Journal of Gastroenterology and Hepatology

JGHF

doi:10.1111/j.1440-1746.2012.07227.x

GASTROENTEROLOGY

N-3 polyunsaturated fatty acid attenuates cholesterol gallstones by suppressing mucin production with a high cholesterol diet in mice

Ja Kyung Kim,*[,1] Soo Min Cho,[†,1] So Hee Kang,[‡] Eunjung Kim,[§] Hee Yi,[†] Eun Sun Yun,[†] Dong Goo Lee,[†] Hee Jung Cho,[†] Yong Han Paik,* Yang Kyu Choi,[¶] Seung Joo Haam,[§] Ho Chul Shin[†] and Dong Ki Lee*

Department of *Internal Medicine, [‡]Medical Research Center, Gangnam Severance Hospital, Yonsei University College of Medicine, Department of [§]Chemical and Biomolecular Engineering, Yonsei University, and Departments of [†]Veterinary Pharmacology and Toxicology and [¶]Laboratory Animal Medicine, College of Veterinary Medicine, KonKuk University, Seoul, Korea

Key words
cholesterol gallstone, high cholesterol diet, mucin, phopholipid, n-3 polyunsaturated fatty acid.

Accepted for publication 16 May 2012.

Correspondence
Mr Dong Ki Lee, Department of Internal Medicine, Gangnam Severance Hospital, Yonsei University College of Medicine, 712 Eonjuro Gangnam-gu, Seoul 135-270, Korea.
Email: dklee@yuhs.ac

[1]These authors contributed equally and share first authorship.

Abstract

Background and Aim: The increasing prevalence of cholesterol gallstone (CG) disease has become an economic burden to the healthcare system. Ursodeoxycholic acid (UDCA) is the only established medical agent used to dissolve gallstones. In investigating novel therapeutics for CG, we assessed the preventive effects of n-3 polyunsaturated fatty acids (n-3PUFA) on the formation of CG induced by feeding a lithogenic diet (LD) containing high cholesterol levels to mice.

Methods: Mice were divided into the following six groups: (A) regular diet (RD); (B) RD+n-3PUFA; (C) LD; (D) LD+n-3PUFA; (E) LD+UDCA; (F) LD+n-3PUFA+UDCA. After RD/LD feeding for 2 weeks, n-3PUFA or UDCA was administered orally and the diet maintained for 8 weeks. The levels of phospholipids and cholesterol in bile, CG formation, gallbladder wall thickness, MUC gene expression in gallbladder were analyzed.

Results: No stone or sludge was evident in the RD groups (Groups A, B). Mice in the n-3PUFA treatment (Groups D, F) showed significantly lower stone formation than the other LD groups (Groups C, E). The combination treatment of n-3PUFA and UDCA suppressed stone formation more than mono-therapy with n-3PUFA or UDCA. Bile phospholipid levels were significantly elevated in the Group F. Hypertrophy of the gallbladder wall was evident in mice fed LD. MUC 2, 5AC, 5B and 6 mRNA expression levels were significantly elevated in the LD-fed group, and this was suppressed by n-3PUFA with or without UDCA.

Conclusions: N-3PUFA attenuated gallstone formation in mouse, through increasing the levels of bile phospholipids and suppressing bile mucin formation.

Introduction

Cholesterol gallstone (CG) disease begins in the liver and is associated with an increased production of abnormal bile with excess cholesterol relative to bile salts and phospholipids. In developed countries, CGs are one of the most prevalent and most costly digestive diseases. The Western diet consists of a high level of total calories, cholesterol, saturated fatty acids, refined carbohydrates, proteins, and low fiber and is known to be related to cholesterol cholelithiasis.[1,2] Westernization of the Asian diet has resulted in an increased incidence of CGs.[3]

In Western diets, the ratio of n-6 to n-3 polyunsaturated fatty acids (PUFAs) ranges from 15–16:1 compared with the healthy range of 1–4:1.[4] Although a high intake of n-6 PUFAs shifts the physiological state to one that is prothrombotic, n-3 PUFAs display hypolipidemic properties[5,6] and, as such, negatively influences hepatic lipogenesis.[7] Additionally, n-3 PUFAs have been reported to display both anti-inflammatory and antithrombotic properties. Because precipitation of cholesterol from supersaturated bile is the key stage in gallstone formation,[8] we assumed that dietary fat modification could affect gallstone formation through changes in the composition of bile.

Ursodeoxycholic acid (UDCA) is the only currently established medical treatment for CG.[9] Here, we investigated the effects of n-3 PUFAs on CG formation and the hepatobiliary system, including the composition of bile and the expression of mucins (common abnormalities in gallstone patients) in a mouse model, to assess the potential use of n-3 PUFAs as an alternative treatment for CG disease.

