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Endurance Exercise Training Reduces Gallstone Development in Mice

Kenneth R. Wilund^{1,2}, Laura A. Feeney¹; Emily J. Tomayko², Hae R. Chung¹, Kijin Kim³.

¹Department of Kinesiology and Community Health and ²Division of Nutritional Sciences, University of Illinois, Urbana, IL. ³Department of Physical Education, Keimyung University, Daegu, Korea.

Short title: Exercise and Gallstones

To whom correspondence should be sent:
Kenneth R. Wilund
University of Illinois
Department of Kinesiology and Community Health
906 S. Goodwin Avenue
Urbana, IL 61801
Email: kwilund@uiuc.edu

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ABSTRACT

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43 Gallstones form when the ratio of bile cholesterol to bile acids and phospholipids is elevated,
44 causing cholesterol to precipitate. Physical *inactivity* is hypothesized to increase gallstone
45 development, but experimental evidence supporting this is lacking, and potential mechanisms for
46 the anti-lithogenic effects of exercise have not been described. The purpose of this study was to
47 examine the effect of endurance exercise training on gallstone formation and the expression of
48 genes involved in bile cholesterol metabolism in gallstone-sensitive (C57L/J) mice. At 10
49 weeks, 50 male mice began a lithogenic diet and were randomly assigned to an exercise-training
50 (EX) or sedentary (SED) group (n=25 per group). Mice in the EX group ran on a treadmill at
51 ~15m/min for 45 minutes/day for 12 weeks. At sacrifice, gallstones were collected, pooled by
52 group, and weighed. The weight of the gallstones was 2.5-fold greater in the SED mice
53 compared to EX (143 mg vs. 57 mg, respectively). In the EX mice, hepatic expression of the
54 LDLr, SRB1, and Cyp27 was increased by ~2-fold ($p < 0.05$ for each). The LDLr and SRB1
55 increase cholesterol clearance by LDL and HDL particles, respectively, while Cyp27 promotes
56 the catabolism of cholesterol to bile acids. Taken together, these data indicate that exercise
57 promotes changes in hepatic gene expression that increase cholesterol uptake by the liver, but
58 simultaneously increase the catabolism of cholesterol to bile acids, effectively reducing
59 cholesterol saturation in the bile. This suggests a mechanism by which exercise improves
60 cholesterol clearance from the circulation while simultaneously inhibiting gallstone formation.

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63 **Keywords:** cholesterol, bile, physical activity

64 INTRODUCTION

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

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

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

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

87 exercise training studies in rats have examined cholesterol metabolism in the liver and bile(11,
88 27, 34), but rats do not have gallbladders, therefore gallstone development studies are not
89 possible in this model. Recently, it was discovered that certain inbred strains of mice rapidly
90 form gallstones when placed on a high-fat diet supplemented with cholic acid (15). These
91 gallstone-susceptible strains have been used to identify genetic contributions to gallstone disease
92 (13), but not the impact of exercise-training on gallstone formation. Therefore, we used a 12-
93 week exercise training intervention in a gallstone-susceptible mouse strain (C57L/J) to
94 investigate if exercise would prevent or attenuate the development of gallstones in mice
95 consuming a lithogenic diet. Furthermore, we examined the expression of several genes
96 associated with hepatic cholesterol metabolism in order to elucidate potential mechanisms by
97 which exercise might be impacting gallstone formation. We hypothesized that exercise-training
98 would attenuate gallstone formation in mice, possibly by modulating hepatic gene expression in
99 ways that increase the solubilization of cholesterol in the bile.

