Efficacy of Lidan Tang on high-fat-diet induced gallstone in mice and possible mechanism

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Supported by the National Natural Science Foundation of China (the Mechanism of Treating Cholelithiasis Based on the Theory of Biliary Diseases Treated by Differentiating Syndromes of the Liver, No. 81473570), the Drug Innovation Major Project (No. 2018ZX09711001-002-007, 2018ZX09711001-003-005, 2018ZX09711001-009-013), Beijing Key Laboratory of New Drug Mechanisms and Pharmacological Evaluation Study (BZ0150)

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Accepted: April 2, 2020

Abstract

OBJECTIVE: To investigate efficacy of Lidan Tang (LDT) on gallstone induced by high fat diet in mice, and to study its underlying mechanism.

METHODS: Mice were fed with high fat diet every day and treated with LDT (9.01 times of human clinical dosage). Mice were randomly divided into 6 groups as control group, gallstone model group (high-fat diet), positive control ursodeoxycholic acid (UDCA) group (80 mg · kg⁻¹ · d⁻¹, i.g.), LDT low dose group (6 kg/d, i.g.), LDT middle dose group (12 kg/d, i.g.), and LDT high dose group (24 kg/d, i.g.). The whole experiment was lasted for 4 weeks. The levels of ALT, AST, LDH, CHO, HDL-C and LDL-C in serum were measured, the pathological sections were observed by hematoxylin-eosin staining, the activities of antioxidant enzymes were measured by kits, and the proteins related to oxidative stress and lipid transport were detected by Western blot analysis.

RESULTS: LDT could significantly reduce the contents of ALT and AST in serum and improve the pathological tissue of liver. LDT could significantly reduce the content of MDA and LPO, and increase
the level of GSH and GSH-PX in liver tissue. The data of Western blot showed that LDT had antioxidant effect promoting Keap1/Nrf2 pathway and regulated the process of lipid transport, which was statistically significant. In addition, LDT treatment inhibited the expression of ATP-binding cassette transporters ABCG5/8 in liver, and reduced cholesterol transport from the hepatocytes to the gallbladder.

CONCLUSION: LDT has protective effect on gallstones induced by high fat diet in mice, which might be based on the protective effect on liver, including enhancing the antioxidant capacity of liver and reducing the production of lipid peroxides.

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**Keywords:** Cholelithiasis; ATP-binding cassette transporters; Oxidative stress; Lipids; Lidan Tang

**INTRODUCTION**

Gallstones disease is a common problem caused by the presence of stones in the gallbladder. According to the chemical composition of stones, gallstones can be divided into 3 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. Based on a systematic review of gallstone diseases, 10%-20% population of adults have gallstones, and over 20% of the patients will develop symptoms like abdominal pain, cholecystitis or obstructive jaundice. Gallbladder hypomotility is an early event in gallstone disease and is presumed to be a "trigger" that promotes stone formation. Dysfunction of gallbladder motility is believed to increase the transit time of supersaturated bile and permits cholesterol crystallization and continue growth. Furthermore, cholesterol gallstones can cause biliary colic, acute cholecystitis, pancreatitis and other complications. In brief, 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. Protein in bile can also promote the cholesterol crystal nucleate. An additional factor is the dysfunction of gallbladder motility.

Most patients with cholesterol gallstones are willing to receive a conservative treatment, including UDCA. It was reported to clinically reduce the incidence of biliary pain and acute cholecystitis in patients with symptomatic gallstones. Although UDCA provides benefit to patients, this approach suffers from low efficacy, a long period for the onset of action and a common recurrence of stone formation. Hence, better medicines for treating cholesterol gallstones are needed.

Gallbladder is attached to the short lobe of liver and belongs to each other in meridians, forming the relationship between exterior and interior. Usually, gallstones located in gallbladder, but the source of the disease is in the liver; if the liver function is insufficient or disorder, then the function of gallbladder will be affected as bile conversion deficient and bile secretion maladjusted. The production of abnormal bile is the pathological basis of gallstone formation. In recent years, Traditional Chinese Medicine (TCM) has achieved a good effect in the treatment of cholelithiasis, with the advantages of less side effects and low price. Lidan Tang (LDT) decoction is a prescription for surgical treatment of cholelithiasis in Beijing Integrated traditional Chinese and Western Medicine Hospital.

