# Prevention of gallstones by Lidan Granule: Insight into underlying mechanisms using a guinea pig model

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**Abstract.** The aim of the study was to examine the mechanism of action of Lidan Granule (LDG) for the prevention of gallstones using a guinea pig model. One hundred guinea pigs were divided into five groups randomly: control (standard diet and saline), model [lithogenic diet (LD) and saline], LDG-H (LD and 2 g/kg of LDG), LDG-L (LD and 1 g/kg of LDG), and ursodeoxycholic acid (UDCA) (LD and UDCA) as the positive control. At 6 weeks, the rate of gallstone formation and weight of the adrenal gland were recorded and serum levels of inflammatory cytokines were measured. Levels of corticotrophin-releasing hormone (CRH) in the hypothalamus, adrenocorticotropic hormone (ACTH) in the hypophysis, and serum cortisol were determined. Bile components were tested with colorimetry. At 6 weeks, the rate of gallstone formation was significantly decreased in the LDG-H (14.29%) and LDG-L (21.43%) groups compared to the model group (81.25%; P<0.01). LDG treatment decreased the serum levels of interleukin (IL)-1, IL-6, and tumor necrosis factor-α (P<0.01). LDG decreased bile cholesterol and increased bile acid and phospholipid levels in the bile (P<0.01). LDG treatment recovered the function of the hypothalamic-pituitary-adrenal (HPA) axis by increasing the expression of CRH (P<0.01) and ACTH (P<0.05). LDG made the bile less lithogenic, improved the function of the HPA axis, and regulated the expression of inflammatory cytokines for the prevention of cholelithiasis.

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## Introduction

Gallstone disease is one of the most common gastrointestinal disorders encountered in clinical practice (1,2). Gallstones are often associated with the bile duct or intrahepatic stones, which have difficulty clearing the biliary system and are associated with a high rate of recurrence (3). Thus, prevention of the formation and recurrence of gallstones is necessary (4-7).

Gallstones are formed for many reasons. One cause is inflammation, which often changes the bile acid composition and accelerates stone formation (8,9). Previous findings have shown that inflammatory cytokines such as lipopolysaccharide (LPS), tumor necrosis factor (TNF), and interleukin-1 (IL-1) can inhibit the production of bile acid through classic and bypass pathways (9-11). Inflammatory cytokines can disturb cholate transport in the hepatocytes and reduce the secretion of bile salts and phospholipids (9).

Apart from inflammation, an imbalance in bile components such as cholesterol, bile acids, and lecithin, also contributes to gallstone formation. As the concentration of cholesterol in bile increases, the bile begins to be saturated with cholesterol, and at a certain level, the cholesterol begins to crystallize. McNeilly *et al* (12) found that bile acids are involved in the regulation of glucocorticoid metabolism within the liver of female patients (12,13). The increased level of bile acids during cholestasis may induce the downregulation of the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis plays a role in the body's response to stress by mediating the secretion of the adrenocorticotropic hormone (ACTH). Clinical studies have shown that gallstone disease is associated with dysregulation of the HPA axis with abnormal secretion of serum corticosterone (14). Thus, homeostasis of the HPA axis is important for the prevention of pigment gallstones.

In traditional Chinese medicine, gallstone refers to 'rib pain' or 'liver bilges', and the treatment of gallstones is based on the principle of 'liver-dispersing and bile discharging,' i.e., stone clearance and restoration of liver function. Lidan Granule (LDG) is formulated on the basis of these principles and consists of 15 types of Chinese herbs. It has been used for many years in clinical practice to treat and prevent gallstones (4,15,16). The aim of the present study was to investigate

Table I. Components and efficacy of LDG.