Methods

Animals and diets. C57BL/6J (male, 12 weeks old) mice were purchased from the Central Lab. Animal (Seoul, Korea). Mice were divided into the following six groups (14 mice in each group): (i) regular diet (RD); (ii) RD+n-3 PUFA; (iii) lithogenic diet (LD); (iv) LD+n-3 PUFA; (v) LD+UDCA; (vi) LD+n-3 PUFA+UDCA. After 4 weeks of acclimatization and 2 weeks of adaptation to LD/RD, n-3 PUFA with or without UDCA was given for 8 weeks maintaining LD/RD. The LD (DYET#102136, Dyets, Bethlehem, PA, USA) consisted of 15% anhydrous Milkfat, 2.0% corn oil, 1.0% cholesterol, 0.5% cholic acid, and contained 4379.70 kcal/kg. The RD (Mouse2, Samyang Oil and Feed, Seoul, Korea) consisted of 6.33% of crude fat, and contained 3470 kcal/kg. N-3 PUFA (70 mg/kg per day, Omacor, Pronova Biocare, Sandefjord, Norway) and UDCA (20 mg/kg per day, Ursa, Daewoong Pharm., Seoul, Korea) and was diluted in 0.75% Tween-80 and administered orally by sonde for 8 weeks. Serum and bile were collected and stored at −80°C prior to use. The liver and gallbladder were separated and divided into two samples, one of which was frozen in liquid nitrogen and the other fixed in 10% formalin. The experimental protocol was reviewed by the ethical committee (IACUC No. KU09049).

Macroscopic and microscopic studies of the gallbladder and gallstones.

Stone formation assessment. The gallstone formation was assessed into a 6-score system: 0 = clear bile; 1 = little sludge; 2 = wide-spread sludge; 3 = large levels of sludge; 4 = a few small formed stones; 5 = several formed stones; 6 = full of formed stones. Average scores of each group were compared.

