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Endurance Exercise Training Reduces Gallstone Development in Mice

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Short title: Exercise and Gallstones

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ABSTRACT

Gallstones form when the ratio of bile cholesterol to bile acids and phospholipids is elevated, causing cholesterol to precipitate. Physical *inactivity* is hypothesized to increase gallstone development, but experimental evidence supporting this is lacking, and potential mechanisms for the anti-lithogenic effects of exercise have not been described. The purpose of this study was to examine the effect of endurance exercise training on gallstone formation and the expression of genes involved in bile cholesterol metabolism in gallstone-sensitive (C57L/J) mice. At 10 weeks, 50 male mice began a lithogenic diet and were randomly assigned to an exercise-training (EX) or sedentary (SED) group (n=25 per group). Mice in the EX group ran on a treadmill at ~15m/min for 45 minutes/day for 12 weeks. At sacrifice, gallstones were collected, pooled by group, and weighed. The weight of the gallstones was 2.5-fold greater in the SED mice compared to EX (143 mg vs. 57 mg, respectively). In the EX mice, hepatic expression of the LDLr, SRB1, and Cyp27 was increased by ~2-fold ($p < 0.05$ for each). The LDLr and SRB1 increase cholesterol clearance by LDL and HDL particles, respectively, while Cyp27 promotes the catabolism of cholesterol to bile acids. Taken together, these data indicate that exercise promotes changes in hepatic gene expression that increase cholesterol uptake by the liver, but simultaneously increase the catabolism of cholesterol to bile acids, effectively reducing cholesterol saturation in the bile. This suggests a mechanism by which exercise improves cholesterol clearance from the circulation while simultaneously inhibiting gallstone formation.

Keywords: cholesterol, bile, physical activity

INTRODUCTION

Gallbladder disease affects 10-25% of adults in the United States(8). It has the second highest direct cost of any digestive disease at $5.8 billion annually and results in over 800,000 hospitalizations per year(24). Cholesterol gallstones form when the ratio of cholesterol to bile acids and phospholipids exceeds critical levels, causing the cholesterol to precipitate in the bile(32). Known risk factors for gallstones include age, high energy intake, obesity, non-insulin dependent diabetes, hyperlipidemia, rapid weight loss, female gender, and parity(7, 8).

Physical inactivity has been suggested to increase the risk of gallstone(29), but experimental evidence supporting this hypothesis is lacking. Several observational studies in humans have shown an inverse relationship between the prevalence of gallstones and physical activity levels(6, 16, 17, 20), though several others have not(2, 14). To date, there have been no longitudinal studies in humans or animals to directly examine the impact of endurance exercise-training on gallstone development.

In addition, the mechanisms by which physical activity or exercise may influence biliary cholesterol and gallstone development are unclear. As obesity is a known risk factor for gallstone disease, exercise-mediated prevention of obesity and/or reduction in body weight may play a role. Exercise also has been shown to increase gastric emptying rates(30), which may improve the impaired gastrointestinal motility that often occurs in gallstone disease. Furthermore, physical activity could directly affect bile cholesterol solubility by altering the expression of genes or proteins involved in hepatic cholesterol, phospholipid, and bile acid synthesis or secretion into the bile, though this hypothesis has never been thoroughly examined.

Longitudinal exercise-training studies in appropriate animal models may help elucidate potential mechanisms by which physical activity could impact gallstone development. Several

exercise training studies in rats have examined cholesterol metabolism in the liver and bile(11, 27, 34), but rats do not have gallbladders, therefore gallstone development studies are not possible in this model. Recently, it was discovered that certain inbred strains of mice rapidly form gallstones when placed on a high-fat diet supplemented with cholic acid (15). These gallstone-susceptible strains have been used to identify genetic contributions to gallstone disease (13), but not the impact of exercise-training on gallstone formation. Therefore, we used a 12-week exercise training intervention in a gallstone-susceptible mouse strain (C57L/J) to investigate if exercise would prevent or attenuate the development of gallstones in mice consuming a lithogenic diet. Furthermore, we examined the expression of several genes associated with hepatic cholesterol metabolism in order to elucidate potential mechanisms by which exercise might be impacting gallstone formation. We hypothesized that exercise-training would attenuate gallstone formation in mice, possibly by modulating hepatic gene expression in ways that increase the solubilization of cholesterol in the bile.

