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Original article:

URSODEOXYCHOLIC ACID LOWERS BILE LITHOGENICITY BY REGULATING SCP2 EXPRESSION IN RABBIT CHOLESTEROL GALLSTONE MODELS

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ABSTRACT

Aims: We designed this study to get insight into the disorder of lipid metabolism during cholesterol gallstone formation and evaluate the effect of ursodeoxycholic acid on the improvement of bile lithogenicity and on expression of lipid related genes.
Methods: Rabbit cholesterol gallstone models were induced by high cholesterol diet. Bile, blood and liver tissues were obtained from rabbits after 0, 1, 2, 3, 4 and 5 weeks. Bile and blood lipids were measured enzymatically. 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), cytochrome P450, family 7, subfamily A, polypeptide 1 (CYP7A1) and sterol carrier protein 2 (SCP2) mRNA expressions were detected by using quantitative real-time RT-PCR. Cholesterol saturation index (CSI) was calculated by using Carey table to represent the bile lithogenicity.
Results: Rates of gallstone formation of the 4 and 5 week treatment groups were 100 %, but that of the ursodeoxycholic acid treatment group was only 33.3 %. Expression of HMGCR and SCP2 mRNA in the 4 week group was upregulated and that of CYP7A1 mRNA decreased as compared with the 0 week group. Ursodeoxycholic acid could significantly extend nucleation time of bile and lower CSI. Ursodeoxycholic acid could reduce the expression of SCP2, but couldn't influence expression of HMGCR and CYP7A1.
Conclusions: Abnormal expression of HMGCR, CYP7A1 and SCP2 might lead to high lithogenicity of bile. Ursodeoxycholic acid could improve bile lipids and lower bile lithogenicity, thereby reducing the incidence of gallstones. So it might be a good preventive drug for cholesterol gallstones.

Keywords: cholesterol gallstones, HMGCR, CYP7A1, SCP2 mRNA, bile lipids, CSI, rabbit models

INTRODUCTION

Gallstone disease is one of the most common gastrointestinal diseases. Worldwide prevalence rates scatter between 5 % and 20 %, but may be as high as 70 % in female American Indians. Gallstone disease is a multifactorial disease based on a complex interaction of environmental and genetic factors (Lammert and Sauerbruch, 2005; Portincasa et al., 2006; Marschall and Einarsson, 2007). More than 90 % of gallstones consist mainly of cholesterol and are

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formed within the gallbladder. Cholesterol hypersaturation of bile is a prerequisite for the formation of such stones. Hypersecretion of cholesterol in bile leading to the formation of lithogenic bile is believed to be the major cause of cholesterol gallstones (Venneman and van Erpecum, 2010). Furthermore, these changes are closely related to the disorders of lipid metabolism in liver. However, during the formation of cholesterol gallstones, different links in the disturbance of cholesterol metabolism and their effects in lithogenesis still have many controversies.

Bile formation is essential for the removal of excess dietary cholesterol. Cytochrome P450, family 7, subfamily A, polypeptide 1 (CYP7A1) catalyzes the first rate-limiting reaction of cholesterol catabolic pathway converting cholesterol to bile salts which are essential for keeping cholesterol molecules solubilized in bile. Some investigators observed tendencies for reduced CYP7A1 activity in gallstone disease patients (Reihnér et al., 1991; Ito et al., 1996). Xie et al. (2009) found that the AU-rich RNA binding-protein Apobec-1 mediated post-transcriptional regulation of murine CYP7A1 expression and increased susceptibility to diet-induced gallstone formation. Other research indicated that there was no difference of cholesterol 7α-hydroxylase mRNA expression in gallstone susceptible mice, but in the gallstone resistant mice the expression level of 7α-hydroxylase mRNA increased (Tazuma et al., 1998). Khanuja et al. (1995) found the regulation of the rate-limiting enzyme in cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR), may be pivotal in determining the occurrence and severity of cholesterol hypersecretion and hence lithogenicity of gallbladder bile. Lammert et al. (1999) found that HMGCR in cholesterol synthesis was not down-regulated in C57L mice fed a lithogenic diet which contains large amounts of cholesterol. Caroli-Bosc et al. (2001) found that increased activity of HMGCoA reductase corrsponds with a rise in hepatic cholesterol synthesis, so that the Cholesterol Saturation Index of bile would also go up. Some researchers found the overexpression of sterol carrier protein 2 (SCP2) might accelerate the transportation of cellular cholesterol, increase the cholesterol in the bile and promote the formation of gallstones (Ito et al., 1996; Fuchs et al., 1998). Our former finding indicated that SCP2 might be one of the genetic factors contributing to cholesterol gallstone formation (Cui et al., 2011).

