

# Effect of eicosapentaenoic acid on cholesterol gallstone formation in C57BL/6J mice

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**Abstract.** The present study investigated the preventive effect of  $\omega$ -3 fatty acids against cholesterol gallstone (CG) formation. CG formation was induced in C57BL/6J mice using a lithogenic diet (LD). The mice were divided into four treatment groups: i) LD, ii) LD plus eicosapentaenoic acid (EPA), iii) LD plus docosahexaenoic acid (DHA) and iv) LD plus EPA plus DHA. Subsequent to feeding the mice the LD for four weeks, EPA and/or DHA (70 mg/kg/day) were orally administered for eight weeks. The mice in the EPA treatment groups exhibited significantly less gallstone formation than those in the LD group. By contrast, DHA treatment only slightly suppressed gallstone formation. The expression of mucin 2, 5AC, 5B and 6 was significantly decreased in the gallbladders of mice in the EPA groups (70-90%) and the LD plus DHA group (30-50%), compared with that in the mice in the LD group. In addition, the mRNA expression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase was significantly decreased in the livers of mice in the EPA treatment group compared with that in the livers of mice in the LD group. In conclusion, EPA was found to have a dominant anti-lithogenic effect in C57BL/6J mice.

## Introduction

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are essential fatty acids belonging to the group of  $\omega$ -3 fatty acids, which are primarily found in coldwater fish. These polyunsaturated fatty acids (PUFAs) have an important role in the functions of the body and are converted into hormone-like substances known as prostaglandins and leukotrienes (1). EPA has been shown to provide health benefits in patients with coronary heart disease, high blood pressure, mental health conditions, such as schizophrenia, and inflammatory disorders, including rheumatoid arthritis (2-6). DHA is a key component of brain tissue and the retina of the eye (7,8). Low levels of DHA cause reduced serotonin levels in the brain, which may result in depression, attention deficit hyperactivity disorder, cognitive decline and Alzheimer's disease (9-11).

It is well established that the key factors associated with cholesterol gallstone (CG) formation are biliary cholesterol supersaturation and gallbladder mucin hypersecretion. The supersaturation of cholesterol in the bile may result from a high-cholesterol diet or hepatic cholesterol overproduction. The elevation of biliary cholesterol concentration leads to the hypersecretion of mucin and the aggregation of cholesterol crystals (12,13). Several mucin (MUC) genes, including MUC2, MUC5AC, MUC5B and MUC6, are expressed in the gallbladder mucosa (14). The upregulation of these MUC genes leads to increased mucin concentrations, which increase bile viscosity and lead to the formation of a gel matrix that can entrap cholesterol crystals in the gallbladder (15-16).

The only established medical treatment for CG is ursodeoxycholic acid (UDCA). However, a number of studies have demonstrated that the therapeutic efficacy exhibited by UDCA lacks consistency and is not entirely satisfactory (17-19). Despite numerous attempts to develop novel CG drugs, there have been no satisfactory findings. In our previous study (20), it was found that a medical combination of  $\omega$ -3 PUFAs originating from fish oil had a preventive effect against CG formation. This medical combination consisted primarily of EPA and DHA. Therefore, the present study aimed to investigate which of these two  $\omega$ -3 PUFAs is responsible for the anti-lithogenic effect observed on CG formation using a mouse model.

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**Abbreviations:** EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PUFAs, polyunsaturated fatty acids; CG, cholesterol gallstone; LD, lithogenic diet; HMGCR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase; MUC, mucin; UDCA, ursodeoxycholic acid

**Key words:** EPA, DHA, cholesterol gallstone, anti-lithogenic, fish oil

## Materials and methods

**Materials.** EPA (90.2%) and DHA (80.9%) were obtained from Chemport, Inc. (Naju, Korea). The lithogenic diet (LD) was purchased from Dyets, Inc. (cat no. 102136; Bethlehem, PA, USA).