| English | Latin | Species | Effect |
|---|---|---|---|
| Oriental wormwood | *Artemisia capillaris* Thunb | *Origanum* L. | Clear away heat and promote diuresis |
| Hawthorn fruit | *Crataegus pinnatifida* Bunge | *Crataegus* L. | Aid digestion, increase appetite |
| Rice sprout | *Setaria italica* | Rice | Aid digestion, increase appetite |
| Germinated barley | *Hordeurn vulgare* L. | *Gramineae* L. | Aid digestion, increase appetite |
| Green orange peel | *Citrus reticulata* Blanco | *Citrus* L. | Relieve food retention |
| Tangerine peel | *Citrus reticulata* Blanco | *Citrus* L. | Dispel moisture and eliminate phlegm |
| Medicated leaven | *Massa Medicata Fermentata* | Powder | Digestion |
| Cyperus tuber | *Cyperus rotundus* L. | *Cyperus* Linn. | Relieve chest and abdominal pain |
| Radish seed | Semen Raphani | *R. sativus* L. | Relieve abdominal distention |
| Caulis perillae | *Perilla frutescens* (L.) Britton. | *P. frutescens* L. Britt. | Relieve nausea |
| Turmeric root | *Curcuma longa* L. | *Curcuma* L. | Relieve abdominal pain |
| Rhubarb | *Rheum palmatum* L. | *Rhubarb* L. | Clear heat-fire |
| Pinellia tube | *Pinellia ternata* (Thunb.) Breit. | *Pinellia* Ten. | Antiemetic effects |
| Chinese honeylocust fruit | *Gleditsia sinensis* Lam. | *Gleditsia* Linn | Aid digestion; increase appetite |

LDG, Lidan Granule.

Table II. Components of lithogenic diet food.

| Name | Dosage (g/kg) | Name | Dosage (g/kg) | Name | Dosage (g/kg) |
|---|---|---|---|---|---|
| Corn flour | 136.3 | Alfalfa meal | 416.5 | Cellulose | 20 |
| Salt | 10 | Whole wheat | 90.9 | Cholesterol | 1 |
| Flour | 90.7 | Yeast powder | 10 | Vitamin C | 0.05 |
| Soy bean flour | 90.9 | Lard oil | 20 | Cholic acid | 0.4 |
| Fish meal | 63.6 | Sucrose | 20 | Casein | 20 |

the role of LDG in the treatment and prevention of pigment gallstones and to explore the underlying mechanisms using a guinea pig model.

## Materials and methods

*Drugs and reagents*. LDG was provided by the Department of the Integrative Medicine, Huashan Hospital, Fudan University (Shanghai, China), and the components and efficacy of LDG are described in Table I. Ursodeoxycholic acid (UDCA) was purchased from Sanwei Changjiang Biochemical Pharmaceutical Factory (Shanghai, China), and the enzyme-linked immunosorbent assay (ELISA) kits were obtained from eBioscience, Inc. (San Diego, CA, USA). The lithogenic diet food (the components of which are listed in Table II) was purchased from Trophic Animal Feed High-tech Co., Ltd. (Jiangsu, China). LDG and UDCA were diluted in sterile saline. Animals in the LDG-H (2 g/kg/day, determined by the dosage used for humans clinically), LDG-L (1 g/kg/day), and UDCA (50 mg/kg/day) groups were fed twice per day.

*Animals and treatments*. One hundred male guinea pigs (weighing 230-250 g) were obtained from the Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). Animals were maintained in a temperature- (20±2˚C) and humidity- (50-60%) controlled facility upon arrival on a 12-h light/dark cycle (lights on from 7:00 a.m. to 7:00 p.m.) and given access to food and water *ad libitum*. Animals were randomly divided into five groups. The control group mice were fed a chow diet, whereas mice in the other groups were fed a high cholesterol lithogenic diet (17,18). The LDG-H group was fed with a lithogenic diet and LDG (2 g/kg/day), given orally, and the LDG-L group was fed the lithogenic diet and LDG (1 g/kg/day), given orally (Fig. 1). The UDCA group was fed UDCA (50 mg/kg/day), and the model group was fed with lithogenic diet and saline. Experiment animals were housed for a minimum of 7 days prior to the start of the experiment to adapt to the environment. Experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Fudan University.