METHODS

Animals, diets, and exercise

Fifty male C57L/J mice were used in this study. C57L/J (Stock # 000668) breeder pairs were purchased from Jackson Labs (Bar Harbor, ME) to establish a breeder colony. All animals were housed in plastic cages in a temperature-controlled room with 12h light/12h dark cycles and provided *ad libitum* access to food and water.

All mice were weaned at 4 weeks of age and placed on a standard chow diet. At 10 weeks of age, all mice were weighed, had blood drawn from the retroorbital vein after an overnight fast,

and then randomly assigned to an exercise (EX) or sedentary (SED) group (n=25 per group). All mice were then switched to a lithogenic "Paigen" diet(22), consisting of standard chow feed (Harlan Teklad, powder diet #8640) supplemented to a final concentration of 21% fat (using milkfat), 1.25% cholesterol, and 0.5% cholic acid. The EX mice began their 12 week exercise training which consisted of treadmill running 5 days per week at approximately 12-15 m/min, gradually increasing to 45 minutes of running by the end of the first week. Past research has shown that this running speed corresponds to approximately 65% VO_2max in this strain of mice(25). After 12 weeks, all mice were again weighed, had blood drawn from the retroorbital vein after an overnight fast, and then sacrificed for tissue collection and analysis as described below. All experiments were approved by the University of Illinois at Urbana-Champaign IACUC.

Plasma lipid analysis

Plasma was collected by centrifugation from blood samples taken at baseline and sacrifice and frozen at -80^{o}C until analyzed. Plasma cholesterol and triglyceride levels were measured by standard enzymatic methods using commercially-available assay kits (Wako Chemicals USA, Inc., Richmond, VA)

Gallstone measurements

At sacrifice, the gallbladders were removed from each animal and cut open to remove the gallstones. Due to the small size and low weight of the gallstones in many individual animals, stones from all animals in each group of mice (SED and EX) were pooled and weighed. The weight of the intact gallbladders (containing gallstones and bile) from individual animals was not

recorded prior to removing the stones because it was evident that some of the gallbladders had recently contracted, so contained little bile. As a result, the weight of the intact gallbladders would not have been informative.

Gene expression analysis

At sacrifice, the liver and intestine were removed and snap-frozen in liquid nitrogen until analyzed. Prior to freezing, the intestine was divided into three sections of equal length to represent the duodenum, jejunum, and ileum. The duodenum and jejunum are the primary sites of cholesterol absorption(19), so the ileum was discarded. RNA was isolated from liver, duodenum, and jejunum from 10 animals in each group using RNA-STAT 60 (Tel-test Inc., Friendswood, TX). DNAse treatment was performed using an RNAeasy mini kit (Qiagen, Inc., Turnberry, CA). Reverse transcription reactions were performed with 2ug of RNA using TaqMan Reverse Transcription Reagents (Applied Biosystems, Inc., Foster City, CA). cDNA was stored at -20°C prior to analysis.

Real-time PCR was performed on an Mx3000P QPCR System (Stratagene, Inc., La Jolla, CA) using the Brilliant SYBR Green QPCR Master Mix (Stratagene, catalogue #600548). Primers used are as follows: Cyclophilin (sense, 5'-TGGAGAGCACCAAGACAGACA-3'; antisense, 5'-TGCCGGAGTCGACAATGAT-3'). Low density lipoprotein receptor (LDLr) (sense, 5'-AGGCTGTGGGCTCCATAGG-3'; antisense, 5'-TGCGGTCCAGGGTCATCT-3'). Scavenger receptor class B type 1 (SRB1) (sense, 5'-TCCCCATGAACTGTTCTGTGGA-3'; antisense, 5'-TGCCCGATGCCCTTGA-3'). ATP binding cassette transporter (ABC) G5 (sense, 5'-TGGATCCAACACCTCTATGCTAAA-3'; antisense, 5'-GGCAGGTTTTCTCGATGAACTG-3'). ABCG8 (sense, 5'-TGCCCACCTTCCACATGTC-3';

