

Role of osteopontin in diet-induced brown gallstone formation in rats

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Abstract

Background: Although osteopontin (OPN) is expressed in the liver and pigment gallstones of patients with hepatolithiasis, its role in pigment gallstone formation remains unclear. This study aimed to explore the function of OPN in pigment gallstone formation.

Methods: Rats were fed a chow diet (CD) or lithogenic diet (LD) for 10 consecutive weeks; blocking tests were then performed using an OPN antibody (OPN-Ab). Incidence of gallstones and levels of several bile components, OPN, tumor necrosis factor alpha (TNF- α), and cholesterol 7 α -hydroxylase (CYP7A1) were analyzed. To determine TNF- α expression in hepatic macrophages and both CYP7A1 and bile acid (BA) expression in liver cells, recombinant rat OPN and recombinant rat TNF- α were used to treat rat hepatic macrophages and rat liver cells, respectively. Chi-square or Fisher exact tests were used to analyze qualitative data, Student *t*-test or one-way analysis of variance were used to analyze qualitative data.

Results: Incidence of gallstones was higher in LD-fed rats than in CD-fed rats (80% vs. 10%, $P < 0.05$). BA content significantly decreased in bile ($t = -36.08$, $P < 0.01$) and liver tissue ($t = -16.16$, $P < 0.01$) of LD-fed rats. Both hepatic OPN protein expression ($t = 9.78$, $P < 0.01$) and TNF- α level ($t = 8.83$, $P < 0.01$) distinctly increased in the LD group; what's more, CYP7A1 mRNA and protein levels ($t = -12.35$, $P < 0.01$) were markedly down-regulated in the LD group. Following OPN-Ab pretreatment, gallstone formation decreased (85% vs. 25%, $\chi^2 = 14.55$, $P < 0.01$), liver TNF- α expression ($F = 20.36$, $P < 0.01$) was down-regulated in the LD group, and CYP7A1 expression ($F = 17.51$, $P < 0.01$) was up-regulated. Through CD44 and integrin receptors, OPN promoted TNF- α production in macrophage ($F = 1041$, $P < 0.01$), which suppressed CYP7A1 expression ($F = 48.08$, $P < 0.01$) and reduced liver BA synthesis ($F = 119.4$, $P < 0.01$).

Conclusions: We provide novel evidence of OPN involvement in pigmented gallstone pathogenesis in rats.

Keywords: Pigment gallstone; Rat model; Osteopontin; TNF- α ; CYP7A1

Introduction

Gallstone disease (GSD), the most common type of biliary disorder, affects 10% to 20% of the adult population worldwide.^[1,2] Various complications associated with GSD often result in heavy financial burdens to the individual, their families, and society in general.^[3-5] GSD is associated with genetic and metabolic factors, increasing age, and the female gender.^[6] According to the chemical composition of stones, GSD can be divided into cholesterol, pigment, or mixed stones. Although many studies have reported on cholesterol stone pathogenesis, the pathological mechanism behind pigment stones, often occurring in the intrahepatic bile duct, is still undetermined. To date, three hypotheses concerning pigment stone pathogenesis have been proposed^[1]: bacterial and parasite infections, bile duct obstruction or stricture and cholestasis, and alteration of lipid metabolism and bile components. At present, the content alteration of hepatocyte metabolism is of notable interest.^[7,8]

Osteopontin (OPN), a multifunctional cytokine and extracellular matrix protein,^[9] has been shown to closely relate to hepatolithiasis due to its distinctly high expression in the epithelium of stone-laden intrahepatic bile ducts.^[10] A previous study demonstrated that OPN serves as a core protein in cholesterol gallstone formation.^[11] Interestingly, previous findings by Yang *et al*^[12] suggested that OPN inhibits cholesterol gallstone formation through suppression of the calcium ion-induced pro-nucleation effect. Another study indicated that OPN deficiency protects rats from cholesterol gallstone formation by reducing intestinal cholesterol absorption through the inhibition of Niemann-Pick C1-Like 1 expression.^[13] Overall, as OPN plays an important role in gallstone formation, investigating its function in pigment stone formation is warranted. To provide novel therapeutic or preventive insights into pigment gallstones, our study aimed to observe alterations in bile components and OPN expression using a diet-induced rat brown gallstone model.

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Methods

Ethical Approval

All experimental procedures were conducted in accordance with animal care guidelines and approved by the Peking University Institutional Review Board (No. LA2019327).