This study is aimed to investigate the lipid changes in blood and bile in order to find out the relationship between the disorder of lipid metabolism and the formation of cholesterol gallstones. We also evaluated the effect of ursodeoxycholic acid on the improvement of lithogenicity of rabbit model bile and expression of lipid related genes.

MATERIALS AND METHODS

Animals, diets and treatment

This study was approved by the Animal Care and Use Committee in Tianjin Nankai Hospital. Male and female white Japanese rabbits were obtained from the Experimental Animal Center of Tianjin Medical University. They were divided randomly into 0 week group (0W) as a control group and as 1 to 5 week groups (1W, 2W, 3W, 4W, 5W) as five experimental groups, and ursodeoxycholic acid treatment group (U) each constisting of seven rabbits. Rabbits in control group were given a normal diet, but those in experimental groups were fed with 1.2 % cholesterol diet for one to five weeks (1W to 5W). Rabbits in the ursodeoxycholic acid treatment group (U) were fed with 1.2 % cholesterol diet for four weeks and were simultaneously orally treated with ursodeoxycholic acid (75 mg/kg) by gavage (Liu and Jun, 1997). All animals were maintained in separate cages with free access to water.

Blood, bile and liver sampling

At the end of each experiment, following an overnight fasting, animals were anesthetized. After the collection of samples,

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rabbits were euthanatized. Blood samples were collected for the measurement of blood lipids and portions of liver were obtained for the histological analysis and the analysis of gene expression. Bile specimen was aspirated from gallbladder and kept for subsequent analysis. The gallbladder was cut open under the microscope and the gallstones and cholesterol crystals were evaluated.

Identification of gallstones and cholesterol crystals

Criteria for gallstones:

If gallstone particles were found by inspection with the naked eye or large dense clumps of cholesterol crystals were seen under the microscope, samples were considered positive for gallstone formation.

Quantitative criteria for cholesterol crystals:

A portion of fresh gallbladder bile was centrifuged, the sediment smears were observed microscopically, and cholesterol crystals were quantified according to Juniper standard (Block and Priest, 1967).

Nucleation time of gallbladder bile

Biles were centrifuged 100,000 x g at 37 °C for 2 hours in order to remove the cholesterol crystals. Biles were taken and put in the incubator at 37 °C and were observed every day under the polarizing microscope. The time when cholesterol monohydrate crystals appeared was named nucleation time and the observation time was no more than 21 days (Holan et al., 1979).

Analysis of blood lipids

Plasma triglycerides (TG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) were measured on Hitachi 704 Analyzer (Roche Diagnostics, Indianapolis) with enzymatic methods using the manufacturer's reagents and instructions.

Analysis of bile lipids

The concentrations of biliary cholesterol, phospholipids, and total bile acids were measured using enzymatic techniques (Roda et al., 1975; Qureshi et al., 1980; Talalay, 1960). Cholesterol Saturation Index (CSI) was calculated by using the Carey table (Carey, 1978).

Quantitative real-time RT-PCR

Total RNA was prepared from liver tissue by using the Trizol method. Briefly, 1 µg RNA was reverse-transcribed by using random primer hexamers. Amplification was carried out with an ABI 7300 real-time PCR system (Applied Biosystems) using SYBR green I. The reference gene was glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primer sequences for rabbit HMGCR (forward 5'-ATGGAATTCCATGGCTGGGAGCATAGGAG-3' and reverse 5'-TCCTTGAACACCTAGCATCTGC-3'), CYP7A1 (forward 5'-ATGGAATTCCATCTCAAGCAAACACCATTCC-3' and reverse 5'-TGCATTAACTGTGGATAAAGAGC-3'), and SCP2 (forward 5'-GCCAACGAACTCCTCACTTAT-3' and reverse 5'-TTGCCATTCTTCACATCTACCA-3') were designed to span an intronic sequence and were validated by PCR and gel analysis.

Microscopy/Histology

Liver and gallbladder tissues were fixed with formalin and embedded with paraffin. The sections were stained with hematoxylin and eosin (HE), and observed under a light microscope. Three HE sections from each group were scored (Zeymer et al., 1992). Slides were examined at high magnification. Liver cells with steatosis and total cells were counted.