antisense, 5'-ATGAAGCCGGCAGTAAGGTAGA-3'). ABCB4 (sense, 5'-CTTGAGGCAGCGAGAAACG-3'; antisense, 5'-GGTTGCTGATGCTGCCTAGTT-3'). Cyp7a1 (sense, 5'-AGCAACTAAACAACCTGCCAGTACTA-3'; antisense, 5'-GTCCGGATATTCAAGGATGCA-3'). Sterol 27 hydroxylase (Cyp27) (sense, 5'-GGAGGGCAAGTACCCAATAAGA-3'; antisense, 5'-TGCGATGAAGATCCCATAGGT-3'). Sterol 7-a hydroxylase (Cyp7a1) (sense, 5'-AGCAACTAAACAACCTGCCAGTACTA-3; antisense, 5'-GTCCGGATATTCAAGGATGCA-3'). 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) (sense, 5'-CTTGTGGAATGCCTTGTGATTG-3'; antisense, 5'-AGCCGAAGCAGCACATGAT-3'). HMG-CoA reductase (HMGCR) (sense, 5'-GCCGTGAACTGGGTCGAA-3'; antisense, 5'-GCATATATAGCAATGTCTCCTGCAA-3'). ABCB11 (sense, 5'-AAGCTACATCTGCCTTAGACACAGAAA-3'; antisense, 5'-CAATACAGGTCCGACCCTCTCT-3'). Niemann-Pick C1-Like 1 (NPC1L1) (sense, 5'-TGGACTGGAAGGACCATTTCC-3'; antisense, 5'-CTCATAATGGTGCAGTTCTTGTTGT-3'). Reaction conditions for all primers were 95°C, 10min, 1 cycle; 95°C, 30sec, 60°C, 1min, 72°C, 1min, 40 cycles.

The Mx3000P software was used to analyze real-time PCR results. Cyclophilin was used as the normalizing gene and fold changes were calculated using the $2^{-\Delta\Delta CT}$ method(18). The relative expression of each gene of interest in the SED group was arbitrarily set at 1.0.

Statistics

All statistics were performed using SPSS version 12.0 (Chicago, IL). Independent samples t-tests were used to analyze group differences in all variables measured. All data is expressed as mean ± standard deviation (S.D.). A p-value of ≤ 0.05 was accepted as statistically

significant**.** No statistical analysis was performed on the gallstone weights because only pooled sample weights were available.

RESULTS

Body weights and plasma lipids

Body weights and plasma lipid levels for animals in the SED and EX groups are shown in Table 1. Body weight did not differ between the groups at baseline. However, mice in the EX group gained significantly less weight than SED mice during the intervention (6.7 ± 3.6 grams vs. 12.9 ± 5.6 grams, $p < 0.01$), resulting in significant difference in body weights at sacrifice ($p < 0.01$). There were no significant differences in triglyceride levels between the groups at baseline or final testing. Plasma cholesterol levels were slightly higher in the SED mice at baseline ($p < 0.01$), but did not differ between EX and SED at final testing.

Gallstone development

At sacrifice, the gallbladder was removed and the gallstones from all mice in each group were pooled and weighed. The pooled weight of the gallstones was 2.5-fold greater in the SED mice than in the EX mice (Figure 1A). Representative samples of gallbladders from a SED and EX mice are shown in Figure 1B.