*Serum, bile, and histological sample preparation*. The animals were sacrificed by an overdose of sodium pentobarbital (50 mg/kg body weight, i.p.), and blood samples were obtained from the aorta abdominal and clotted for 2 h at room temperature before centrifugation (1,006.2 x g at 4˚C for 10 min). The supernatant was subsequently collected and stored at -80˚C for measurement of the levels of IL-6, IL-1, TNF-α, and cortisol (CORT) in the peripheral blood. The guinea pigs were decapitated, and the brain tissues were

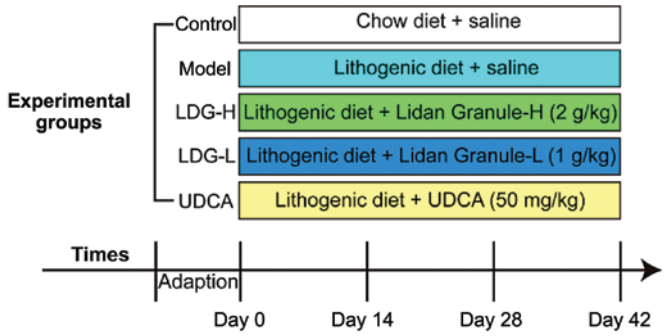


Figure 1. Schematic representation of the experiment.

removed and stored on ice. The bilateral hippocampus and hypophysis were rapidly and carefully removed using curved tweezers. Once snap frozen in liquid nitrogen, the brain tissue samples were stored at -80˚C until use. The adrenal glands of guinea pigs were also removed and weighed carefully.

Bile samples were obtained from dissected gall bladders, stored at -20˚C, and diluted 1:5 with distilled water prior to analysis by ultra-performance liquid chromatography-mass spectrometry (UPLC-MS).

*Physical state score (PSS).* The physical states of the guinea pigs in the different groups were assessed as previously described (19). The physical states of guinea pigs were evaluated weekly until the end of the experiment. The coat state was recorded on a scale from 1 to 3 as follows: guinea pigs in a good state were scored as 3 (the fur was smooth with no tousling), animals in a bad state were scored as 1 (dirty fur on most of the body), and those with a coat state between 1 and 3 were scored as 2. Each measurement was scored by another experimenter blinded to the treatment group.

*Serum CORT, corticotrophin-releasing hormone (CRH), IL-6, and ACTH ELISA.* Serum levels of CORT, IL-6, IL-1, and TNF-α were measured using a commercially available enzyme competitive ELISA test kit following the manufacturer's instructions (eBioscience, Inc.).

*Statistical analysis.* Data were presented as mean ± standard error of the mean and analyzed with SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). The statistical significance of differences between the means of groups was determinant by one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) for post-hoc comparisons. The rate of different groups were compared with the Chi-square test. $P<0.05$ was considered to indicate a statistically significant difference.

## Results

*Effect on weight, PSS change, and the rate of gallstone formation.* During 6 weeks of exposure to a lithogenic diet, guinea pigs in the model group showed a mild increase in weight, whereas those on low concentration (1 g/kg) or high concentration (2 g/kg) of LDG showed an obvious increase in weight after 2 weeks (Fig. 2A).