Statistical analysis

All data were expressed as mean ± standard deviation. Differences between control and experimental groups were evaluated by Chi-square test and one-way analysis of variance (ANOVA). $P<0.05$ was considered statistically significant ($\alpha=0.05$).

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RESULTS

Analysis of rabbit gallstones

Gallstones in gallbladder were not found in any animal of the 0W group fed with routine diet (Figure 1B). However, cholesterol gallstones were found in the experimental groups fed with dietary cholesterol. All gallstones appeared as small brown black stones with the naked eye and in some cases microliths could be observed under the 20 x light microscope (Figure 1C). The rate of gallstones gradually increased with diet time and those of 4W and 5W groups were 100 %, but the rate of ursodeoxycholic acid treatment group was 33.3 % (Figure 1A). The rates of gallstone formation between groups can be considered as statistical significance, $\chi^2 = 27.453$, $\upsilon = 5$, $P < 0.01$ (bilateral) (Table 1).

Under the 100X light microscope (Figure 1D), the examination of rabbit gallbladder bile showed that the cholesterol monohydrate crystals increased gradually with cholesterol diet time. The system of Juniper and Burson for counting microlithiasis was employed to evaluate the formation of gallstones (Table 2). The differences between 6 groups was significant, $\chi 2 = 33.728$, $\upsilon = 5$, $P < 0.01$ (bilateral).

Histological analysis of liver and rabbit gallbladder after the treatment of ursodeoxycholic acid

The percent of liver cells with steatosis was calculated by positive cells/total cells x100 and results are given in mean ± standard error. The section of liver in 4W group showed a lot of fat were accumulated in the liver parenchyma, there were more liver cells with steatosis in the 4W group (88.1 % ± 2.3 %) (Figure 1I) than in the 0W group (2.2 % ± 0.3 %) (Figure 1H) ($P < 0.05$). Compared with the 4W group, the steatosis was alleviated in the U group (7.2 % ± 1.4 %) (Figure 1J) ($P<0.05$). The section of gallbladder in the 4W group (Figure 1F) showed some proliferation of epithelium and some infiltration of inflammatory cell, but those in the 0W group (Figure 1E) and U group (Figure 1G) showed only few of them.