Animals and sample collection

Male Sprague-Dawley rats (6–8 weeks of age, specific pathogen-free; Beijing Biotechnology, Beijing, China) were housed in a controlled environment ($22 \pm 2^\circ\text{C}$, $60\% \pm 10\%$ relative humidity) and exposed to a 12-h light-dark cycle. A total of 20 rats were randomly divided into chow diet (CD) ($n = 10$) and lithogenic diet (LD) ($n = 10$) groups, in which they were fed the corresponding diet for 10 weeks. LD was designed as a modified version of the formula published by Kong *et al*^[14] with its detailed components shown in Supplementary Table 1, <http://links.lww.com/CM9/A552>. In the blocking experiment, 80 rats were divided into four groups ($n = 20$), with rats in the blocking group injected twice a week for 9 weeks with $30 \mu\text{g}$ of rabbit anti-rat OPN antibody (OPN-Ab) (Bioss, Beijing, China) via tail veins. To eliminate any non-specific effects of the OPN-Ab, non-specific IgG (NS-IgG; Bioss) was used as a control in this intervention. At week 10, rats were euthanized via cervical dislocation following an intraperitoneal injection of pentobarbital sodium (20 mg/kg); next undergoing laparotomy via a midline incision. After collecting 0.5 mL of bile using a 1.0-mL syringe to puncture the common bile duct, this duct was surgically removed for microscopic examination. For protein isolation, some liver specimens were immediately frozen in liquid nitrogen (-196°C). For the histological assay, the remainder of the liver samples was fixed in 4% paraformaldehyde overnight. Ten additional rats were required to be euthanized at the beginning of the experiment to measure baseline hepatic OPN expression.

Cell culture and treatment

Rat primary liver macrophage (ScienCell, Carlsbad, CA, USA) and rat liver cells (BRL cell line; ATCC, Manassas, VA, USA) were cultured in Dulbecco modified Eagle medium (Gibco, Grand Island, NY, USA), containing 10% fetal bovine serum, and maintained at 37°C and 5% CO_2 . Next, cells were grown in six-well plates to 80% confluency and later treated with drugs. Liver macrophages were stimulated using normal saline or 60 ng/mL of recombinant rat OPN (rrOPN; Cloud-Clone, Houston, TX, USA) for 6 h. Specifically, they were pretreated with a CD44 antibody (CD44-Ab; Abcam, Cambridge, UK) or CWHM12 (Abmole Bioscience Inc, Houston, TX, USA; a novel small molecule inhibitor of αV integrins) for 1 h, to block the OPN receptor; NS-IgG (Bioss) was used as the control. BRL cell lines were treated separately with recombinant rat tumor necrosis factor alpha (rrTNF- α ; Abcam) at different concentrations and times.

Biochemical analysis of lipid and bile acid (BA) from bile and liver

Bile, liver homogenate, and liver cell supernatant were prepared for biochemical analysis. BA, total cholesterol,

total bilirubin, conjugated bilirubin, and unconjugated bilirubin levels were measured using a microplate reader (Bio-Rad Laboratories Inc., CA, USA) and commercial biochemical kits, according to the manufacturer's protocol. Phospholipid content was assayed using a phospholipid enzyme-linked immunosorbent assay (ELISA) kit (Jianglai Chem, Shanghai, China). Liver cell supernatant TNF- α content was determined using the TNF- α ELISA kit (Multi Sciences, Zhejiang, China). All biochemical measures in this study were repeated three times. The common bile duct was surgically removed to check for gallstones, defined as microscopically (original magnification $\times 40$) visible stones, and the composition of these stones was analyzed using Fourier transform infrared spectroscopy (FTIR) (Thermo Scientific, Nicolet iN10, MA, USA).

Real-time quantitative polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from frozen liver samples and liver cells using TRIZOL reagent (Invitrogen, CA, USA), and reverse transcription was performed using a Super-Script III RT kit (Invitrogen). A qRT-PCR was conducted using SYBR qPCR mix (Invitrogen). Gene-specific primer sequences were as follows: forward primer: $5'\text{-CGCACA-TAAAGCTCGGGAAA-3'}$; reverse primer: $5'\text{-TTGCTT-GAGATGCCAGAGA-3'}$. β -actin was employed as the housekeeping gene. Amplification was performed using an ABI 7500HT Fast Real-Time PCR System (Applied Biosystem, CA, USA). Cholesterol 7 α -hydroxylase (CYP7A1) mRNA levels were quantified relative to β -actin.

Western blotting

Proteins were obtained from liver tissues and liver cells using radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China), while protein concentration was assayed using a bicinchoninic acid (BCA) protein assay kit (Beyotime). Proteins were separated using sodium salt-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After blocking, membranes were incubated overnight with primary antibodies at 4°C (anti-TNF- α [1:1000; Bioss]; anti-OPN [1:1000; Abcam]; anti-CYP7A1 antibodies [1:1000; Invitrogen]; anti- β -actin [1:2000; Abcam]). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit (1:10,000; Jackson, PA, USA) was used as the secondary antibody. Finally, membranes were visualized using enhanced chemiluminescence (Beyotime) and analyzed on Image J 1.8.0 (National Institutes of Health, Bethesda, MD, USA).