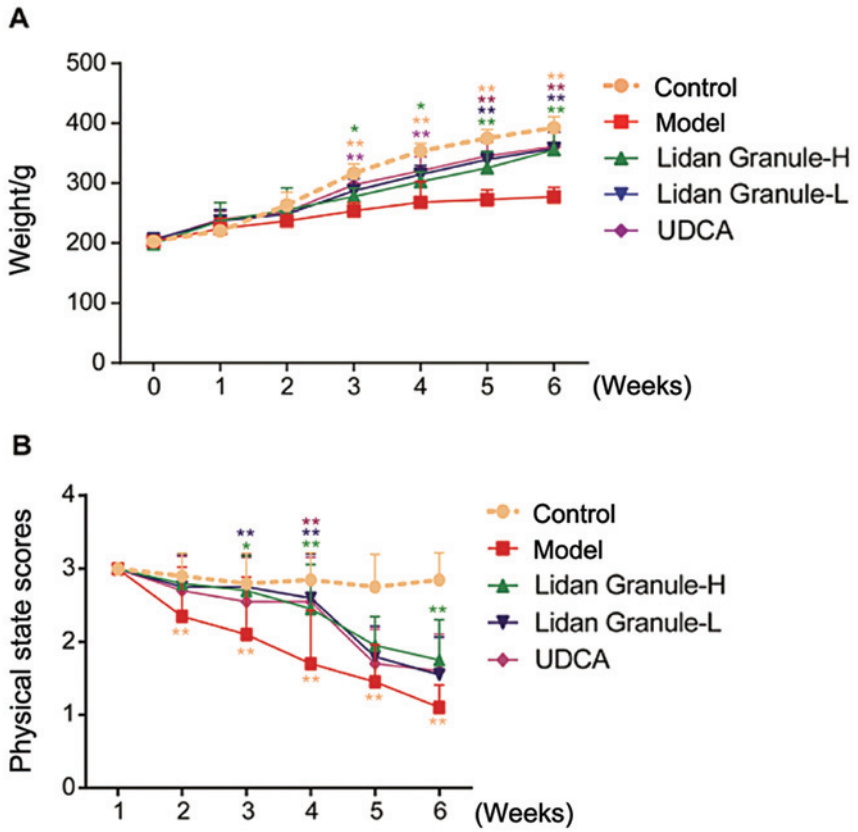


Figure 2. Trends in weight and PSS score of the guinea pigs. (A) Weight changes in the guinea pigs during the experiment. (B) The PSS score trend in the guinea pigs during the 6-week experiment. PSS, physical state score.

Table III. Rates of gallstone formation in the different groups.

| Group | No. of animals | No. of gallstones | Rate of gallstone formation (%) |
|---|---|---|---|
| Control | 18 | 1 | 5.56[b] |
| Model | 16 | 13 | 81.25 |
| LDG-H | 14 | 2 | 14.29[b] |
| LDG-L | 14 | 3 | 21.43[b] |
| UDCA | 13 | 4 | 30.77[a] |

Analysis by Chi-square test, compared with model group. [a]P<0.05, [b]P<0.01. LDG, Lidan Granule; UDCA, ursodeoxycholic acid.

Six weeks later, the differences in weights between the model and LDG groups were significant (both P<0.01). The PSS score of the model group guinea pigs decreased during the 6-week experiment, and the PSS score was statistically different between the control and model groups (P<0.01) beyond the second week. The PSS scores for the treatment groups were reduced significantly during the fourth and fifth weeks (Fig. 2B).

Six weeks later, the rate of gallstone formation was 5.56% among 18 guinea pigs in the control group (Table III). For the model group, the stone formation rate was 81.25%, which was significantly higher than that in the control group (P<0.01; Table III). Four of 13 guinea pigs developed gallstones in the UDCA group (P<0.05), and the stone formation rates in the LDG-H and LDG-L groups were 14.29% (P<0.01) and 21.43% (P<0.01), respectively. The rates were lower than the stone formation rate observed in the model group (Table III).

*Effect on histopathological assessment of liver tissues*. After 6 weeks of treatment, normal hepatocytes with blue-stained nuclei located in the center of the cell and arranged in cord-like patterns were evident in the control group (Fig. 3A and A1). In the pigment gallstone model group, hepatic steatosis in the form of fat globules in the hepatocytes with an eccentric nucleus and loose cytoplasm was observed. Many inflammatory cells were dispersed around the central vein, and small Mallory bodies were identified at higher magnification (Fig. 3B and B1). Compared to the control group, the LDG-H (2 g/kg/day; Fig. 3C and C1) and LDG-L (1 g/kg/day; Fig. 3D and D1) groups showed fewer inflammatory cells, and no obvious ballooning degeneration. Similar findings were observed in the UDCA group (Fig. 3E and E1), except for the presence of ballooning degeneration in a few areas around the central vein.