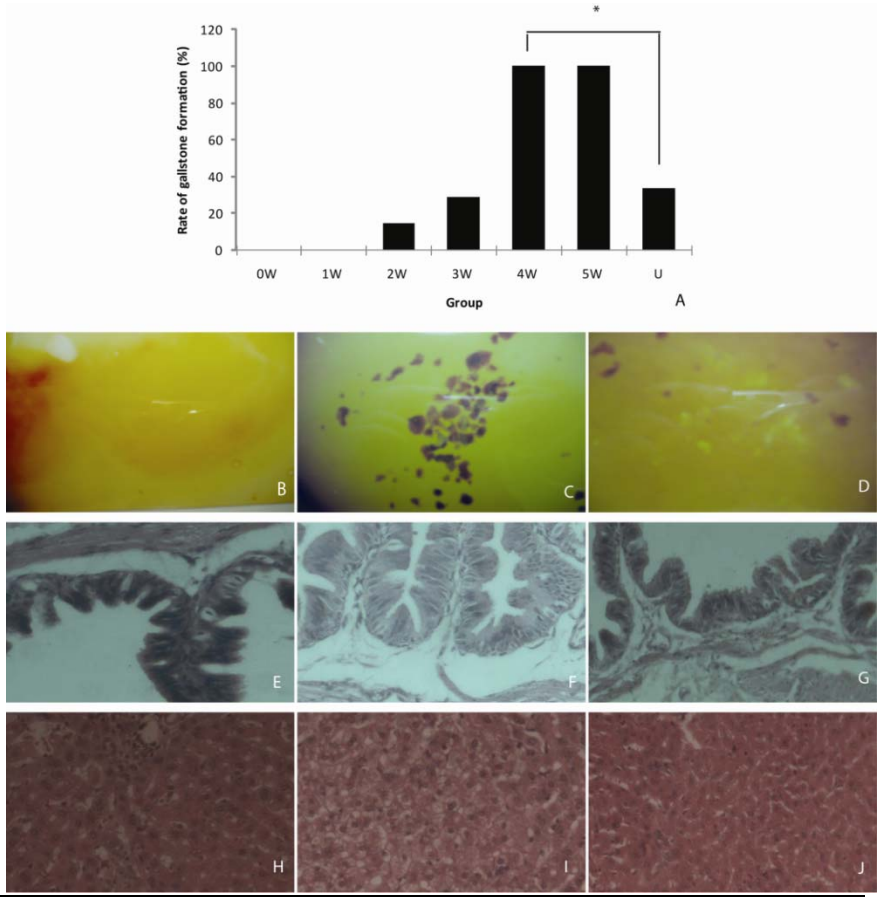

Figure 1: Gallstone formation and histological examination of gallbladder and liver. A. Quantification of gallstone formation in rabbit models by means of high cholesterol diet. **B-D**. Detection of stone particles in bile fluid: (**B**) normal bile of 0W group, (**C**) stone particles of 4W group, and (**D**) cholesterol crystals of 4W group. **E-G**. Histological analysis of gallbladder thin sections stained with HE: (**E**) 0W group, (**F**) 4W group showing some proliferations of epithelium and some infiltrations of inflammatory cell, and (**G**) U group. **H-J.** Histological examination of liver thin sections stained with HE: (**H**) 0W group, (**I**) 4W group, and (**J**) U group showing less liver cells with steatosis than 4W group. 0-5 W, 0-5 week; U, ursodeoxycholic acid treatment.

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Table 1: Analysis of gallstone formation in rabbits

Group	Number of rabbit	Number of gallstone rabbit	Rate of gallstone formation (%)*
0W Group	7	0	0
1W Group	7	0	0
2W Group	7	1	14.3
3W Group	6	2	28.6
4W Group	7	7	100
5W Group	6	6	100
U Group	6	2	33.3**

*The differences between 7 groups was significant, χ^2=27.453, υ =6, P <0.01 (bilateral). 0-5 W, 0-5 week; U, ursodeoxycholic acid treatment
** Compared with 4W group, P<0.05

Table 2: Analysis of cholesterol crystal formation in rabbits

Group	0W Group	1W Group	2W Group	3W Group	4W Group	5W Group	U Group
-	6	1	0	0	0	0	0
+	1	6	6	0	0	0	6
++	0	0	1	2	1	1	2
+++	0	0	0	3	2	1	0
++++	0	0	0	1	4	4	0

The differences between 7 groups was significant, χ^2=33.728, υ =6, P<0.01 (bilateral). 0-5 W, 0-5 week; U, ursodeoxycholic acid treatment

Changes of plasma TG, TC, HDL-c and LDL-c

As shown in Figure 2D, the concentrations of plasma TC and LDL-c gradually increased with the prolonged feeding time of dietary cholesterol (P<0.05). The plasma levels of TC (16.07 ± 1.04 mmol/L) and LDL-c (5.18 ± 0.51 mmol/L) in the 4W group were higher than those (4.17 ± 0.85, 2.46 ± 0.89 mmol/L) in the 0W group (P<0.05). HDL-c showed a decreasing tendency without statistical significance (P>0.05) whereas TG showed an increasing tendency also without statistical significance (P>0.05). There were no differences about plasma TC, TG, HDL-c and LDL-c between the 4W and the U group (P>0.05).

Analysis of bile cholesterol, bile acids and pospholipids

As shown in Figure 2A, the bile cholesterol of the 4W and 5W groups significantly increased by about 2 times as compared with the control group. The bile cholesterol of the 4W group (2.17 ± 0.37 mmol/L) was higher than that of the 0W group (1.20 ± 0.65 mmol/L) (P<0.05). Total bile acids showed a slight increase during the dietary cholesterol feeding in the 2W and 3W groups, but then decreased in 4W and 5W groups, and total bile acids of 4W and 5W groups significantly decreased as compared with the control group (P<0.05). Total bile acid of the 4W group (6.36 ± 0.17 mmol/L) was lower than that of the 0W group (7.29 ± 0.26 mmol/L) (P<0.05). Bile phospholipids showed a slight increase during the dietary cholesterol feeding in 2W and 3W groups, and then decreased in the 4W and 5W groups, but there was no significant difference for bile phospholipids between 4W group and the control group (P>0.05). Ursodeoxycholic acid could reduce the bile cholesterol from 2.17 ± 0.37 mmol/L (4W group) to 1.66 ± 0.067 mmol/L (U group) (P<0.05) and significantly increased the bile acids from 6.36 ± 0.17 mmol/L (4W group) to 11.23 ± 0.25 mmol/L (U group) (P<0.05). However, it couldn't influence the bile phospholipid level.