Histopathological and immunohistochemical analyses

Liver specimens were fixed, embedded in paraffin wax, and cut into $4\text{-}\mu\text{m}$ thick sections. Sample slides were incubated with anti-OPN-Ab (1:100; Abcam), followed by a 30-min incubation with HRP-labeled goat anti-rabbit secondary antibody (1:2000; Jackson). Specimens were examined using a Leica DM3000 microscope (Leica, Solms, Germany) at a $\times 10/40$ objective; images were acquired using a DEI-750 camera (Optronics Engineering, Jerusalem, Israel). Images of immunohistochemistry (IHC) stained slides were digitally scanned and captured as color

images. Image analysis was performed using Image-Pro Plus (version 6.0; Media Cybernetics, FL, USA).

Statistical analysis

All statistical analyses were performed using SPSS (version 18.0; SPSS Inc., Chicago, IL, USA). Qualitative data were presented as frequency, while quantitative data were reported as mean \pm standard deviation. Chi-square or Fisher exact tests, followed by *post hoc* analysis with Bonferroni adjustment for multiple comparisons, were used to analyze qualitative data. The Student's *t* test was used for two-group quantitative data. Quantitative data of multiple groups were compared using one-way analysis of variance, followed by *post hoc* analysis using the least significant difference test. Correlations were performed using Pearson test. A $P < 0.05$ was considered to be statistically significant.

Results

Biliary and hepatic expression of biochemical indices and pigment gallstone formation

Microscopic examination of the choledochus revealed gallstone as shown in Figure 1A. Eight of ten rats developed gallstones after 10 weeks of LD feeding, while penetrance of CD-fed rats was one of ten ($P < 0.05$). Furthermore, according to the results of gallstone FTIR spectroscopy and the standard infrared spectrum of bilirubin, gallstones collected from LD-fed rats were classified as brown gallstones, as they showed corresponding characteristic absorption bands [Figure 1B and 1C]. In pathological sections, as shown in Figure 2, blur of the hepatic plate-like structure, lymph node infiltration, and ballooning degeneration of hepatocytes were found in the liver tissue of LD-fed rats, while CD-fed rats presented with only mild liver cell degeneration around the central vein. Furthermore, LD-fed rats showed a significant increase in the number of macrophages, compared with CD-fed rats ($t = 9.72$, $P < 0.01$).

In addition, biliary and hepatic expression levels of biochemical indices in CD- and LD-fed rats were analyzed. LD-fed rats exhibited significant reductions in both BA and phospholipid levels, as well as increases in total bilirubin and unconjugated bilirubin levels, in both bile and liver

tissue samples, compared with CD-fed rats (data not shown). Importantly, these changing bile composition trends in the LD-induced pigment stone rat model were similar to those in patients with pigment stones.

Hepatic expression of OPN, TNF- α , and CYP7A1

Next, we investigated the expression of hepatic OPN, TNF- α , and CYP7A1 in brown gallstone formation. Results of the immunohistochemical examination indicated that OPN expression significantly increased in LD-fed rats compared with that in CD-fed rats ($t = 5.71$, $P < 0.01$) [Figure 3A and 3B]. Furthermore, as shown in Figure 3C and 3D, the results of the western blot showed a significant increase in OPN expression in the LD group compared with that in CD and baseline groups ($F = 121.54$, $P < 0.01$). In contrast, OPN expression between CD and baseline groups did not differ ($t = 0.48$, $P = 0.76$). Moreover, the results of the western blot showed that TNF- α expression significantly increased in LD-fed rats, compared with that in CD-fed rats ($t = 8.83$, $P < 0.01$) [Figure 4A and 4B]. However, as shown in Figure 4C and 4D, CYP7A1 protein and mRNA expression levels significantly decreased in LD-fed rats, compared with that in CD-fed rats ($t = -12.35$, $P < 0.01$). Furthermore, Pearson correlation analysis showed that OPN expression was positively associated with TNF- α ($r = 0.869$, $P < 0.01$) and negatively associated with CYP7A1 ($r = -0.899$, $P < 0.01$) expression. An obvious negative correlation was observed between TNF- α and CYP7A1 expression ($r = -0.903$, $P < 0.01$). These indications suggest that LD-fed rats show distinctive alterations in OPN, TNF- α , and CYP7A1 expression compared with CD-fed rats, providing insight into the mechanism behind diet-induced brown pigment gallstones.

Blocking test

To further verify the effects of OPN on pigment gallstone formation, a blocking test was conducted using OPN-Ab, with NS-IgG as the control. Results showed that the gallstone formation rate in the LD + OPN-Ab group was significantly lower than that in the LD group (25% [5 of 20] *vs.* 85% [17 of 20], $\chi^2 = 14.55$, $P < 0.01$) and the LD + NS-IgG group (25% [5 of 20] *vs.* 75% [15 of 20], $\chi^2 = 14.00$, $P < 0.01$). As shown in Figure 5A, the expression levels of OPN significantly increased in the