*Effect of LDG on HPA axis activation*. As shown in Fig. 4, 6 weeks of the lithogenic diet increased the CORT concentration in serum (P<0.01) compared to that in the control group and decreased the level of CRH in the hypothalamus (P<0.01) and expression of ACTH in the hypophysis (P<0.01). The lithogenic diet also had some effect on the weight of the adrenal gland (P<0.01). Six weeks of treatment with LDG (2 and 1 g/kg/day) significantly improved the disturbance in the HPA axis caused by the lithogenic diet. Similarly, UDCA had a positive effect on the level of CORT in the peripheral blood (P<0.01) and the weight of the adrenal gland (P<0.01).

*Effect of LDG on IL-6, IL-1, and TNF-α levels in peripheral blood*. The levels of IL-6, IL-1, and TNF-α in the peripheral blood were increased after 6 weeks on a lithogenic diet. Following treatment with high- or low-dose LDG, decreases in the serum concentration of IL-6 (Fig. 5A; P<0.01), IL-1 (Fig. 5B; P<0.01), and TNF-α (Fig. 5C; P<0.01) were observed.

*Effect of LDG on the bile components*. As expected, the lithogenic diet increased the concentration of cholesterol, direct bilirubin (DBIL), and indirect bilirubin (IBIL) in the bile and reduced the bile concentrations of bile acids and phospholipids (Fig. 6A and D). However, LDG significantly reduced the cholesterol, IBIL, and DBIL concentrations in the bile (Fig. 6B; P<0.01). At the same time, LDG increased the acid concentration in the bile (Fig. 6A; P<0.01) after 6 weeks of treatment.

## Discussion

The results of the present study suggest that a lithogenic diet can induce the formation of gallstones and disturb the HPA

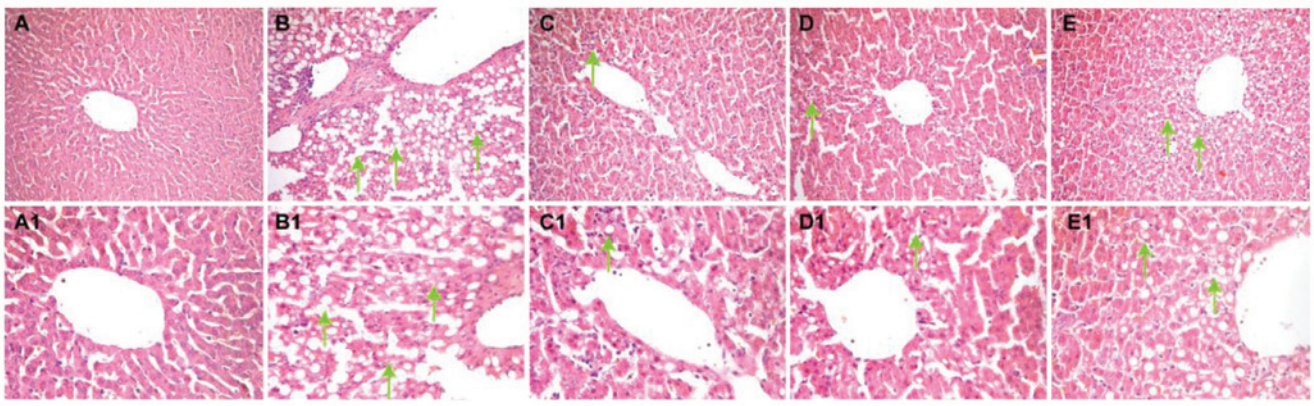


Figure 3. Histopathological findings in the liver of different groups. (A and A1) Sections of normal liver showing normal hepatocytes arranged in a cord-like pattern. (B and B1) Histological sections showing enlarged fatty hepatocytes with loose cytoplasm (green arrows) and eccentric nucleus (C and C1). (D and D1) Sections of liver after 6 weeks of exposure to LDG. (E and E1) Less loose cytoplasm and some ballooning degeneration in hepatocytes of the UDCA group (green arrows). LDG, Lidan Granule; UDCA, ursodeoxycholic acid.