**Animals and diets.** Male 12-week-old C57BL/6J mice were purchased from Central Lab Animal, Inc. (Seoul, Korea) and bred in a laboratory animal breeding room at the College of Veterinary Medicine of Konkuk University (Seoul, Korea). The mice were divided into the following four groups of 10 mice: A) LD, B) LD plus EPA, C) LD plus DHA and D) LD plus EPA plus DHA. The acclimation period was four weeks. Subsequent to being fed the LD for four weeks, EPA and/or DHA was orally administered to the mice at a dose of 70 mg/kg/day for eight weeks. The LD feeding was continued during this period. The LD contained 1.0% cholesterol and 0.5% cholic acid. EPA (70 mg/kg/day) and DHA (70 mg/kg/day) were diluted in 0.75% Tween-80 and administered orally by gavage for eight weeks. The LD group was treated with 0.75% Tween-80 as a vehicle control. The animal experiments were approved by the Institutional Animal Care and Use Committee of Konkuk University. Mouse blood was collected from the vena cava and separated serum samples were stored in a -80°C biofreezer. The liver and gallbladder were isolated and frozen in liquid N<sub>2</sub> until required for analysis.

**Blood chemical analysis.** The plasma levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), total cholesterol, high-density lipoprotein (HDL)-cholesterol, phospholipids and triglycerides were determined using an automated Hitachi Clinical Analyzer (model 7020; Hitachi, Ltd., Kobe, Japan).

**Stone formation assessment.** Gallstone formation was assessed using a six-point system as follows: 0, clear bile; 1, little biliary sludge; 2, widespread biliary sludge; 3, high levels of biliary sludge; 4, a few small stones; 5, several stones; and 6, full of stones. The average scores of each group were compared.

**Quantitative polymerase chain reaction (qPCR) analysis for MUC genes, low-density lipoprotein receptor (LDLR) and 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR).** Total RNA was extracted from mouse gallbladder tissue using TRIzol® reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and dissolved in RNase-free water. Reverse transcription was performed using superscript III reverse transcriptase (Invitrogen Life Technologies). The mRNA levels of MUC2, -5AC, -5B and -6, and LDLR and HMGCR were quantified using TaqMan® PCR with GAPDH as an internal control. qPCR was performed using a Chromo4 Real-Time PCR Detection System (BioRad, Hercules, CA, USA). The relative abundances of the target genes were calculated using the comparative threshold cycle method. The qPCR primers and fluorogenic probes were purchased from Metabion (Martinsried, Germany) and were as follows: MUC2, NM\_023566; MUC5AC, NM\_010844; MUC5B, NM\_028801; MUC6, NM\_181729; LDLR, NM\_010700; HMGCR, NM\_008255 and GAPDH, NM\_008084.

**Statistical analysis.** Data are expressed as the mean ± standard deviation. Student's t-tests were performed to compare the treatment groups. A value of P<0.05 was considered to indicate a statistically significant difference.

## Results

**Effects of EPA and DHA on the liver weight-to-body weight ratio and plasma aminotransferase and lipid levels.** To assess the effects of EPA and DHA on the liver, the liver weight-to-body weight ratio and plasma aminotransferase levels were analyzed. The liver weight-to-body weight ratio was not significantly different among the different treatment groups. Fig. 1 shows that the serum ALT and AST levels were unchanged by treatment with EPA or DHA. In addition, no significant differences were observed in the levels of plasma lipids, including HDL-cholesterol, phospholipids and triglycerides, among the treatment groups. Although the level of cholesterol was significantly increased in the LD plus DHA group relative to that in the control LD group, this difference was <20%.

**Effects of EPA and DHA on gallstone formation.** Gallstone formation was observed in the different treatment groups. Gross analysis of the gallbladder revealed that the mice in the EPA treatment groups (groups B and D) exhibited significantly lower stone formation (P<0.01) than those in the LD plus DHA treatment group (group C), which exhibited only slightly suppressed stone formation (Fig. 2). Combined EPA and DHA treatment (group D) showed no significant synergistic effect on preventing gallstone formation. The mean scores for the stone formation grades were 4.38, 1.75, 3.0 and 2.13 for the LD, LD plus EPA, LD plus DHA and LD plus EPA plus DHA groups, respectively.