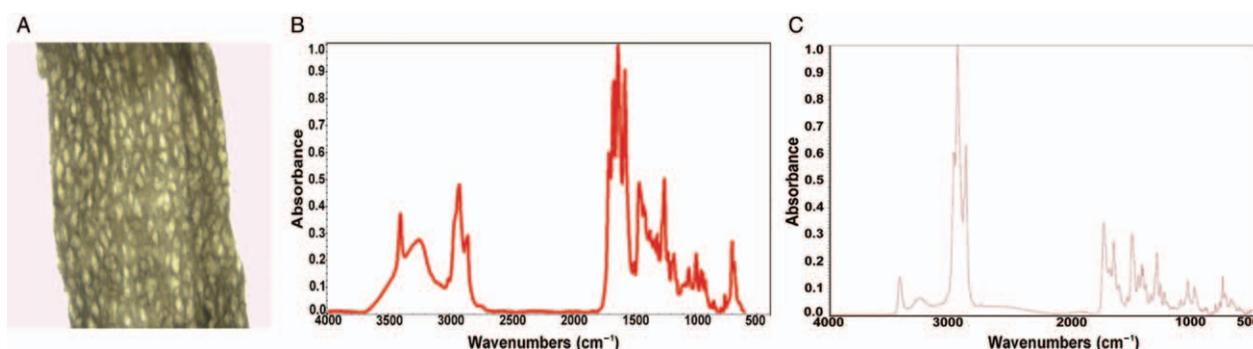


Figure 1: (A) Microscopic view of bile duct gallstones in LD-induced rats (original magnification $\times 40$). (B) The result of FTIR spectroscopy of gallstones collected from LD-fed rats. (C) The standard infrared spectrum of bilirubin. FTIR: Fourier transform infrared; LD: Lithogenic diet.

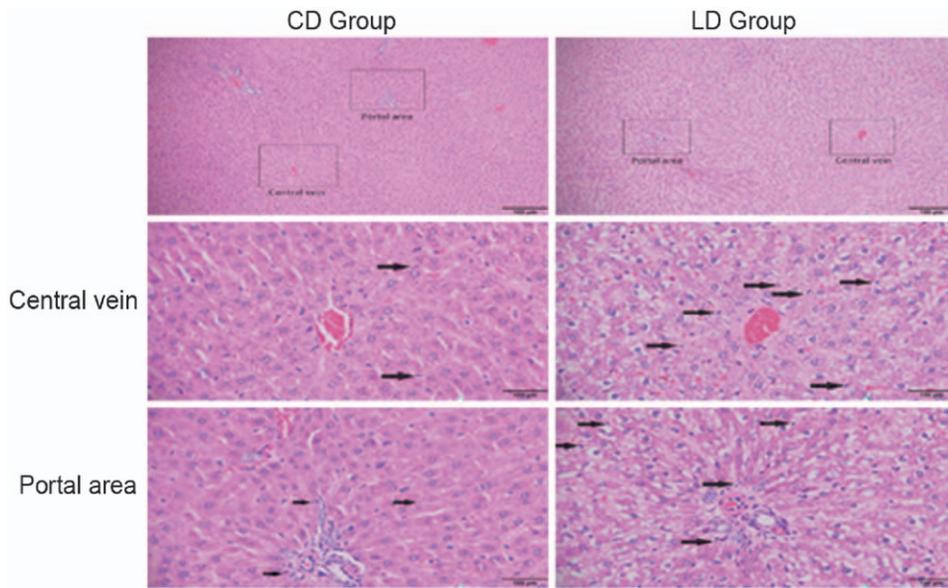


Figure 2: Histology examinations of liver tissue from CD- or LD-fed rats (original magnification $\times 100$), central vein area and portal area (original magnification, $\times 400$); black arrows point to macrophages.

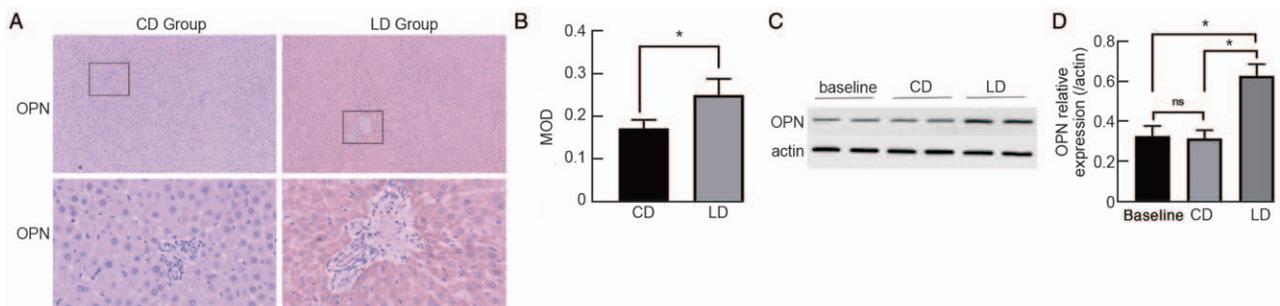


Figure 3: Expression of hepatic OPN in rats from baseline, CD, and LD groups. (A) Hepatic specimens shown are immunohistochemically stained for OPN (original magnification, $\times 100/400$). (B) OPN immunohistochemical MOD differed significantly between CD- and LD-fed rats. (C) Hepatic proteins isolated from baseline, CD, and LD group rats were analyzed using western blotting for OPN protein expression. β -actin was the control for gel loading. (D) Western blotting quantification for OPN. Relative average protein level was determined using densitometry. Data are expressed as the mean \pm SD ($n = 10$ per group). * $P < 0.05$. CD: Chow diet; LD: Lithogenic diet; MOD: Mean optical density; ns: No significance; OPN: Osteopontin; SD: Standard deviation.