LDT is a formula from TCM, which has been used in China for years. Although LDT has been reported to have significant pharmacology function, it is not known the whole mechanism in treating gallstone. At present, LDT had become a dialectical prescription for the treatment of gallstones. In this study, we aimed to investigate LDT’s efficacy on gallstone induced by high fat diet in mice, and to study its underlying mechanism.

**METHODS**

**Lidan Tang decoction**

LDT decoction is a special prescription for surgical treatment of cholelithiasis in Beijing Integrated traditional Chinese and Western Medicine Hospital, and its cured effect is obvious clinically. LDT prescription: Chaiku (Radix Bupleuri Chinensis) 10 g, Yinchen (Herba Artemisia Capillaris) 10 g, Zhishi (Fructus Aurantii Immaturus) 10 g, Muxiang (Radix Aucklandiae) 10 g, Jinqiancao (Herba Lysimachiae) 10 g, Baishao (Radix Paeoniae Alba) 15 g, Houpu (Radix Rhei Palmati) 6 g, Wucao (Radix Linderae Aggregate) 10 g, and Chuanxiong (Rhizoma Chuanxiong) 10 g.

**Animals and treatment**

Male C57BL/6 mice (specified pathogen free obtained from Vital River Laboratories, Beijing, China) were used in a temperature and light control room, and free to water. Control group mice were fed a standard chow diet, other groups including model and LDT groups, were fed to a high-cholesterol lithogenic diet (15% total fat, 1.25% cholesterol and 0.5% cholic acid). 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 Peking Union Medical College and Chinese Academy of Medical Sciences.

Mice were randomly divided into six groups. LDT groups were given 6, 12, and 24 kg/d as low, middle and high dosages. UDCA group mice were given 80 mg/kg UDCA by intragastric administration. Control and model group mice were treated with isometric water. Every day the mice were treated once by gavage, and the whole experiment lasted for 4 weeks. Surgery was performed on mice rapidly. Blood was collected by eye bleeding, and the serum was separated. Gallbladder was removed, and the bile was obtained. The livers were collected and stored.

**Serum lipid analysis**

Blood serum total alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), cholesterol (CHO), high density lipoprotein cholesterol (HDL-C), and low density lipoprotein cholesterol (LDL-C) concentrations were measured once by 40FR automatic biochemical analyzer (BioSino Biotechnology and Science, Inc., Beijing, China).

**Hematoxylin-eosin (HE) staining**

HE staining was used to detect inflammatory cell infiltration in gallbladder wall. The paraffin-embedded tissues were cut at 4 μm in thickness using a rotary microtome, and a standard HE stain was performed. Slices were examined and captured on a microscope system (Eclipse Ni-U, Nikon Instruments Inc, Tokyo, Japan).

**Hepatic oxidative stress and antioxidant enzyme analysis**

Hepatic MDA, LPO, GSH and GSH-PX content were determined by the Assay Kits (Beyotime Institute of Biotechnology, Jiangsu, China).

**Western blot analysis**

Proteins of cholesterol transporters and oxidative stress which regulate cellular cholesterol homeostasis were measured as described previously. Briefly, liver samples from mice were fractionated by protein preparation kit (Applygen Technologies Inc., Beijing, Jiangsu, China), and separated proteins were used for western blot analysis. Briefly, samples were separated by 10% SDS-PAGE then transferred to PVDF membrane (Millipore, CA, USA), and immunoblotted with polyclonal antibodies. Bands were visualized by an enhanced chemiluminescence procedure (Applygen Technologies Inc., Beijing, China) and quantified with a Microplate Reader (Thermo Fisher Scientific Inc., Waltham, MA, USA) and an ImageQuant Las 4000 mini (GE Healthcare Life Sciences, Shanghai, China).