**Effects of EPA and DHA on the expression levels of MUC genes, LDLR and HMGCR.** It has been suggested that the downregulation of MUC gene expression causes a decrease in bile viscosity in the gallbladder, which attenuates CG formation (15,17). The expression of MUC2, -5AC, -5B and -6 was significantly reduced in the gallbladders of the mice in the EPA groups (70-90%) and the LD plus DHA treatment group (30-50%), compared with that in the gallbladders of the mice in the LD group (Fig. 3). No significant differences were observed in the mRNA expression of LDLR in the liver among the mice in the different treatment groups (data not shown). As shown in Fig. 4, the mRNA expression of HMGCR was significantly decreased in the livers of the mice in the LD plus EPA, LD plus DHA and LD plus EPA plus DHA treatment groups compared with that in the mice in the LD group (P<0.01). HMGCR expression is an index of cholesterol uptake and synthesis in the liver.

## Discussion

EPA from fish oil has a number of biological effects, including the reduction of the severity of cardiovascular diseases, such as atherosclerosis, through modulating lipid metabolism, as well as the inhibition of inflammatory processes (21,22). In the present study, EPA was found to have a dominant anti-lithogenic effect in C57BL/6J mice. Previous animal studies (24-26) have attempted to develop ω-3 PUFAs as dominant anti-lithogenic agents. Magnuson *et al* (23) reported that dietary fish oil inhibited

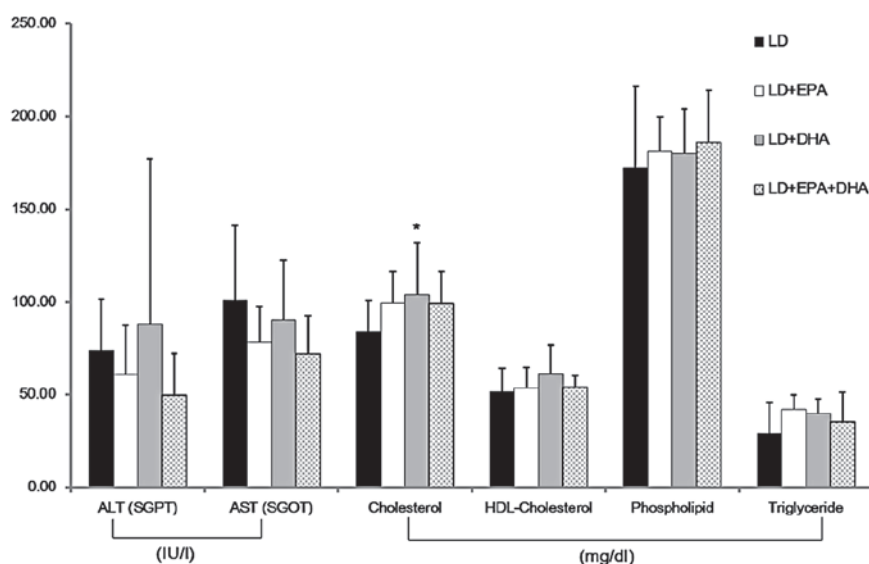


Figure 1. Effects of EPA and DHA on the levels of aminotransferases and lipids in the plasma. The level of cholesterol was significantly increased in the LD+DHA treatment group. The levels of ALT, AST, HDL-cholesterol, phospholipid and triglyceride showed no significant differences among the treatment groups. Data are presented as the mean  $\pm$  standard deviation (n=5). \*P<0.05 versus the LD group. LD, lithogenic diet; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ALT, alanine aminotransferase; SGPT, serum glutamic pyruvic transaminase; SGOT, serum glutamic oxaloacetic transaminase; AST, aspartate aminotransferase; HDL, high-density lipoprotein.