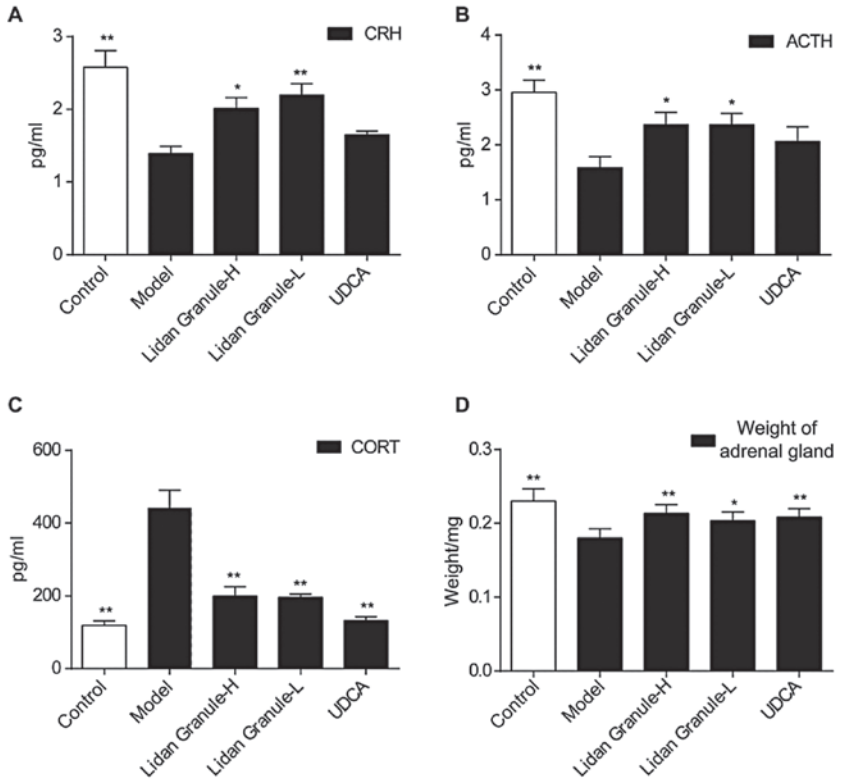


Figure 4. LDG administration partially improves the lithogenic diet-induced disturbance in the HPA axis. (A) LDG increased the expression of CRH and (B) ACTH but (C) reduced the production of CORT. (D) The effect of LDG on the weight of the adrenal gland. Data are the mean ± SEM. $^{*}$P<0.05, $^{**}$P<0.01 vs. model. LDG, Lidan Granule; HPA, hypothalamic-pituitary-adrenal; CRH, corticotrophin-releasing hormone; ACTH, adrenocorticotropic hormone; CORT, cortisol.