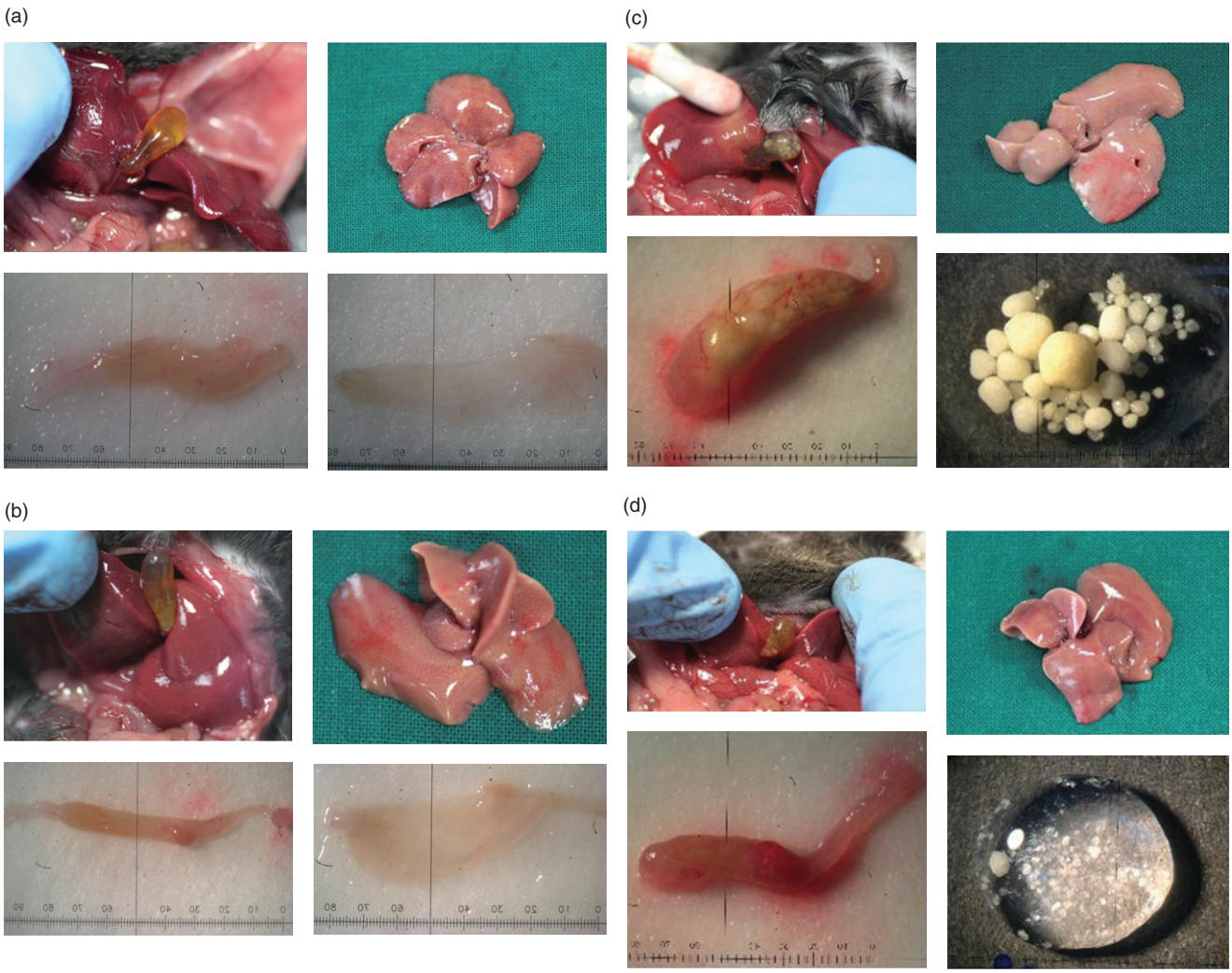

Figure 1 Gross findings of liver, gallbladder and gallstones (a–f) and grading of gallstone formation (g). (a) regular diet (RD); (b) RD+n-3 polyunsaturated fatty acids (n-3 PUFA); (c) lithogenic diet (LD); (d) LD+n-3 PUFA; (e) LD+ursodeoxycholic acid (UDCA); (f) LD+n-3 PUFA+UDCA. Mice fed with n-3 PUFA displayed significantly fewer gallstones. (g) Lithogenic diet induced significant grades of gallstones while n-3 PUFA administration significantly lowered gallstone formation**;** 0 = clear bile; 1 = little sludge; 2 = wide-spread sludge; 3 = large levels of sludge; 4 = a few small formed stones; 5 = several formed stones; 6 = full of formed stones.