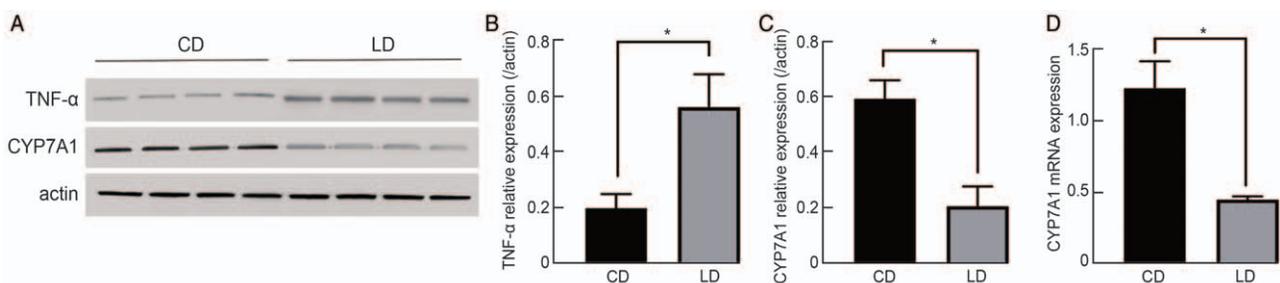


Figure 4: Expression of hepatic TNF- α and CYP7A1 in CD- and LD-fed rats. (A) Hepatic proteins isolated from CD- and LD-fed rats were analyzed using western blotting for TNF- α and CYP7A1 protein expression. β -actin was the control for gel loading. (B, C) Western blotting quantification for TNF- α and CYP7A1. Relative average protein level was determined using densitometry. (D) qRT-PCR analysis of hepatic CYP7A1 mRNA levels in the liver tissue of CD- and LD-fed rats. Data are expressed as the mean \pm SD ($n = 10$ per group). * $P < 0.05$. CD: Chow diet; CYP7A1: Cholesterol 7 α -hydroxylase; LD: Lithogenic diet; qRT-PCR: Real-time quantitative polymerase chain reaction; SD: Standard deviation; TNF- α : Tumor necrosis factor alpha.

LD, LD + OPN-Ab, and LD + NS-IgG groups, compared with those in the CD and baseline groups ($F = 48.24$, $P < 0.01$); however, OPN expression levels were not significantly different between LD, LD + OPN-Ab, and

LD + NS-IgG groups ($F = 0.03$, $P = 0.89$). OPN expression levels between CD and baseline groups were not significantly different ($t = 0.51$, $P = 0.62$), while TNF- α protein expression levels in the LD + OPN-Ab group were