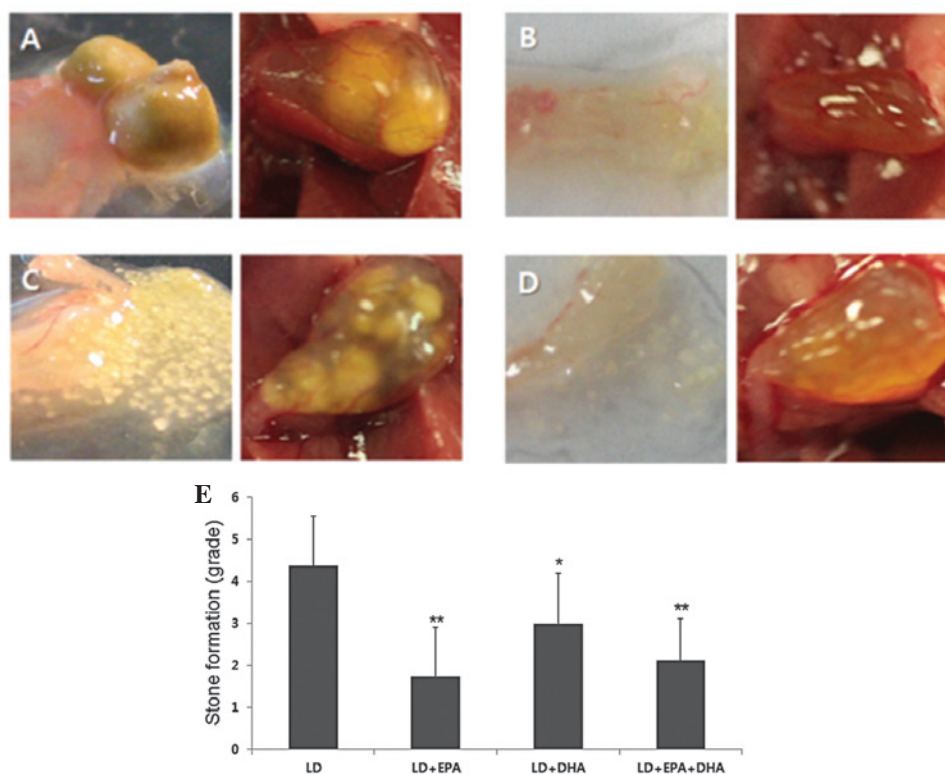


Figure 2. Gross findings for the gallbladder and gallstones. (A) LD, (B) LD+EPA, (C) LD+DHA and (D) LD+EPA+DHA groups (Left image: Postseparation; magnification, x20. Right image: Preseparation, magnification, x10). Mice fed EPA exhibited significantly fewer gallstones than those in the LD+DHA group. (E) Grading of gallstone formation. Mice in the LD+EPA and LD+EPA+DHA treatment groups showed significantly lower gallstone formation than those in the LD+DHA treatment group. Mice were graded according to the following scale: 0, clear bile; 1, little biliary sludge; 2, widespread biliary sludge; 3, high levels of biliary sludge; 4, a few small stones; 5, several stones and 6, full of stones. Data are presented as the mean  $\pm$  standard deviation (n=10). \*P<0.05 and \*\*P<0.01 versus the LD group. LD, lithogenic diet; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

solid cholesterol crystal precipitation and gallstone formation in prairie dogs. Furthermore, Scobey *et al* (24) demonstrated that fish oil consumption led to a decrease in gallstone formation and

the cholesterol saturation index (CSI) in African green monkeys. Mizuguchi *et al* (25) also presented animal data indicating that the repeated administration of EPA to hamsters decreased the