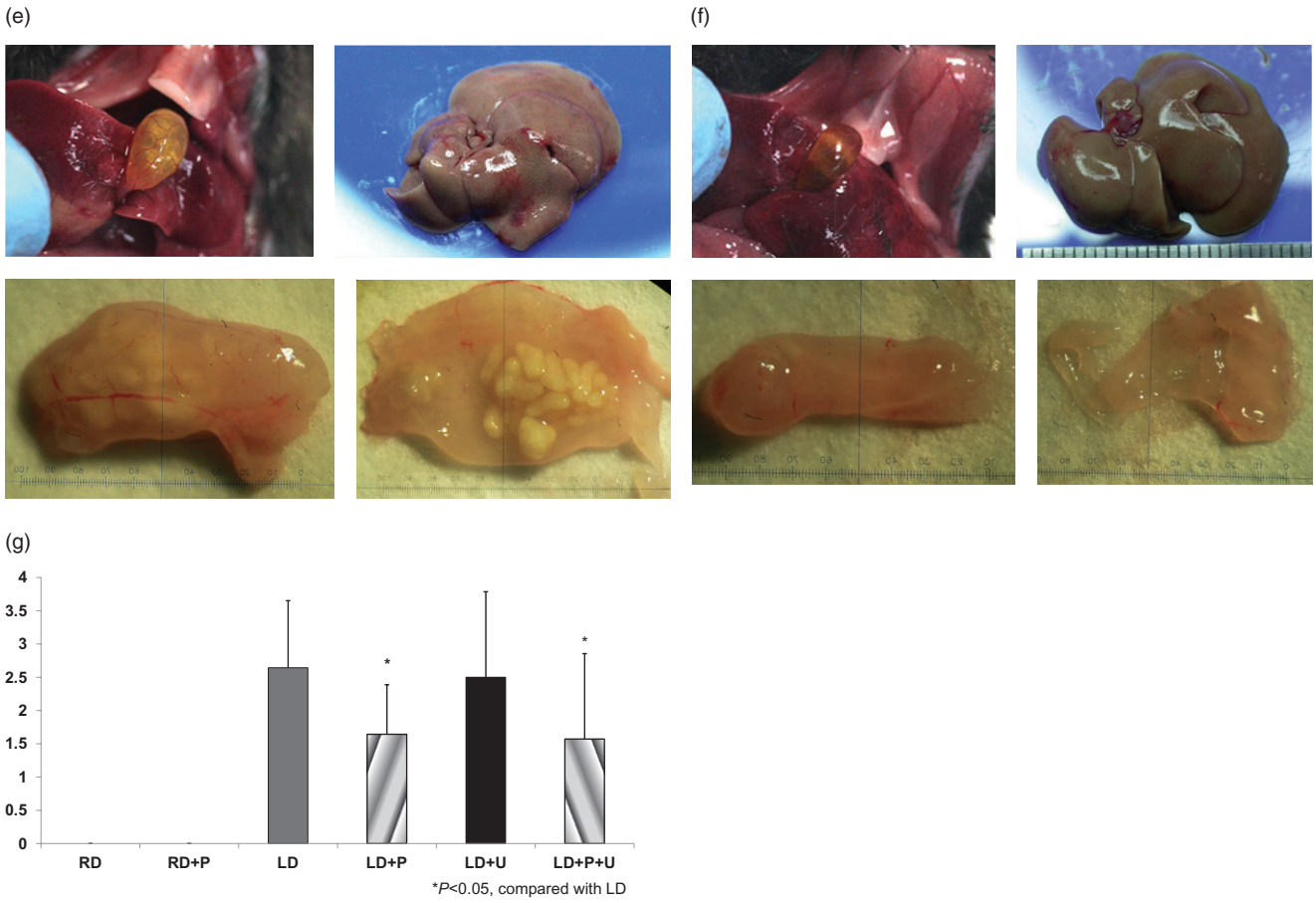

Figure 1 *Continued*

Hematoxylin and eosin staining. Fixed tissue was processed routinely for paraffin sections. Gallbladder tissues were fixed in 10% formalin for 2 days and sliced into six strips. All tissue strips were embedded in paraffin and cut into 4-μm sections. Sections were deparaffinized, rehydrated and stained with hematoxylin and eosin (H&E). Stained sections were dehydrated, cleaned, mounted, and subsequently examined by light microscopy.

Quantification of total bile phospholipids and cholesterol. Total phospholipid and cholesterol levels in bile were determined via a colorimetric assay (BioAssay Systems, Hayward, CA, USA). Briefly, bile samples were diluted 10-fold and treated with working reagent. The absorbance was measured using a microplate spectrometer (Epoch, BioTek, Winooski, VT, USA) at 570 nm and 340 nm for the quantification of phospholipids and cholesterol, respectively.

Quantitative real-time polymerase chain reaction assay for MUC gene. From gallbladder tissue, total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and treated with RNase-free DNase (Promega, Madison, WI, USA) for 30 min at 37°C. The RNA was then cleaned using an RNeasy kit (Qiagen, Germantown, MD, USA). A reverse transcription reaction was performed using a commercially available High Capacity cDNA Archive Kit (Applied Biosystems, Carlsbad, CA, USA). MUC 2, MUC 5ac, MUC 5b and MUC 6 mRNA were quantitated using TaqMan polymerase chain reaction (PCR) with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an mRNA internal control. The PCR reaction and analysis were performed using the Applied Biosystems 7300 software (Applied Biosystems). The relative abundance of the target genes was obtained using the comparative threshold cycle (C_t) method. All PCR primers and fluorogenic probes were purchased from Applied Biosystems: Muc2 (Mm00458299_m1), Muc5ac (Mm01276725_g1), Muc5b (Mm00466346_m1), Muc6 (Mm00725165_m1), and GAPDH (Mm00725165_m1).

Statistical analyses. All data are expressed as means ± standard error (SE). Statistically significant differences among the groups were assessed using Student's *t*-test, or the Mann-Whitney *U*-test. Statistical significance was defined as a *P*-value ≤ 0.05. Analyses were performed using the SPSS (18.0) software.

Results

Effects of n-3 PUFAs on gallstone formation, liver and body weight. Figure 1a–f demonstrates that following LD, the livers showed a hypertrophic and yellow color change. The

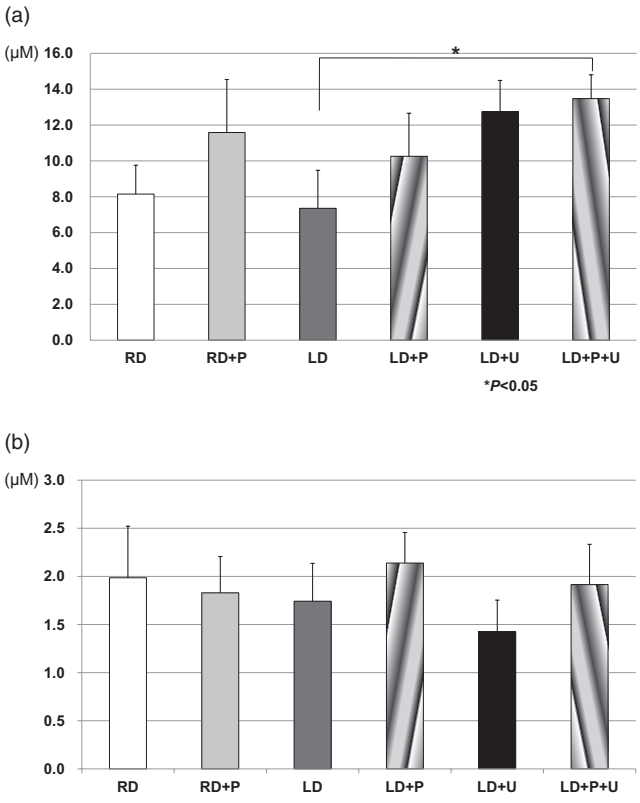

Figure 2 Biliary phospholipids (a) and cholesterol (b) concentrations of individual gallbladder bile. N-3 polyunsaturated fatty acids (n-3 PUFA) and ursodeoxycholic acid (UDCA) administration significantly increased phospholipids in gallbladder bile.