**Statistical analysis**

All data are expressed as the mean ± standard error of mean (SEM). One-way analysis of variance was conducted using Prism 7.0 software (GraphPad Software Inc, San Diego, CA, USA) in the tests. A P < 0.05 was considered statistically significant.

**RESULTS**

**Body weight and liver/body weight changes**

The whole preparation process of LDT was shown in Figure 1A. As shown in Figure 1B, LDT treatment did not change the weight of these mice. However, after given LDT, liver/body weight had significantly decreased compared to model group (Figure 1C).

**General evaluation of gallstone formation**

Gallstones formation was analyzed and calculated after the gallbladders were removed. The bile was muddy, and all the mice in model group formed gallstones (Figure 2). In contrast, no gallstones were observed in control group. In different dosages of LDT and UDCA groups, the total number of mice formed gallstones were decreased. Also, the gallstones were also relatively small compared to model group.

**Lidan Tang reduced abnormal serum lipid levels**

The levels of ALT, AST, LDH, CHO, HDL-C, and LDL-C in serum were determined. The data presented in Figure 3 showed that there was a significant increase in ALT, AST, LDH, CHO, and LDL-C and a significant decrease in HDL-C/LDL-C ratio in serum of mice compared with control group. All of these were reversed in mice administrated LDT and UDCA.

**Lidan Tang prevented inflammatory cells infiltration**

The HE staining showed liver tissue inflammation in model group. Compared to control group, there were more inflammatory cells infiltrating the blood vessels in model group. However, LDT and UDCA reduced the inflammation in liver and protected liver cells (Figure 4).

**Lidan Tang regulated hepatic lipid peroxidation**

The liver of lithogenic diet feeding mice showed an increase in MDA and LPO, and a decrease in GSH and GSH-PX compared with control group. High dose of LDT groups showed significantly decreased MDA and LPO, and increased GSH and GSH-PX levels (Figure 5).

**Lidan Tang modulated the expression of lipid transport related proteins**

We next evaluated whether LDT inhibited cholesterol accumulation in liver through regulating the expression of transporters. As expected, we found that the amount of ABCG5 and ABCG8 were significantly increased in model group mice compared to control.
However, LDT and UDCA treatment significantly decreased these levels of ABCG5 and ABCG8 compared with those in model group. In contrast, ABCB11 and pAMPK/AMPK were decreased but after given LDT and UDCA, ABCB11 and pAMPK/AMPK were increased significantly (Figure 6).

Lidan Tang adjusted the expression of antioxidant stress related proteins

Finally, we detected the antioxidant stress related proteins as Keap1, Nrf2, HO-1, and NQO1. High fat diet induced the downregulation of Keap1/Nrf2 pathway and its downstream proteins. LDT and...
UDCA treatment rescued these proteins, especially high dose of LDT (Figure 7).

**DISCUSSION**

In the study, C57BL/6 mice were fed a lithogenic diet for 4 weeks as reported before. LDT played an important role in the prevention of cholesterol gallstones formation and our results indicate the effect of LDT 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. It is found in our research that cholesterol crystals appeared in the gallbladder bile in model group. However, LDT or UDCA administration reduced the crystals area in gallbladder bile.

UDCA has been used clinically to dissolve stones and to protect the liver by inhibiting cholesterol secretion into bile. As natural product, UDCA exhibits antioxi-
Figure 5 Effect of LDT in hepatic lipid peroxidation induced by lithogenic diet in mice
A: MDA; B: LPO; C: GSH; D: GSH-PX. Mice were randomly divided into six groups. LDT groups were given 6, 12, and 24 kg/d as low, middle and high dosages. UDCA group mice were given 80 mg/kg UDCA by intragastric administration. Control and model group mice were treated with isometric water. Every day the mice were treated once by gavage, and the whole experiment lasted for 4 weeks. LDT: Lidan Tang; UDCA: ursodeoxycholic acid; MDA: methane dicarboxylic aldehyde; LPO: lipid peroxide; GSH: glutathione; GSH-PX: glutathione peroxidase. Data represents mean ± standard error of mean (n = 10). *P < 0.05, **P < 0.01 vs control group; ***P < 0.005, ****P < 0.001 vs model group.