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Analysis of bile nucleation time and CSI

Bile nucleation times of all animals were gradually reduced from 0 to 5 weeks (Figure 2B). The bile nucleation time of the 4W group (8 ± 4 days) was significantly shorter than that of the 0W group (21 ± 6 days) ($P<0.05$). Compared with 4W group (8 ± 4 days), the bile nucleation time of U group (15 ± 5 days) was extended ($P<0.05$). CSIs increased with the prolonged feeding time of dietary cholesterol (Figure 2C). CSI of the 4W group (1.58 ± 0.09) was significantly higher than that of the 0W group (0.34 ± 0.08) ($P<0.05$). CSI in the U group (0.45 ± 0.026) significantly decreased compared with the 4W group (1.58 ± 0.085) ($P<0.05$).

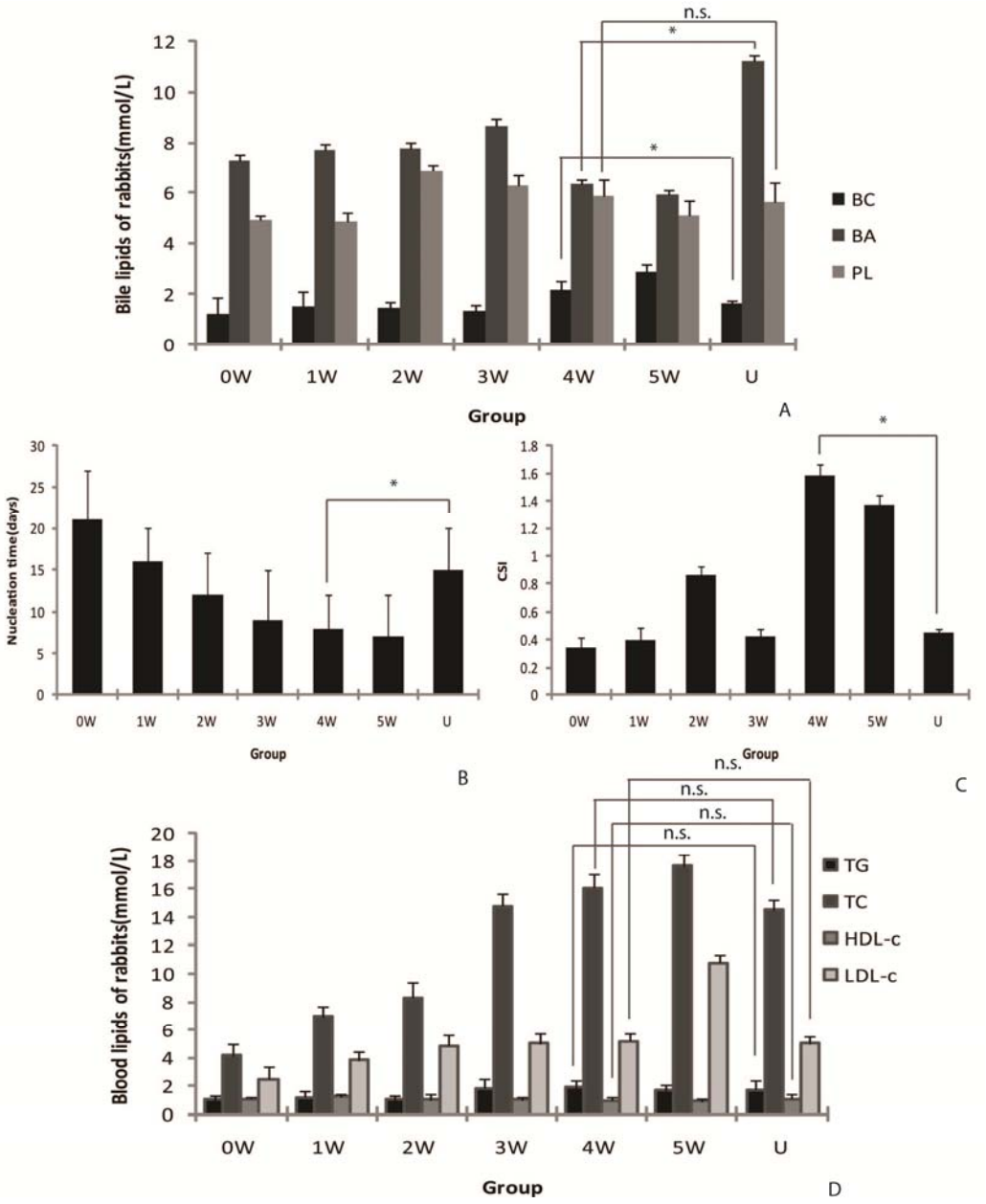

Figure 2: Analysis of bile lipids, nucleation time, CSI and blood lipids in rabbit gallstone models. A. Analysis of bile lipids, showing that the U group had a higher BA level than the 4W group (*) and a lower BC level than the 4W group (*). **B.** Analysis of bile nucleation time. **C.** Analysis of bile CSI, showing that the U group had a lower CSI than the 4W group (*). **D.** Analysis of blood lipids, showing that there were no differences in plasma TC, TG, HDL-c and LDL-c between 4W and U group (n.s.). "*" indicates $P<0.05$. "n.s." indicates "not significant". BC, bile cholesterol; BA, bile acids; PL, phospholipid; TG, triglyceride; TC, total cholesterol; HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; 0-5 W, 0-5 week; U, ursodeoxycholic acid treatment

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The mRNA expression of HMGCR, CYP7A1 and SCP2 in liver tissue

As shown in Figure 3, HMGCR mRNA levels were increased with the prolonged feeding time of dietary cholesterol ($P<0.05$), but there was a valley in the 2W and 3W groups. The HMGCR mRNA level of the 4W group (4.0587 ± 0.1011) was higher than that of the 0W group (1.12 ± 0.068) ($P<0.05$). CYP7A1 mRNA levels gradually decreased with the prolonged feeding time of dietary cholesterol ($P<0.05$). The CYP7A1 mRNA level of