liver to body weight ratio and serum cholesterol levels were higher in the LD groups (Groups C–F) than the RD groups (Groups A, B). Among the LD groups, the liver to body weight ratios in LD+UDCA and LD+n-3 PUFA+UDCA groups were significantly lower than those in the LD and LD+n-3 PUFA groups.

Both stones and sludge were not evident in the RD groups (Groups A, B). Among the LD groups, the mean CG formation score was 2.64, 1.64, 2.5 and 1.57 in LD, LD+n-3 PUFA, LD+UDCA and LD+n-3 PUFA+UDCA groups, respectively (Fig. 1g). Mice receiving n-3 PUFA treatment (Groups D, F) showed significantly lower stone formation than the other LD groups (Groups C, E). Despite the combination treatment of n-3 PUFA and UDCA suppressing stone formation to a greater level than monotherapy with n-3 PUFA or UDCA, no statistically significance difference was evident. When the mass of dried gallstones was analyzed in the individual groups, the dried stone mass in the LD group was the heaviest. The dried stone mass was markedly reduced in the groups receiving n-3 PUFA±UDCA administration (data not shown).

Effects of omega-3 PUFA on biliary cholesterol composition. Figure 2 demonstrates that biliary phospholipid levels increased in the n-3 PUFA administrated group regardless of the diet. Compared with the LD group, the bile phospholipid levels in the LD+n-3 PUFA+UDCA group displayed a significant increase ($P = 0.02$). The biliary cholesterol levels did not change among the groups.

Effect of omega-3 PUFA on gallbladder mucosa. Figure 1 demonstrates that under LD feeding conditions, the transparency of the gallbladder decreased compared with the RD feeding group. In the LD group, the mucosal epithelium of the gallbladder showed hypertrophic changes and the height of gallbladder wall increased compared with the RD group (Fig. 3). Treatment of n-3 PUFA with or without UDCA restored the mucosal change under LD into a transparent and slim gall bladder wall (Figs. 1, 3).

Effects of omega-3 PUFA on MUC gene expression of the gallbladder. As shown in Figure 4, the data are expressed relative to the mRNA levels of the mucins in mice, fed LD and receiving a RD without n-3 PUFA. Treatment with n-3 PUFA (70 mg/kg per day) resulted in a significantly decreased expression of MUC2, MUC5AC, MUC5B, and MUC6 genes in the gallbladder of mice in the LD group. It seems likely that these changes result in a decreased viscosity of bile, which attenuated the formation of CG.

Discussion

The present study demonstrates that n-3 PUFA in the presence or absence of UDCA prevents gallstone formation in a lithogenic state in the presence of a hypercholesterol diet. The ability of dietary n-3 PUFA to protect against CG formation has been suggested from observations where dramatic increase in the complications of cholesterol cholelithiasis have been observed in Canadian Eskimos, accompanying the shift from a predominantly fish-based diet to a Westernized-type diet.[10] This epidemiological observation is supported by evidence in human studies, where healthy subjects fed n-3 PUFA supplements (1.5 g n-3 PUFA per day over 6 weeks) showed a decrease in their biliary cholesterol saturation index (CSI) and percentage concentration of cholesterol in bile, with no change in phospholipids or bile acids.[11] In animal models, African green monkeys[12] and prairie dogs[13] have been used to demonstrate the anti-lithogenic effects of fish oil. However, n-3 PUFA effects, such as increase in biliary phospholipids and suppression of MUC gene expression even with LD, had not been documented in an experimental mouse model. As n-3 PUFA in fish oil displays very few adverse effects, it is readily applicable to human studies.

Adding UDCA is of concern. Until now, UDCA is the only proven medical treatment for the dissolution of CGs,[14] that is also effective in the prevention of gallstone formation and recurrence.[14] UDCA therapy strongly decreases α1-acid glycoprotein, haptoglobin, immunoglobulin (Ig) A, IgG, γ-glutamyl transpeptidase, and aminopeptidases N protein levels, and nucleation-promoting activity in human gallbladder bile.[15] However, mucin, IgM and β-glucuronidase are not significantly decreased following UDCA treatment.