Figure 6 Effect of LDT in the expression of ABCG5/ABCG8, ABCB11 and pAMPK/AMPK by western blot induced by lithogenic diet in mice
A: ABCG5; B: ABCG8; C: ABCB11; D: pAMPK/AMPK. Mice were randomly divided into six groups. LDT groups were given 6, 12, and 24 kg/d as low, middle and high dosages. UDCA group mice were given 80 mg/kg UDCA by intragastric administration. Control and model group mice were treated with isometric water. Every day the mice were treated once by gavage, and the whole experiment lasted for 4 weeks. 1: control; 2: model; 3: LDT-L; 4: LDT-M; 5: LDT-H; 6: UDCA. LDT: Lidan Tang; UDCA: ursodeoxycholic acid; ABCG5: ATP-binding cassette transporter G5; ABCG8: ATP-binding cassette transporter G8; ABCB11: ATP-binding cassette sub-family B member 11; pAMPK: phosphorylase AMP-activated protein kinase; AMPK: AMP-activated protein kinase. The protein expression data for ABCG5, ABCG8, ABCB11, and pAMPK/AMPK were presented as mean ± standard error of mean (n = 6). *P < 0.001 vs control group; **P < 0.001, ***P < 0.05 vs model group.
Our results indicated, the levels of ALT, AST, LDH, CHO, HDL-C and increased cholesterol gallstones formation. High cholesterol diet can lead to high blood lipid and high-cholesterol diet as positive drug. UDCA protect cells in the gallbladder in mice fed with a 30 d. cholesterol gallstones after treatment with UDCA for 4 weeks. Gallbladder muscle cell isolation was performed from mice with cholesterol gallstones treated with UDCA for 4 weeks. UDCA also functions to neutralize toxic hydrophobic bile acids. Previous study found that after treatment of UDCA for 4 weeks, gallbladder muscle cells isolated from patients with cholesterol gallstones had significant lower levels of oxidative stress and inflammation, and restored the contractility of smooth muscle cells as well. Another study found a diminished number of activated macrophages infiltrated in the muscular layer of the gallbladder in patients with cholesterol gallstones after treatment with UDCA for 30 d. In the study, we showed the UDCA effectively protect cells in the gallbladder in mice fed with a high-cholesterol diet as positive drug.

High cholesterol diet can lead to high blood lipid and increased cholesterol gallstones formation. Therefore, the levels of ALT, AST, LDH, CHO, HDL-C and LDL-C in serum were detected. Our results indicated that the contents of ALT, AST, LDH, CHO, and LDL-C were elevated, while HDL-C was decreased in serum of model group. However, all these changes can be relieved by administrating LDT or UDCA, which showed that LDT can improve lipid metabolic (Figure 3).

The formation of cholesterol gallstones was associated with the disorder of liver metabolism and cholesterol transport from the hepatocytes to the gallbladder. Therefore, the healthy liver could ensure normal secretion of bile and transport. In this study, LDT decreased MDA and LPO (Figure 5A, 5B), and increased the levels of GSH and GSH-PX induced by lithogenic diet, which improved the liver peroxidation (Figure 5C, 5D). From the histology in HE staining (Figure 4), we found that liver in model group damaged seriously, hepatic cord arrangement disorder. However, LDT or UDCA treatment group reduced liver damage in a dose-dependent manner. In other words, LDT can protect liver cells from lithogenic diet injury. The major lipid components of bile-cholesterol and phospholipids-are transported from the liver to the bile duct by distinct transporters. ABCG5 and ABCG8 are major transporters involved in cholesterol reverse transport in hepatocytes. In addition, ABCB11 encodes for the bile salt export pump located on the canaliculus, anti-inflammatory and cytoprotective properties. It is approved for cholecystolithiasis and is used in many other indications, such as for the treatment of enlarged polycystic liver diseases, intrahepatic cholestasis of pregnancy, primary sclerosing cholangitis and other cholestatic hepatopathies, on the basis of its capacity to counteract inflammation and liver damages. Further, UDCA also functions to neutralize toxic hydrophobic bile acids. Previous study found that after treatment of UDCA for 4 weeks, gallbladder muscle cells isolated from patients with cholesterol gallstones had significant lower levels of oxidative stress and inflammation, and restored the contractility of smooth muscle cells as well. Another study found a diminished number of activated macrophages infiltrated in the muscular layer of the gallbladder in patients with cholesterol gallstones after treatment with UDCA for 30 d. In the study, we showed the UDCA effectively protect cells in the gallbladder in mice fed with a high-cholesterol diet as positive drug.