the 4W group (0.3485 ± 0.0597) was lower than that of the 0W group (1.04 ± 0.039) ($P<0.05$). SCP2 mRNA levels were also gradually increased with the prolonged feeding time of dietary cholesterol ($P<0.05$). The SCP2 mRNA level of 4W group (4.96 ± 0.15) was about 5-fold higher than that of 0W group (1.02 ± 0.17) ($P<0.05$). Our results showed that ursodeoxycholic acid could also reduce the expression of SCP2 from 4.96 ± 0.15 (4W group) to 1.76 ± 0.08 (U group) ($P<0.05$), but couldn't significantly reduce the expression of HMGCR ($P>0.05$) or increase the expression of CYP7A1 ($P>0.05$) compared with the 4W group.

The expression differences of HMGCR, CYP7A1 and SCP2 mRNA between gallstone and non-gallstone groups

As shown in Figure 3D and 3E, we divided all the rabbits fed with high cholesterol diet into two groups, gallstone group (16 rabbits) and non-gallstone group (24 rabbits). Then we detected the mRNA expressions of HMGCR, CYP7A1 and SCP2 in gallstone and non-gallstone groups by using quantitative real-time RT-PCR. HMGCR (2.26 ± 0.075) and SCP2 (2.18 ± 0.179) mRNA expression in the gallstone group was significantly higher than those (1.00 ± 0.088 and 1.08 ± 0.152) in the non-gallstone group ($P<0.05$). CYP7A1 mRNA was expressed lower in the gallstone group (0.699 ± 0.446) than that in the non-gallstone group (1.04 ± 0.24) ($P<0.05$). Furthermore CSI in the gallstone group (1.41 ± 0.08) was significantly higher than that in thenon-gallstone group (0.506 ± 0.073) ($P<0.05$).

DISCUSSION

In this study, we used high cholesterol diet to induce rabbit cholesterol gallstones. The rate of gallstone formation gradually increased with feeding time, and the formation of cholesterol monohydrate crystal also increased gradually. Our experiments indicated that 4 weeks were long enough to set up the rabbit cholesterol gallstone model, which was consistent with other previous studies (Zhao et al., 1998). So we chose 4 week as an optimal time for setting up the gallstone model and observing the preventive effect of ursodeoxycholic acid on cholesterol gallstones.