In this study, the combination of n-3 PUFA and UDCA resulted in a significant increase in bile phospholipids and a decrease in the

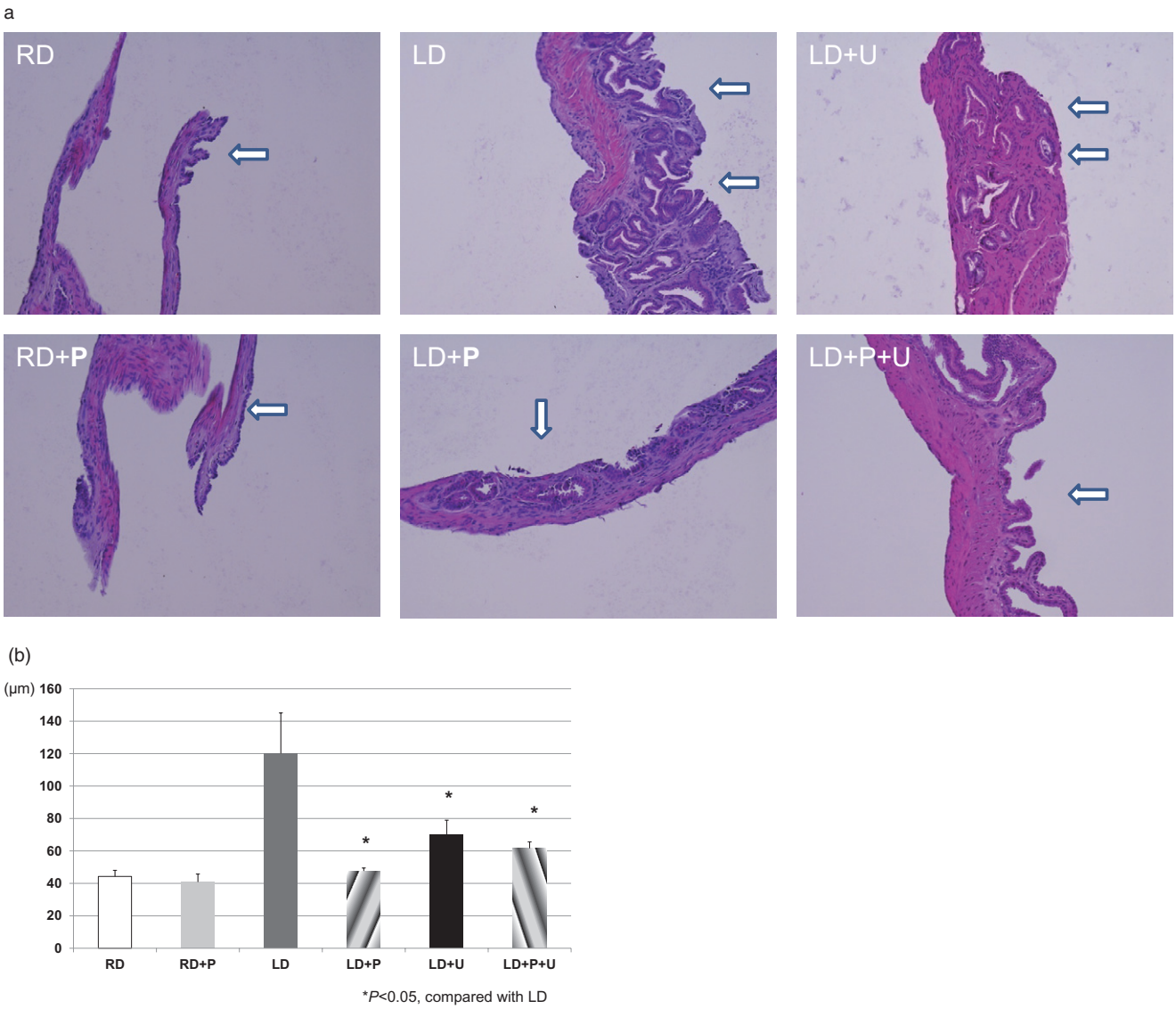

Figure 3 Histological mucosal changes (a) and total wall thickness (b) of the gallbladder. A lithogenic diet induced mucosal hypertrophy while n-3 polyunsaturated fatty acids (n-3 PUFAs) with or without ursodeoxycholic acid (UDCA) administration displayed lower levels of hypertrophied gallbladder wall.

total dried stone weight. Although synergistic preventative effects on stone formation were not proven, a beneficial additive effect may be expected with a longer duration of treatment or in a human clinical setting.

Ezetimibe, a potent cholesterol absorption inhibitor is an agent reported to reduce biliary cholesterol content, presents a promising strategy for the prevention and treatment of CGs.[16] The differential mechanisms of action of n-3 PUFA, UDCA and cholesterol absorption inhibitors suggest that combination treatments may prove effective in the prevention of CG formation.