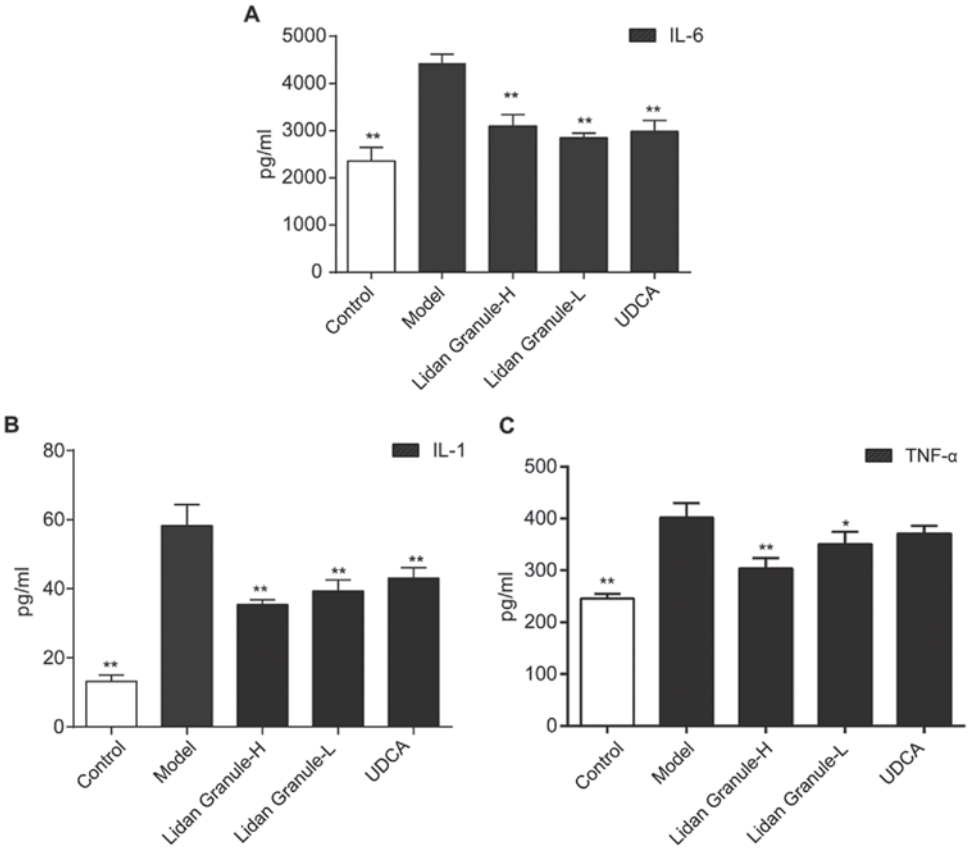


Figure 5. LDG improved the serum levels of IL-6, IL-1, and TNF-α in the guinea pigs. (A) Lithogenic diet induced an increase in IL-6 in the serum. LDG reduced the inflammatory response of the guinea pigs; (B) LDG improved the level of IL-1 in the peripheral blood. (C) LDG decreased the level of TNF-α in the serum. LDG, Lidan Granule; IL, interleukin; TNF, tumor necrosis factor.