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Figure 7 Effect of LDT in the expression of Keap1, Nrf2, NQO1 and HO-1 by Western blot induced by lithogenic diet in mice. A: Keap1; B: Nrf2; C: NQO1; D: HO-1. Mice were randomly divided into six groups. LDT groups were given 6, 12, and 24 kg/d as low, middle and high dosages. UDCA group mice were given 80 mg/kg UDCA by intragastric administration. Control and model group mice were treated with isometric water. Every day the mice were treated once by gavage, and the whole experiment lasted for 4 weeks. 1: control; 2: model; 3: LDT-L; 4: LDT-M; 5: LDT-H; 6: UDCA. LDT: LidanTang; UDCA: ursodeoxycholic acid; Keap: Kelch-like ECH-associated protein 1; Nrf2: nuclear factor erythroid 2-related factor 2; NQO1: NAD(P)H dehydrogenase quinone 1; HO-1: heme oxygenase 1. The protein expression data for Keap1, Nrf2, NQO1, and HO-1 were presented as mean ± standard error of mean (n = 6). "P < 0.01,"P < 0.001 vs control group; "P < 0.01,"P < 0.05,"P < 0.001 vs model group.
cular membrane of hepatocytes. Transport across the canicular membrane by ABCB11 is the limiting step in the hepatocellular transport of bile acids. Therefore, our data suggested that the expression of ABCG5 and ABCG8 increased significantly in lithogenic diet group, which were significantly lower by LDT or UDCA administration (Figure 6). Meanwhile, the decrease of ABCB11 and pAMPK/AMPK were reversed by LDT and UDCA. These data suggested that LDT had a strong effect on regulating lipid transport, in order to maintain the normal function of liver and gallbladder, and showed a certain dose-dependent manner.

Stimulation of cells by xenobiotics or drugs results in the overexpression of ROS and thus oxidative stress. The association of ROS with drug-induced hepatotoxicity is an indication that oxidative stress is one of the major causes of hepatocyte apoptosis and liver dysfunction. The activation of Nrf2 has been linked to hepatotoxicity. After translocation of Nrf2 to nucleus, it interacts with ARE to modulate intracellular antioxidant responses. High cellular levels of ROS activate the dissociation of Nrf2 from Keap1 and its subsequent transfer to the nucleus. In nucleus, Nrf2 binds to ARE and activates the expression of oxidoreductases such as HO-1 and NQO1, resulting in manifestation of its antioxidant effects. Thus, the Nrf2 pathway is considered a major factor regulatory mechanism for reducing oxidative stress. According to our results in Figure 7 we found that LTD can upregulate Keap1/Nrf2 pathway and the downstream enzymes such as HO-1 and NQO1.

In conclusion, we have demonstrated that LDT prevented cholesterol gallstones formation and reduced biliary cholesterol secretion induced by lithogenic diet in C57BL/6J mice. The possible mechanism behind the actions involves the suppression in the expression of hepatic proteins ABCG5, ABCG8 and increase ABCB11, AMPK, Nrf2, HO-1 and NQO1. The cholesterol from bile reabsorption and transport from hepatocytes to the gallbladder is reduced and the level of cholesterol in gallbladder is decreased. Moreover, LDT alleviated the serum lipid and hepatic peroxidation may also protect the liver and decrease the cholesterol crystal formation. However, there may be other LTD’s effects involved in preventing gallstone formation, which needs further confirmation.

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