Regarding the mechanism of action n-3 PUFA, we are particularly interested in the change of mucin as a one of pro-nucleating factors, promoting cholesterol crystallization and acting as a matrix for stone growth. Hypersecretion of gallbladder mucins is a prerequisite for gallstone formation, and is consistently observed in the gallbladder bile of several animal models.[17,18] Our results showed that n-3 PUFA significantly inhibited mucin gene expression in the gallbladder. Although mucin overproduction is important in the pathogenesis of gallstones, the mechanisms triggering mucin production during gallstone formation are still unclear.[8] We suggest that the increased bile phospholipid levels following n-3 PUFA administration, lowers CSI and thus decreases the chemical irritation to the mucosa of the gallbladder. This may result in a decreased expression of the MUC gene and lower mucin formation, promoting nucleation in bile under LD.

Limitations of this study include a lack of data for bile acid, biliary proteins, including mucin, and CSI. The levels of collected bile were low and, as such, measurement of these parameters was not possible. We confirmed the prevention of cholesterol gallstones in situations of increased bile CSI; however, we did not observe

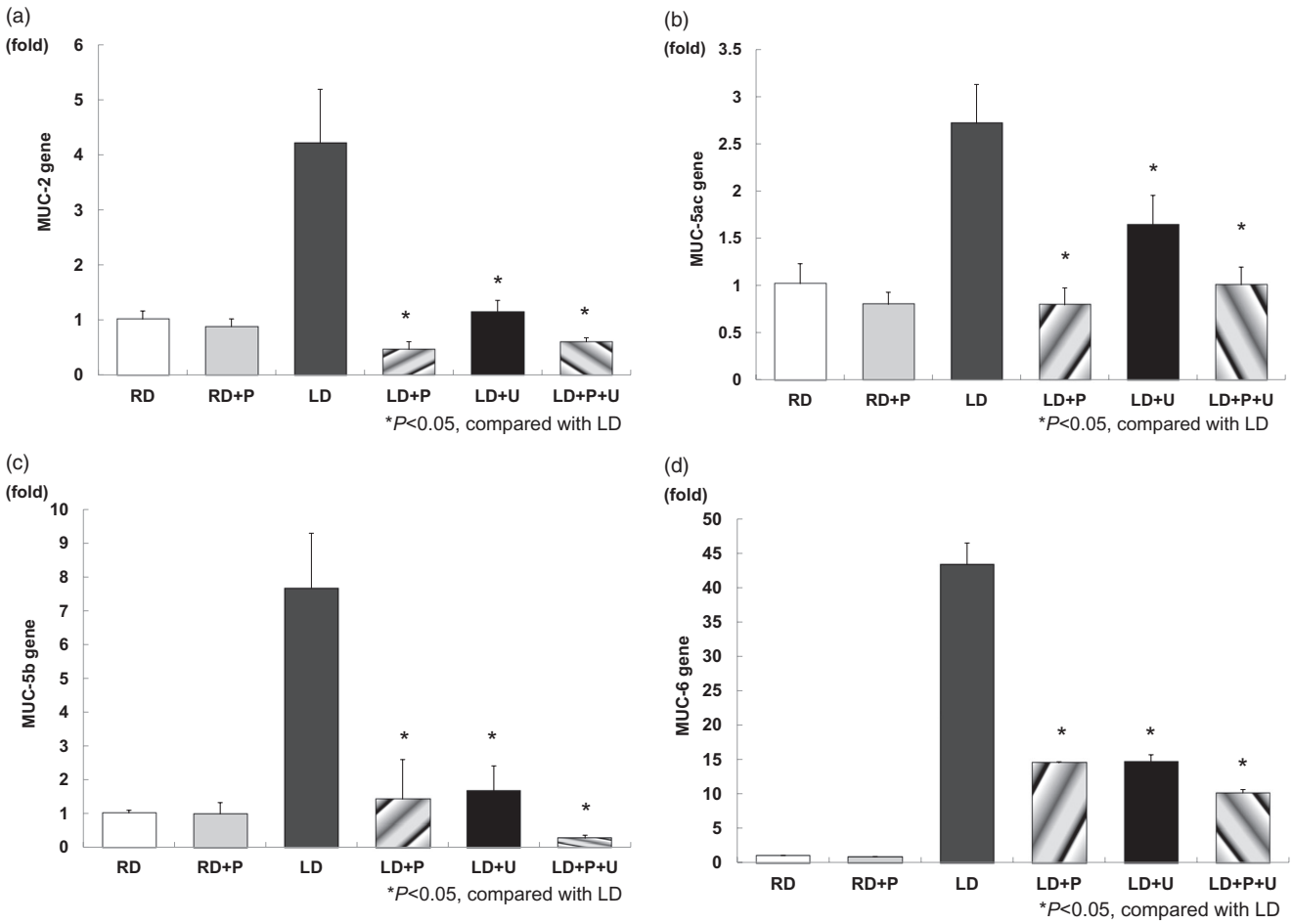

Figure 4 MUC gene expression in the gallbladder. (a) MUC-2; (b) MUC-5AC; (c) MUC-5B; (d) MUC-6. N-3 PUFAs with or without ursodeoxycholic acid (UDCA) administration significantly inhibited multiple MUC gene expression even in the gallbladder with lithogenic diet.

treatment effects of n-3 PUFA. Further clinical studies in situations of gallstone formation, including rapid weight loss, are required.