Data of previous epidemiological studies showed that levels of plasma lipoprotein cholesterol varied in cholesterol gallstone patients (Hayes et al., 1992; Busch and Matern, 1991). In normal people, the plasma LDL-c concentration appeared to be related to biliary cholesterol (Lee et al., 1985). Animal studies showed that in cholesterol fed hamsters with a VLDL-c/HDL-c ratio greater than 1.0, cholesterol gallstone formation occurred easily (Dietschy et al., 1993). We found that plasma TC and LDL-c in the 4W group were 4 and 2 fold those in the 0W control group respectively. LDL-c is a cholesterol-rich lipoprotein (North et al., 2006), and LDL-c cholesterol is the main resource of cholesterol from plasma to the liver. In this model, high cholesterol diet could lead to a large accumulation of cholesterol in the blood. Under this circumstance, cholesterol couldn't be cleared through the bile acid pathway, but it might be secreted into the bile directly.

Our results showed that the cholesterol level of gallbladder bile increased with cholesterol diet time. Under the high cholesterol diet, there was often an impaired bile acid synthesis, so the excessive cholesterol is mainly excreted into the bile and then leads to high lithogenicity of bile and gallstone formation. Bile acids increased in the first

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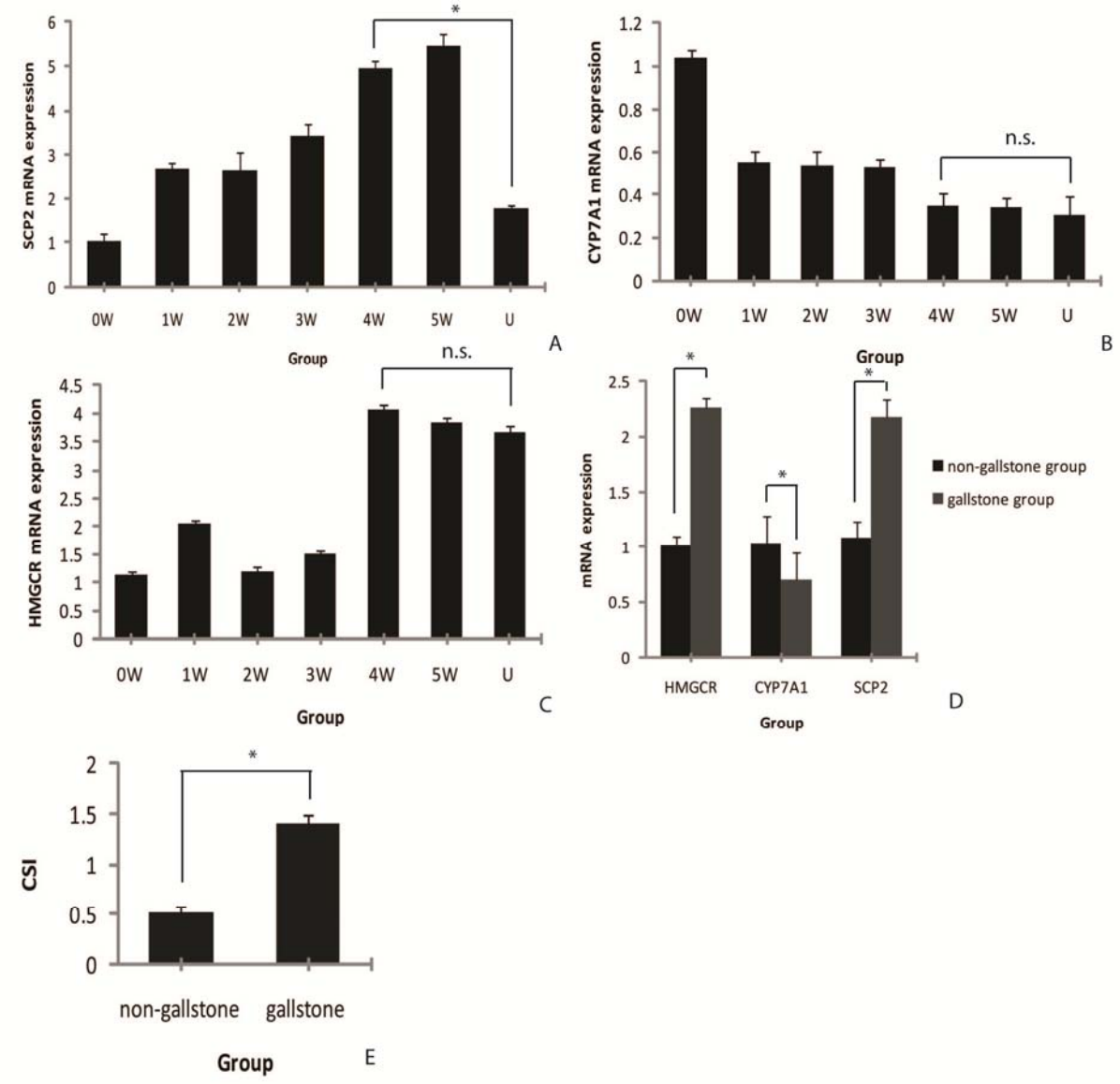

