and fat production (42). In the present study, except BA synthase FXR, CYP7A1 and CYP7B1 and efflux transporters NTCP and organic solute transporter β (OSTβ), nicotine did not change the expression of the efflux transporter BSEP, OATP1, OSTα, MRP2/3/4 and alternative pathway synthase cytochrome P450 Family 27 Subfamily A Member 1 (CYP27A1) and CYP8B1. In addition to bile acid metabolism mediated by the entero-hepatic circulation, a study suggested that attention should be paid to cholesterol metabolism in gallbladder epithelial cells (43), as the gallbladder has the capacity to actively absorb cholesterol from bile and thereby modulate bile cholesterol content. Thus far, the expression of cubilin and megalin, ATP binding cassette subfamily G (ACBG)5/8, scavenger receptor class B type I (SR-BI), has been reported in the apical side of gallbladder epithelial cells (44). However, further elucidation of the mechanism is required.

The qPCR data showed that nicotine could attenuate LD-downregulation of NPC1L1 and reduce the expression of megalin, ABCG5/G8 and SR-BI. In addition, megalin and cubilin, which are expressed on the gallbladder but not on hepatocytes, are also regulated by bile acids and their receptor FXR (16). This indicates that FXR/megalin/cubilin may serve a central role in the pathophysiology of gallstones. In the study of Tsaroucha et al (15), the megalin and cubilin mRNA levels are low in gallstone tissues, but the study of Erranz et al (16) showed megalin protein levels were upregulated by a lithogenic diet. This is mainly because CA, not cholesterol, increases the expression of megalin, but does not affect the expression of cubilin (16). The present study used a similar lithogenic diet, which contained the natural FXR agonists CA. The data demonstrated that FXR/megalin protein expression increased in the LD group during gallstone formation, low or high-dose nicotine could decrease megalin mRNA and protein expression, but cubilin expression did not change. These data indicated that nicotine may act on cholesterol metabolism in gallbladder cells and may be involved in the process of gallstones formation. However, further assessment of the direct effect of megalin and cubilin regulated by nicotine on gallstone formation is required.

There are several limitations to the present study. First, it did not conduct an in-depth analysis for microscopic examination of the gallbladder and list all abnormal findings such as cells, crystals or other components. Second, it did not analyze the difference in stones composition and load between the three groups. Third, although a number of useful aspects of the lithogenic process can be studied using animal models, they are not 100% identical to humans. Moreover, a number of changes apparently associated with gallstone formation may be a function of heredity.

The present study demonstrated that nicotine did not prevent LD-induced gallstone formation. However, nicotine could regulate FXR, sterol regulatory element-binding protein 2, CYP7A1, NTCP and OSTβ in the liver and ABCG5/8, NPC1L1, SR-BI and megalin in the gallbladder. These genes are associated synthesis, transformation and transportation of bile acid. Thus, despite unlikely therapeutic applications, nicotine might have potential beneficial effects for anti-lithogenic activity.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

QG, XL and BY conceived and designed the study. QG, PB and QM contributed to the experiments, acquisition of data and comments and editorial review of the manuscript. YG and JJ analyzed data and revised the manuscript critically for important intellectual content. QG, PB, XL and BY contributed to interpretation of data and drafted the article. All authors read and approved the final version of the manuscript. BY and QG confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The animal experiments were approved by the Institutional Animal Care and Use Committee of Kunming Medical University (approval no. kmmu2021058).

Patient consent for publication

Not applicable.

Competing interests

Note that QG, QM, YG, JJ and XL are employees of the Yunnan Key Laboratory of Tobacco Chemistry, R&D Center of China Tobacco Yunnan Industrial Co. Ltd. who also funded the present study, which explored the effect of nicotine on cholesterol gallstone formation in C57BL/6J mice fed on a lithogenic diet.

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