Gene expression

The expression of selected genes related to hepatic and bile cholesterol metabolism in the liver are shown in Figure 2. There was no difference in hepatic mRNA levels for HMGCS,

ABCB4, ABCB11, or ABCG5 between the SED and EX mice. However, there was a significant increase in the hepatic expression of LDLr (2.5-fold, $p = 0.03$), SRB1 (1.7-fold, $p = 0.04$), and Cyp27 (2.3-fold, $p = 0.002$) in the EX mice relative to SED, and a trend for an increase in the expression of ABCG8 (1.8-fold, $p = 0.1$) and HMGCR (1.9-fold, $p = 0.1$).

The expression of selected genes involved in cholesterol absorption in the intestine are shown in Figure 3. In the duodenum, NPC1L1, ABCG5 and ABCG8 mRNA levels were each reduced by 55% - 65% ($p < 0.05$ for each) in the EX group compared to SED; however, there were no group differences in the expression of these genes in the ileum.

DISCUSSION

The major finding of this study is that 12 weeks of endurance exercise training attenuated gallstone development in a gallstone-susceptible (C57L/J) strain of mice. To our knowledge, this is the first longitudinal study to demonstrate that exercise training reduces gallstones in any model system.

To examine potential mechanisms for the anti-lithogenic effects of exercise training seen in this study, we also measured the hepatic and intestinal expression of selected genes involved in cholesterol metabolism that may impact gallstone development. Cholesterol gallstones may develop due to excessive secretion of cholesterol into the bile; if the ratio of biliary cholesterol to bile acids and phospholipids exceeds critical limits, the cholesterol may precipitate and form stones(5). A number of factors may contribute to biliary cholesterol supersaturation, including increases in cholesterol uptake from the circulation, increased cholesterol biosynthesis in the liver, or reduced catabolism of cholesterol to bile acids(23). Changes in the hepatic expression of genes that regulate these factors could significantly affect gallstone formation. Of particular

interest, we found a significant increase in the hepatic expression the LDLr, SRB1, and Cyp27. The LDLr is responsible for clearing ApoB-containing lipoproteins (LDL and VLDL) from the circulation, while SRB1 promotes selective uptake of HDL-cholesterol into the liver(9). Though increases in the expression of these genes may be expected to increase cholesterol secretion into the bile, the expression of Cyp27 was also elevated in the EX mice. Cyp27 is a rate-limiting enzyme in the conversion of cholesterol to bile acids by the "alternative" pathway(26), so increases in this enzyme may help prevent gallstone formation by increasing the bile acid: bile cholesterol ratio. Taken together, these differences in hepatic gene expression between the EX and SED mice indicate a potential mechanism by which exercise training simultaneously improves cholesterol clearance from the circulation while also inhibiting gallstone formation.

Of note, bile acids are normally synthesized from cholesterol primarily through the "classic" pathway initiated by the enzyme Cyp7a1(26). However, Cyp7a1 is down-regulated by cholic acid feeding in C57L/J mice(21), so expression levels in our animals were too low for a robust analysis. A previous pilot study in our lab in C57BL6 mice found a 4.5-fold increase in Cyp7a1 gene expression after four weeks of voluntary wheel running (K.R. Wilund, unpublished observations), but additional studies are needed to confirm this finding.

Exercise did not induce significant changes in mRNA expression of HMGCR, HMGCS, ABCB4, ABCB11, ABCG5 or ABCG8. HMGCR and HMGCS are the rate limiting enzymes in the de novo synthesis of cholesterol in the liver(10). ABCB4 and ABCB11 promote phospholipid(31) and bile acid(28) secretion, respectively, into bile, while ABCG5 and ABCG8 form a functional dimer that secretes cholesterol into the bile(3). Thus, our data suggests that the reduction in gallstones in the EX mice was not due to reductions in hepatic cholesterol synthesis or secretion into the bile, or increases in biliary phospholipid or bile acid secretion.