Figure 3: The expression of lipid related genes in rabbit gallstone models. A-C. Expression of SCP2 (**A**), CYP7A1 (**B**) and HMGCR mRNA (**C**) during cholesterol gallstone formation: (**A**) The SCP2 mRNA level is lower in the U group than in the 4W group (*); (**B and C**) mRNA levels of CYP7A1 and HMGCR, do not differ between the U and 4W group (n.s.). **D.** Expression of SCP2, CYP7A1 and HMGCR between gallstone and non- gallstone groups. **E.** CSI of the gallstone and non-gallstone groups, showing a higher CSI in the gallstone group (*). "*" indicates P<0.05. "n.s." indicates "not significant". 0-5 W, 0-5 week; U, ursodeoxycholic acid treatment

stage and then decreased with feeding time. The transient increase of bile acids may be due to the accumulation of cholesterol which induced higher expression of CYP7A1 and enhanced the bile acid synthesis, but after that we found that the secretion of bile acids decreased. The possible reasons were that the long-term excessive exogenous cholesterol might inhibit the activity and expression of CYP7A1 and further reduce the bile acid synthesis (Rudel et al., 1994). In our study, we found that bile CSI gradually increased and bile nucleation time was gradually shortened in the process of cholesterol stone formation.

When we compared the gallstone and non-gallstone groups, we found that the expression of HMGCR was elevated in the former, and at the same time CSI was significantly higher in the gallstone group than that in the non-gallstone group. It was found that the expression of HMGCR

mRNA increased with cholesterol diet time. This indicated that high cholesterol diet might induce the overexpression of HMGCR, increase bile CSI and promote the formation of gallstones. We found that the expression of CYP7A1 increased in the 2W and 3W group, and then decreased in the 4W and 5W group. This indicated that the high cholesterol diet may impair expression of CYP7A1 and the expression of CYP7A1 decreased in the gallstone group compared with the non-gallstone group. The reason may be that the conversion of cholesterol to bile acids was blocked, which might cause accumulated cholesterol in hepatocytes to be excreted directly into bile. We also found that the expression of SCP2 increased gradually with feeding time, and the expression of SCP2 was elevated in the gallstone group compared with the non-gallstone group. This indicates that the high cholesterol diet might induce the overexpression of SCP2. Therefore, during the gallstone formation induced by high cholesterol diet, there were abnormal expressions of HMGCR, CYP7A1 and SCP2.

In this study, we chose ursodeoxycholic acid as a preventive drug for gallstone formation and used the 4W group as the control group for comparison. The rate of gallstone formation in the 4W group was 100 %, but the rate in the U group was 33.3 %. Compared with the 4W group, the steatosis was significantly alleviated in the U group. This indicates that ursodeoxycholic acid might improve the steatosis of liver. The serum cholesterol and LDL-c in the U group was not lower than that in the 4W group. Obviously, ursodeoxycholic acid can't improve blood lipid components under the overload of cholesterol in the body. We also found that ursodeoxycholic acid could reduce the gallbladder bile cholesterol and significantly increase the bile acids in bile, but it couldn't influence the bile phospholipid level. Ursodeoxycholic acid could significantly lower CSI of bile, extend the nucleation time of bile and reduce the expression of SCP2, but couldn't influence the expression of HMGCR and CYP7A1 during the formation of gallstones.

In a word, our result was consistent with reports about ursodeoxycholic acid from other researchers. The early and long-term application of ursodeoxycholic acid could prolong cholesterol nucleation time, improve gallbladder emptying (Sharma et al., 1998; van de Heijning et al., 1999), and reduce pain and inflammation (Tomida et al., 1999). We found that ursodeoxycholic acid could increase bile acids and reduce biliary cholesterol, so it can increase the solubility of cholesterol, thereby reducing the incidence of gallstones. Finally ursodeoxycholic acid can also alleviate the steatosis of liver under high cholesterol diet. So it might be a good preventive drug for cholesterol gallstones.

Declaration of funding interests

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