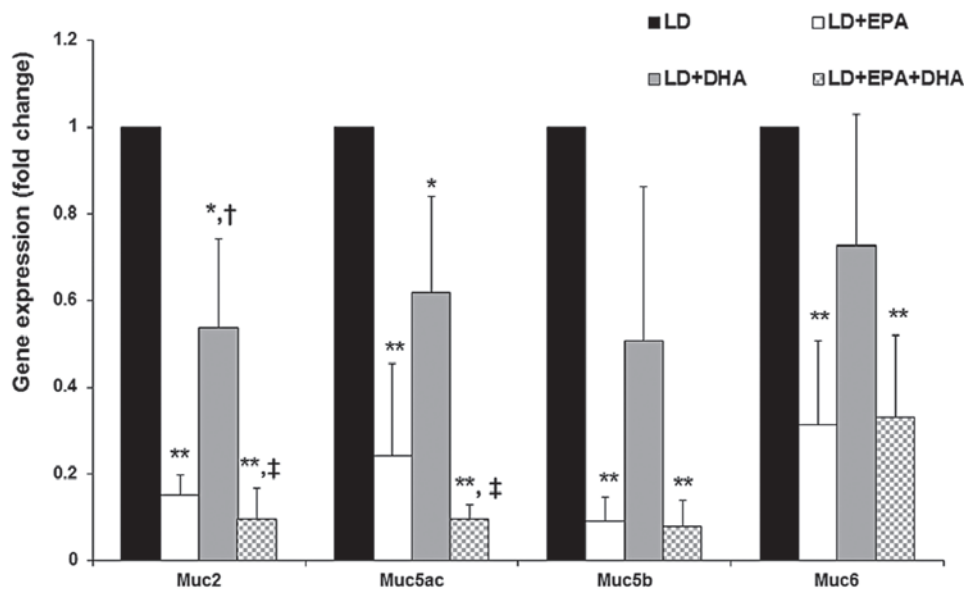


Figure 3. MUC gene expression in the gallbladder. The expression of MUC 2, 5AC, 5B and 6 was measured using a quantitative polymerase chain reaction methodology. The expression of MUC2, -5AC, -5B and -6 was significantly reduced in the gallbladder of the mice in the LD+EPA and LD+EPA+DHA treatment groups compared with that in the LD group. Data are presented as the mean  $\pm$  standard deviation (n=3). \*P<0.05 and \*\*P<0.01 versus the LD group; †P<0.05 versus the LD+EPA group; ‡P<0.01 versus the LD+DHA group. LD, lithogenic diet; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; MUC, mucin.

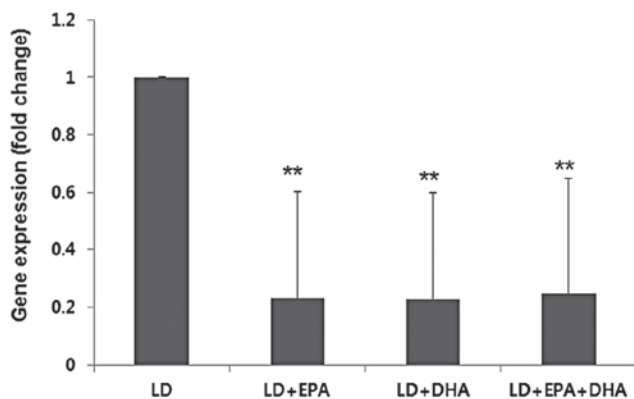


Figure 4. Expression of HMGR in the liver. HMGR expression was measured using a quantitative polymerase chain reaction methodology. The HMGR expression was significantly lower in the livers of the mice in the LD+EPA, LD+DHA and LD+EPA+DHA groups than that in the LD group. Data are presented as the mean  $\pm$  standard deviation (n=3). \*\*P<0.01 versus the LD group. LD, lithogenic diet; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; HMGR, 3-hydroxy-3-methylglutaryl-coenzyme A reductase.

incidence of cholesterol crystallization and gallstone formation. However, the molecular mechanisms responsible for the roles of  $\omega$ -3 PUFAs and EPA in CG formation are yet to be elucidated.

Gallstone formation is primarily caused by the supersaturation of cholesterol in the bile and the hypersecretion of mucin in the gallbladder (26). MUC gene expression is a well-established indicator of gallstone nucleation. In the present study, the expression of secretory MUC genes in the gallbladder was investigated, in order to assess mucin hypersecretion. Treatment with EPA in the presence or absence of DHA was found to significantly decrease the gene expression of MUC2, -5AC, -5B and -6. This finding suggests that EPA can attenuate CG formation, as it is well established that the downregulation of secretory MUC genes results in a decrease in bile viscosity

in the gallbladder. The CSI was not able to be assessed in the present study due to the low volume of bile available in mice. However, Janowitz *et al* (27) reported that fish oil consumption significantly decreased the CSI in humans.

The expression levels of LDLR and HMGCR are considered to be major indicators of cholesterol uptake and synthesis in the liver, respectively (28,29). In the present study, both EPA and DHA were found to significantly inhibit HMGCR expression, suggesting that these fatty acids may affect the availability of cholesterol in the liver. However, no significant difference was observed in the expression of LDLR and HMGCR between mice treated with EPA and those treated with DHA.

In our previous study (20), it was reported that a medical combination of  $\omega$ -3 PUFAs originating from fish oil that contained two major types of PUFAs, EPA and DHA, had a preventive effect against CG formation in C57BL/6J mice. The present study found that EPA has a significantly higher anti-lithogenic effect than DHA in C57BL/6J mice. This anti-lithogenic activity of EPA may be due to the inhibition of nucleation and cholesterol synthesis.

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