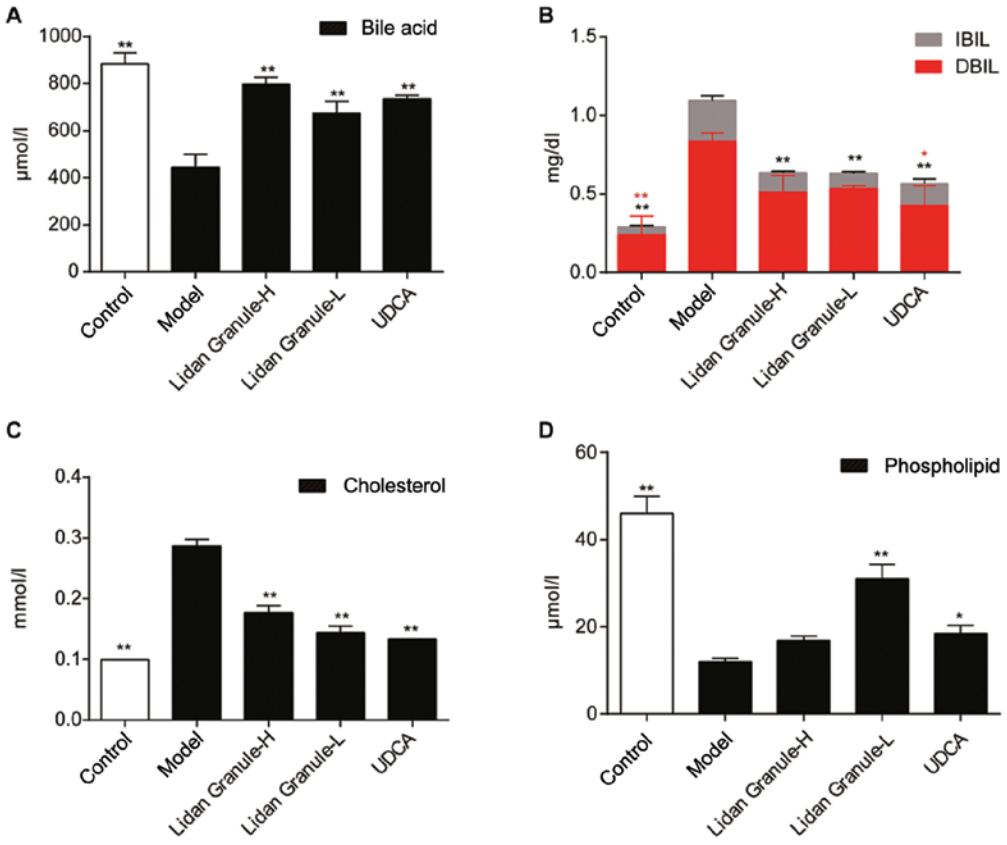


Figure 6. LDG ameliorated the effects of a lithogenic diet on the concentrations of bile acids, DBIL, IBIL, cholesterol, and phospholipids in the bile of guinea pigs. LDG, Lidan Granule; DBIL, direct bilirubin; IBIL, indirect bilirubin.

axis in guinea pigs, whereas LDG can ameliorate the effect of a lithogenic diet on the HPA axis and bile components.

Most gallstones are formed in the bile duct system, where they can block the bile duct and lead to cholestasis (20). During the disease process, cholestasis results in a decrease in liver function, and also, endocrine function (21). The diet is one of the most common pathogenic factors for gallstone formation. Thus, in our study, a lithogenic diet was given to guinea pigs for 6 weeks to induce the formation of pigment gallstones (22). After 6 weeks, serum IL-6, IL-1, and TNF-α levels in the model group were found to be elevated, while the histopathological examination of the livers showed hepatic steatosis and loose cytoplasm, with nuclei pushed to the edge of cells and inflammatory cell infiltration occurring. These findings confirm that the pigment gallstone model is suitable for studying the pathophysiology of pigment stone formation.

LDG has been used as a routine treatment for gallstones for many years, and clinical studies have demonstrated that LDG can promote biliary excretion and litholysis (15). LDG is composed of 15 types of Chinese herbs, namely, oriental wormwood, hawthorn fruit, rice sprout, germinated barley, green orange peel, tangerine peel, medicated leaven, cyperus tuber, radish seed, perilla stem, turmeric root, bitter orange, pinellia tube, and Chinese honey locust fruit, which is based on the Chinese medicinal principle of smoothening liver function, dredging the gallbladder, harmonizing the stomach, and strengthening the spleen (23).

The neuroendocrine system plays an important role in maintaining homeostasis (14). A clinical study has shown that gallstone formation in patients is always followed by endocrine disturbance, and changes in the corticosterone levels may indicate the presence of gallstone disease (24). In our study, after 6 weeks on a lithogenic diet, the CORT level in the model group was increased along with disruption of the HPA axis and a change in the weight of the adrenal gland. LDG treatment can ameliorate the secretion of proteins in the HPA axis, which means that LDG can regulate the neuroendocrine system during the process of gallstone formation.

IL-6 is one of the most important inflammatory biomarkers of cholelith disease (9,25). In our study, serum IL-6 levels in the model group were elevated. Previous findings have shown that IL-6 in combination with other inflammatory cytokines or alone can influence the action of the HPA axis at different levels (26), which may explain the reason HPA axis dysfunction being considered in cholelith disease. LDG can improve the inflammatory state of guinea pigs by reducing the amounts of serum IL-6, IL-1, and TNF-α, and improving the function of the neuroendocrine system at the same time.

Bile components are an important factor for the clinical diagnosis and differential diagnosis of gallstone disease (27,28). In our study, the DBIL of the model group was higher than that of other groups, and it was in agreement with the clinical characteristics of silt-type cholesterol gallstones. After 6 weeks of LDG treatment, the DBIL was reduced significantly in the study group, which proves the dredging action of LDG.

In conclusion, the present study confirmed that cholelith disease is always accompanied by dysregulation of the HPA

axis. LDG cannot only regulate the excretion of inflammatory factors but also improve the function of the neuroendocrine system, and improve the composition of the bile. LDG may influence the lithogenic character of bile via multiple pathways and has a definite role in the prevention and treatment of cholelithiasis.

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