High cholesterol absorption efficiency in the intestine also can enhance gallstone formation(33), so we measured the intestinal expression of three genes, NPC1L1, ABCG5, and ABCG8, that play a critical role in regulating cholesterol absorption in the duodenum and jejunum, the primary sites of cholesterol absorption(12). NPC1L1 promotes cholesterol absorption by transporting cholesterol from the intestinal lumen into enterocytes(1), while ABCG5 and G8 inhibit cholesterol absorption by effluxing cholesterol from enterocytes back into the intestinal lumen. The expression of each of these genes in the duodenum was reduced by approximately 60% in the EX mice compared to SED. While a reduction in NPC1L1 levels should reduce cholesterol absorption, the concomitant reduction in ABCG5 and G8 expression would be expected to enhance cholesterol absorption, so the overall effects of these changes in gene expression are uncertain. However, one potential explanation is that ABCG5 and ABCG8 expression may have decreased in response to the reduction in NPC1L1; meaning if less cholesterol is being transported into enterocytes by NPC1L1, this may reduce ABCG5 and G8 expression as there will be less cholesterol to efflux back into the intestinal lumen. Direct measurements of cholesterol uptake and absorption will be needed to confirm this hypothesis.

There are several limitations to our study: First, due to the small size and weight of gallstones from individual animals, we chose to pool the gallstones from all animals in each group. While this limited our statistical analysis, we felt this was the most accurate way to represent the differences in gallstone weight between the groups. Previous studies have used a variety of methods to estimate gallstone size and weight in mice, but we felt the measurement error using these techniques was too large for a robust analysis. Also, because the SED mice gained significantly more weight than the EX mice, we cannot ensure that the observed changes are due to exercise alone, or due in whole or in part to the reduced weight gain in the EX mice.

However, studies in overweight mice indicate that obesity may not be a risk factor for cholesterol gallstone formation in these animals(4), so we do not believe that the differences in weight gain between the EX and SED mice had a significant effect on gallstone development in this study. Finally, to examine potential mechanisms for the anti-lithogenic effects of the exercise training, we relied on an analysis of mRNA expression in the liver and intestine. Direct measurements of cholesterol absorption and synthesis, biliary lipids, bile secretion and flow rates are among the measurements needed to specifically address these mechanisms.

In conclusion, we have shown that 12 weeks of endurance exercise training attenuates gallstone development in mice. The mechanism for this may lie in changes in expression of several genes related to cholesterol metabolism and bile acid formation. Our exercise training protocol of 45 minutes of running per day, 5 days per week translates to use in a human population. As gallstone disease represents a significant public health problem in the US and around the world, future studies should investigate the impact of endurance exercise training in preventing gallstone development in susceptible human populations.

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Table I

Body weights and plasma lipid levels

	Sedentary	**Exercise**	**p-value**
body weight (g)			
-baseline	25.4 ± 2.4	24.3 ± 1.8	0.09
-final	38.0 ± 6.3	31.3 ± 4.8	< 0.001
-change	12.9 ± 5.6	6.7 ± 3.6	< 0.001
Plasma Triglyceride (mg/dL)			
-baseline	152 ± 49.2	137 ± 42.0	0.33
-final	141 ± 49.6	141 ± 36.0	0.99
-change	-24 ± 70.3	1.8 ± 50.6	0.20
Plasma Cholesterol (mg/dL)			
-baseline	75 ± 17.1	60 ± 10.8	0.003
-final	132 ± 22.4	132 ± 20.4	0.99
-change	60 ± 21.7	74 ± 20.5	0.054