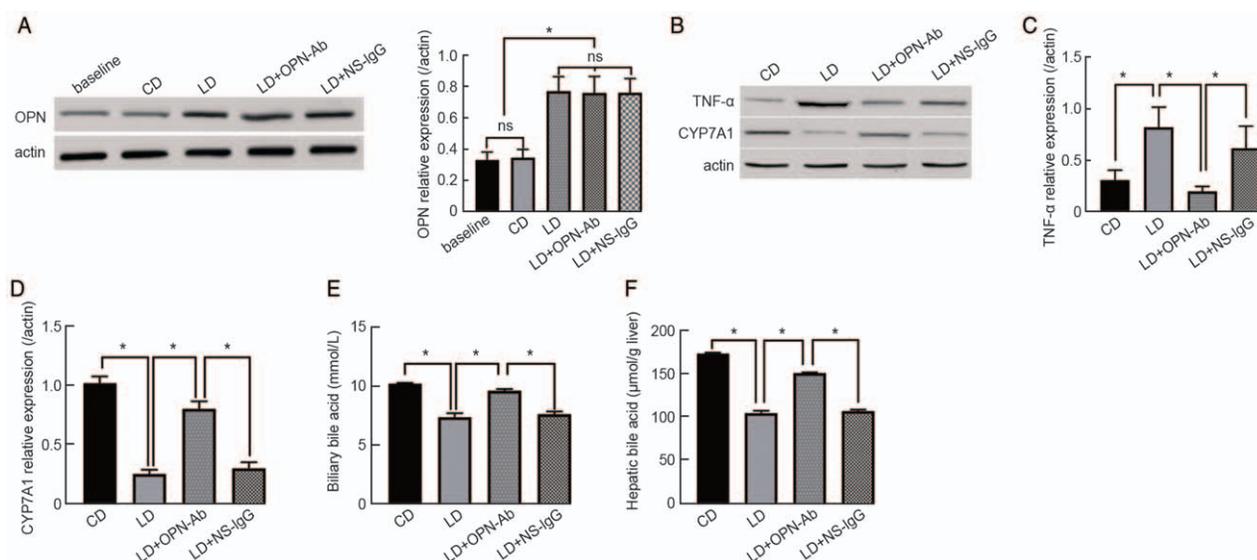


Figure 5: Blocking test with OPN-Ab in LD-fed rats. (A) Hepatic OPN proteins isolated from each experimental group of rats were analyzed using western blotting. β -actin was the control for gel loading. Relative average OPN protein level was determined using densitometry. (B) Hepatic TNF- α and CYP7A1 proteins isolated from each experimental group of rats were analyzed using western blotting. β -actin was the control for gel loading. (C and D) Relative average protein level was determined using densitometry. (E and F) BA content in bile and liver samples from each experimental group of rats. Data are expressed as the mean \pm SD ($n = 6$ per group). * $P < 0.05$. BA: Bile acid; CD: Chow diet; CYP7A1: Cholesterol 7 α -hydroxylase; LD: Lithogenic diet; ns: No significance; NS-IgG: Non-specific IgG; OPN: Osteopontin; OPN-Ab: OPN antibody; SD: Standard deviation; TNF- α : Tumor necrosis factor alpha.

significantly lower than in LD and LD + NS-IgG groups ($F = 20.36$, $P < 0.01$) [Figure 5B and 5C]. As shown in Figure 5B and 5D, CYP7A1 protein expression levels in the LD + OPN-Ab group were significantly higher than in LD and LD + NS-IgG groups ($F = 17.51$, $P < 0.01$). Similarly, as shown in Figure 5E and 5F, BA content in bile and liver tissue of the LD + OPN-Ab group was significantly higher than in LD and LD + NS-IgG groups ($F = 394.8$, $F = 3688$, $P < 0.01$). Blocking tests indicated that the gallstone formation rate decreased, the BA content in liver tissue and bile increased, hepatic TNF- α expression was down-regulated, and hepatic CYP7A1 expression was up-regulated. Therefore, increases in OPN expression were not considered the result, but rather the cause, of gallstone formation. Furthermore, the OPN-Ab was suggested to affect OPN function rather than OPN expression.^[15]

In vitro experimentation

To further verify the role of OPN in promoting macrophages to produce TNF- α , rrOPN and two OPN receptor blockers were used to treat rat liver macrophages cultured *in vitro*. The TNF- α content in cell supernatant was determined using an ELISA. TNF- α content differed between the groups ($F = 1041$, $P < 0.01$) [Figure 6A], with the OPN group showing a significant elevation compared with normal saline ($t = 56.59$, $P < 0.01$), the CWHM12 + OPN group showing a significant reduction compared with the OPN group ($t = -44.21$, $P < 0.01$), and the CD44-Ab + OPN group showing a significant reduction, relative to both OPN ($t = -23.63$, $P < 0.01$) and NS-IgG + OPN groups ($t = -18.40$, $P < 0.01$). The above results suggest that OPN induced rat liver macrophage to produce TNF- α , an effect suppressed by two types of receptor blockers, revealing that OPN promoted macrophage to produce TNF- α through both CD44 and integrin receptors.

To further examine whether TNF- α affected CYP7A1 expression, rrTNF- α was used to stimulate BRL cells, so as to observe CYP7A1 expression in liver cells. The results suggested that CYP7A1 mRNA expression decreased with increasing TNF- α concentration ($F = 101.5$, $P < 0.01$) [Figure 6B]. Compared with the 20 ng/mL group, CYP7A1 mRNA expression in the 50 ng/mL group significantly decreased ($t = -6.52$, $P < 0.01$); no significant differences were detected when compared with the 100 ng/mL group ($t = 0.24$, $P = 0.84$). As shown in Figure 6C, CYP7A1 mRNA expression also decreased with increasing TNF- α treatment time ($F = 48.08$, $P < 0.01$). Compared with the 3 h group, CYP7A1 mRNA expression in the 6 h group significantly declined ($t = -4.78$, $P < 0.01$); no significant differences were observed when compared with the 48 h group ($t = 1.92$, $P = 0.06$). The above results indicate that, to a certain range, TNF- α suppresses CYP7A1 mRNA expression in liver cells in a dose- and time-dependent manner.

To further explore whether TNF- α affects CYP7A1 protein expression, thus suppressing BA synthesis in liver cells, this study used 50 ng/mL of rrTNF- α to treat BRL cells for 6 h, so as to detect CYP7A1 protein expression in liver cells and BA content in liver cell supernatant. The results suggested that TNF- α significantly suppressed CYP7A1 protein expression ($F = 47.92$, $P < 0.01$) and BA synthesis ($F = 119.4$, $P < 0.01$) in liver cells [Figure 6E and 6F].