In conclusion, n-3 PUFA attenuated gallstone formation in a mouse model through increasing bile phospholipid levels and suppressing bile mucin formation. Further clinical studies are now required to assess the effects of n-3 PUFA as a medical treatment for CG disease.

Acknowledgments

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (No. 2009-0071473); and a faculty research grant of Yonsei University College of Medicine (6-2007-0115) .

References

1 Diehl AK. Epidemiology and natural history of gallstone disease. *Gastroenterol. Clin. North Am.* 1991; **20**: 1–19.
2 Mendez-Sanchez N, Zamora-Valdes D, Chavez-Tapia NC, Uribe M. Role of diet in cholesterol gallstone formation. *Clin. Chim. Acta* 2007; **376**: 1–8.
3 Huang YQ, Gu C. [The changing pattern of cholelithiasis in Tianjin]. *Zhonghua Wai Ke Za Zhi* 1987; **25**: 346, 381.
4 Simopoulos AP. Importance of the ratio of omega-6/omega-3 essential fatty acids: evolutionary aspects. *World Rev. Nutr. Diet.* 2003; **92**: 1–22.
5 Simopoulos AP. The importance of the ratio of omega-6/omega-3 essential fatty acids. *Biomed. Pharmacother.* 2002; **56**: 365–79.
6 Simopoulos AP. Essential fatty acids in health and chronic diseases. *Forum Nutr.* 2003; **56**: 67–70.
7 Sekiya M, Yahagi N, Matsuzaka T *et al*. Polyunsaturated fatty acids ameliorate hepatic steatosis in obese mice by SREBP-1 suppression. *Hepatology* 2003; **38**: 1529–39.
8 Wang HH, Portincasa P, Wang DQ. Molecular pathophysiology and physical chemistry of cholesterol gallstones. *Front. Biosci.* 2008; **13**: 401–23.
9 Podda M, Zuin M, Carulli N, Ponz de Leon M, Dioguardi ML. Gallstone dissolution after 6 months of ursodeoxycholic acid (UDCA): effectiveness of different doses. *J. Int. Med. Res.* 1982; **10**: 59–63.
10 Schaefer O. When the Eskimo comes to town. *Nutr. Today* 1971; **6**: 8–16.
11 Wechsler JG, Swobodnik W, Wenzel H, Saal D, Janowitz P, Ditschuneit H. [Effect of omega-3-fatty acids on biliary lipids and lithogenicity]. *Z. Gastroenterol.* 1989; **27**: 254–7.

12 Scobey MW, Johnson FL, Parks JS, Rudel LL. Dietary fish oil effects on biliary lipid secretion and cholesterol gallstone formation in the African green monkey. *Hepatology* 1991; **14**: 679–84.
13 Magnuson TH, Lillemoe KD, High RC, Pitt HA. Dietary fish oil inhibits cholesterol monohydrate crystal nucleation and gallstone formation in the prairie dog. *Surgery* 1995; **118**: 517–23.
14 Villanova N, Bazzoli F, Taroni F *et al*. Gallstone recurrence after successful oral bile acid treatment. A 12-year follow-up study and evaluation of long-term postdissolution treatment. *Gastroenterology* 1989; **97**: 726–31.
15 Van Erpecum KJ, Portincasa P, Eckhardt E, Go PM, VanBerge-Henegouwen GP, Groen AK. Ursodeoxycholic acid reduces protein levels and nucleation-promoting activity in human gallbladder bile. *Gastroenterology* 1996; **110**: 1225–37.
16 Wang HH, Portincasa P, Mendez-Sanchez N, Uribe M, Wang DQ. Effect of ezetimibe on the prevention and dissolution of cholesterol gallstones. *Gastroenterology* 2008; **134**: 2101–10.
17 Wang DQ, Paigen B, Carey MC. Phenotypic characterization of Lith genes that determine susceptibility to cholesterol cholelithiasis in inbred mice: physical-chemistry of gallbladder bile. *J. Lipid Res.* 1997; **38**: 1395–411.
18 Lee SP, LaMont JT, Carey MC. Role of gallbladder mucus hypersecretion in the evolution of cholesterol gallstones. *J. Clin. Invest.* 1981; **67**: 1712–23.