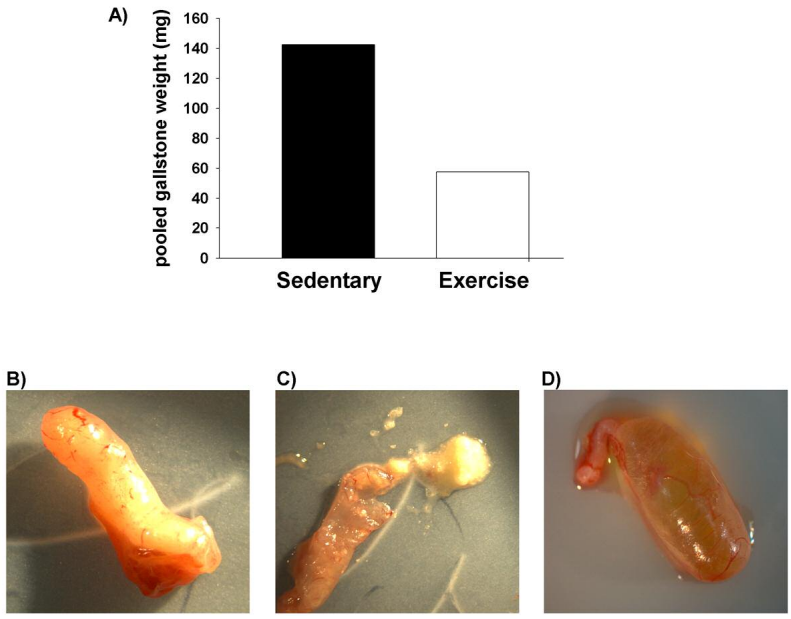
Values are means ± S.D.

Figure Legends

Figure 1. Pooled gallstone weights (a) and representative gallbladders (b-d) from SED and EX mice. Gallstones from all animals in each group (n = 25/group) were pooled and weighed at sacrifice. (b) Intact gallbladder from a mouse in the SED group. c) Gallbladder from the same SED mouse with gallstones removed for display. d) Intact gallbladder from a mouse in the EX group. Note that the gallbladder in this picture is translucent, and no stones are apparent.

Figure 2. Quantitative RT-qPCR of liver (a) and intestine (b) RNA from SED and EX mice. Total RNA was isolated from the liver, duodenum and ileum of ten mice in each group at sacrifice. 2ug of RNA was reverse-transcribed into cDNA which was used for PCR analysis. Cyclophilin was used as the normalizer gene in each tissue. Each value represents the mRNA levels relative to the amount of transcript in the SED mice, which was arbitrarily set to 1.0. Data is expressed as mean ± S.D. * $p \leq 0.05$.

Figure 1



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254x177mm (150 x 150 DPI)

Figure 2A

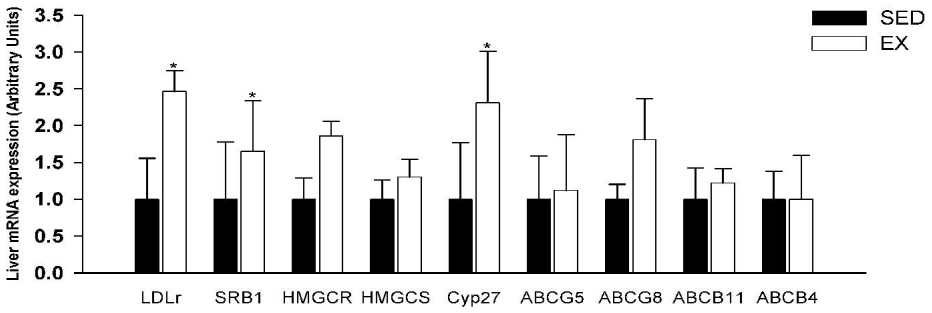
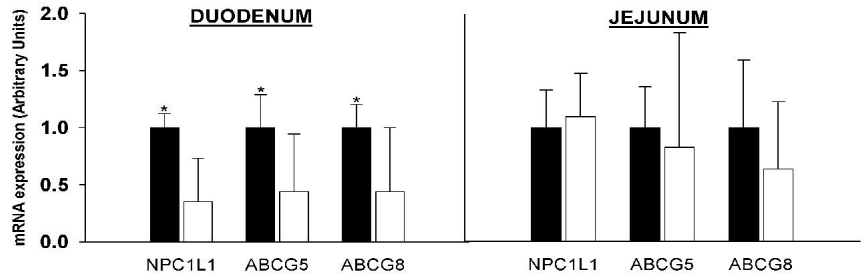


Figure 2B



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