Discussion

Pigment gallstones, typical primary biliary duct stones, reportedly account for up to 10% to 20% of gallstones among patients in China.^[16,17] There are two subtypes of pigment gallstones, black and brown stones, showing

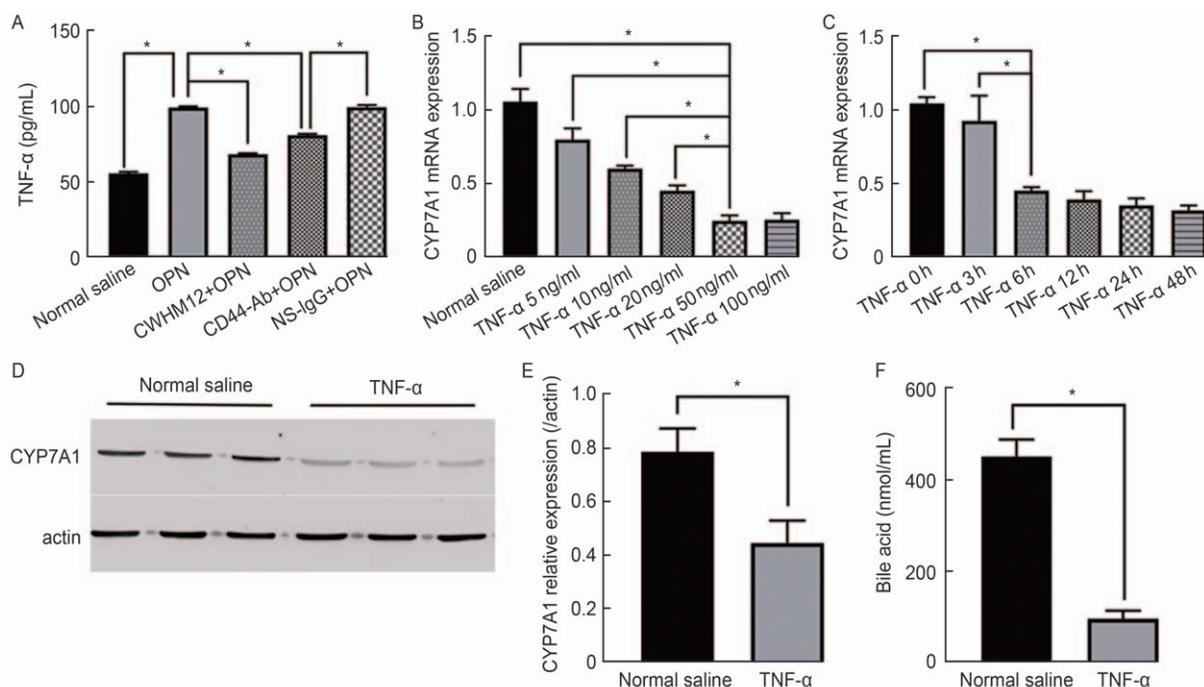


Figure 6: *In vitro* experimentation. (A) TNF- α content in rat macrophage culture supernatant determined using ELISA. (B, C) CYP7A1 mRNA expression in BRL cells was analyzed using qRT-PCR. (D, E) Proteins were isolated from BRL cells and analyzed using western blotting. β -actin was the control for gel loading. The relative average protein level was determined using densitometry. (F) BA content in BRL cell supernatants. Data are expressed as the mean \pm SD ($n = 3$ per group). * $P < 0.05$. BA: Bile acid; CD44-Ab: CD44 antibody; CYP7A1: Cholesterol 7 α -hydroxylase; ELISA: Enzyme-linked immunosorbent assay; ns: No significance; NS-IgG: Non-specific IgG; qRT-PCR: Real-time quantitative polymerase chain reaction; SD: Standard deviation; TNF- α : Tumor necrosis factor alpha.

different chemical compositions and pathogenesis.^[18,19] A previous study by Suzuki *et al*^[20] reported that a pigment gallstone rat model could be established through a low protein diet, resulting in incomplete obstruction of the terminal common bile duct. Another study indicated that the model could be established in mongrel dogs via injection of β -glucuronidase-producing *Escherichia coli* into the spleen, or by narrowing the bile duct with ligature after a 7-day post-operative interval.^[21] Furthermore, hypodermic injection of lincomycin also reportedly induces pigment gallstone formation in guinea pigs.^[22] In our study, we established a brown pigment stone rat model which we induced through a modified LD, with protein content (12%) far lower than in the CD (21%). In our model, the gallstone formation rate was 70% to 85%, similar to that reported by Kong *et al*^[14] (68.24%). Low protein diets have been confirmed to cause malnutrition in rats, leading to abnormal liver metabolism and gallstone formation.^[20,23] Additionally, content alteration of biliary and liver components is closely related to liver metabolism in pigment gallstone progression and has not been previously examined. Therefore, in this study, we focused on the content alteration of BA, phosphatidylcholine (PC), total cholesterol, total bilirubin, and conjugated and unconjugated bilirubin in the bile and liver samples of LD-induced brown pigment gallstone rats. In addition, as pigment gallstones commonly form in the intrahepatic bile duct, and with OPN have been proven closely involved in hepatolithiasis, we also examined OPN expression. Comprehensive assays of bile content alteration and OPN expression provided some insight into the mechanisms involved in brown pigment gallstone progression.

BA, one of the main active components of bile, plays an important role in the metabolism of fat.^[24] PC, composed of glycerol, fatty acid, phosphoryl group, and choline, can effectively promote the exportation of fat in the form of phospholipids, improve fatty acid utilization, and prevent abnormal accumulation of fat, all in the liver.^[25] In our study, a significant decrease in BA and phospholipid content in both bile and liver samples was observed in LD-fed rats, compared with CD-fed rats, implying weakening of fat metabolism capacity and the scavenging ability of abnormal fat accumulation.

Generally, cholesterol is held in bile through the formation of mixed micelles, where bile salts and phospholipids are involved in its expulsion into the intestine, resulting in balanced biliary homeostasis.^[26] In our study, a significant increase in hepatic cholesterol was observed in LD-fed rats, likely due to a weakened capacity to convert cholesterol into BA in the liver; thus finally resulting in an abnormal accumulation of cholesterol and a content decrease of BA. A prerequisite for pigment gallstone formation is increased unconjugated bilirubin and calcium ion content.^[27-29] Our results showed that unconjugated bilirubin was significantly increased in the bile and liver of LD-fed rats. A previous study suggested that lower BA content results in lower solubility of unconjugated bilirubin, promoting its precipitation.^[30-32] Simply, decreased BA content induces the recruitment of unconjugated bilirubin, thereby contributing to brown pigment gallstone formation.

OPN is a glycosylated phosphoprotein found in both tissue and body fluids.^[33] A previous study demonstrated that

OPN was closely related to hepatolithiasis, due to its high expression in the epithelium of stone-laden intrahepatic bile ducts.^[10,34] In our study, results from quantitative IHC and western blot also suggested that hepatic OPN expression was significantly higher in LD-fed rats, implying its great importance in pigment gallstone formation.

Reportedly, OPN is a potent macrophage chemotactic stimulant in macrophage recruitment, resulting in inflammatory cytokine production.^[35] In our study, LD-fed rats presented a blur of hepatic plate-like structures, lymph node infiltration, and ballooning hepatocyte degeneration in liver tissues, suggesting a multitude of infiltrating inflammatory cells, such as lymphocytes and macrophages, and increased inflammatory cytokine production, such as TNF- α . Indeed, TNF- α expression was significantly upregulated in LD-fed rats, compared with CD-fed rats. Obviously, inflammatory reactions emerge as brown pigment stone formation progresses. Correlation analysis, together with the above findings, indicates that OPN and TNF- α expressions display significantly positive correlations. Thus, it was reasonable to speculate that OPN exerts a positive effect on TNF- α production. In our study, after blocking OPN function, hepatic TNF- α was significantly down-regulated in LD-fed rats. *In vitro*, rrOPN significantly increased TNF- α expression in macrophages, while both CD44 and integrin receptors regulated TNF- α expression. A previous study also suggested that increased OPN expression in virus-induced lung injury is related to up-regulated TNF- α and interleukin-6 expression.^[36]

CYP7A1, a rate-limiting enzyme, plays an important role in regulating the BA biosynthesis pathway, which converts cholesterol into BAs in the liver.^[37,38] In our study, CYP7A1 mRNA and protein levels markedly decreased in LD-fed rats. Lacking sufficient CYP7A1 likely hindered the conversion of cholesterol into BA, partially explaining why cholesterol accumulation increased, and BA content decreased, in LD-fed rats; further contributing to the destruction of biliary homeostasis. In addition, our correlation assays revealed that both OPN and TNF- α expressions as significantly and negatively associated with CYP7A1. Previous studies demonstrated that TNF- α down-regulated CYP7A1 gene expression through the c-Jun N-terminal kinase signal transduction pathway.^[39,40] In our study, after blocking OPN function, hepatic CYP7A1 was significantly down-regulated in LD-fed rats. Similarly, experiments performed *in vitro* indicated that TNF- α suppressed CYP7A1 mRNA and protein expression in liver cells, and reduced BA synthesis in liver cells. Thus, it was reasonable to speculate that OPN likely inhibited CYP7A1 expression via up-regulation of TNF- α .

Alteration of biliary and hepatic components, as well as metabolic changes, present in the pigment gallstone rat model had high similarity to alterations observed in gallstone patients. Therefore, the LD-induced rat model can be used for the study of brown pigment gallstones. Furthermore, we speculate that, in brown gallstone progression, OPN not only triggers TNF- α production but also induces the metabolic disorder of biliary and hepatic components through inhibition of CYP7A1 expression via TNF- α . In the near future, further studies

investigating the genetic regulatory mechanisms and signaling pathways involved in this process should be conducted.

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Conflicts of interest

None.

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