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METHODS

103 **Animals, diets, and exercise**

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

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

110 and then randomly assigned to an exercise (EX) or sedentary (SED) group (n=25 per group). All
111 mice were then switched to a lithogenic “Paigen” diet(22), consisting of standard chow feed
112 (Harlan Teklad, powder diet #8640) supplemented to a final concentration of 21% fat (using
113 milkfat), 1.25% cholesterol, and 0.5% cholic acid. The EX mice began their 12 week exercise
114 training which consisted of treadmill running 5 days per week at approximately 12-15 m/min,
115 gradually increasing to 45 minutes of running by the end of the first week. Past research has
116 shown that this running speed corresponds to approximately 65% VO₂max in this strain of
117 mice(25). After 12 weeks, all mice were again weighed, had blood drawn from the retroorbital
118 vein after an overnight fast, and then sacrificed for tissue collection and analysis as described
119 below. All experiments were approved by the University of Illinois at Urbana-Champaign
120 IACUC.

121

122 **Plasma lipid analysis**

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

127

128 **Gallstone measurements**

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

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

136

137 **Gene expression analysis**

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

147 Real-time PCR was performed on an Mx3000P QPCR System (Stratagene, Inc., La Jolla,
148 CA) using the Brilliant SYBR Green QPCR Master Mix (Stratagene, catalogue #600548).
149 Primers used are as follows: Cyclophilin (sense, 5'-TGGAGAGCACCAAGACAGACA-3';
150 antisense, 5'-TGCCGGAGTCGACAATGAT-3'). Low density lipoprotein receptor (LDLr)
151 (sense, 5'-AGGCTGTGGGCTCCATAGG-3'; antisense, 5'-TGCGGTCCAGGGTCATCT-3').
152 Scavenger receptor class B type 1 (SRB1) (sense, 5'-TCCCCATGAACTGTTCTGTGGA-3';
153 antisense, 5'-TGCCCGATGCCCTTGA-3'). ATP binding cassette transporter (ABC) G5 (sense,
154 5'-TGGATCCAACACCTCTATGCTAAA-3'; antisense, 5'-
155 GGCAGGTTTTCTCGATGAACTG-3'). ABCG8 (sense, 5'-TGCCCACCTTCCACATGTC-3';

156 antisense, 5'-ATGAAGCCGGCAGTAAGGTAGA-3'). ABCB4 (sense, 5'-
 157 CTTGAGGCAGCGAGAAACG-3'; antisense, 5'-GGTTGCTGATGCTGCCTAGTT-3').
 158 Cyp7a1 (sense, 5'-AGCAACTAAACAACCTGCCAGTACTA-3'; antisense, 5'-
 159 GTCCGGATATTCAAGGATGCA-3'). Sterol 27 hydroxylase (Cyp27) (sense, 5'-
 160 GGAGGGCAAGTACCCAATAAGA-3'; antisense, 5'-TGCGATGAAGATCCCATAGGT-3').
 161 Sterol 7-a hydroxylase (Cyp7a1) (sense, 5'-AGCAACTAAACAACCTGCCAGTACTA-3;
 162 antisense, 5'-GTCCGGATATTCAAGGATGCA-3'). 3-hydroxy-3-methylglutaryl-coenzyme A
 163 reductase (HMGCR) (sense, 5'-CTTGTGGAATGCCTTGTGATTG-3'; antisense, 5'-
 164 AGCCGAAGCAGCACATGAT-3'). HMG-CoA reductase (HMGCR) (sense, 5'-
 165 GCCGTGAACTGGGTCGAA-3'; antisense, 5'-GCATATATAGCAATGTCTCCTGCAA-3').
 166 ABCB11 (sense, 5'-AAGCTACATCTGCCTTAGACACAGAAA-3'; antisense, 5'-
 167 CAATACAGGTCCGACCCTCTCT-3'). Niemann-Pick C1-Like 1 (NPC1L1) (sense, 5'-
 168 TGGACTGGAAGGACCATTTCC-3'; antisense, 5'-CTCATAATGGTGCAGTTCTTGTGTG-
 169 3'). Reaction conditions for all primers were 95°C, 10min, 1 cycle; 95°C, 30sec, 60°C, 1min,
 170 72°C, 1min, 40 cycles.

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

174

175 **Statistics**

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

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

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RESULTS

184 **Body weights and plasma lipids**

185 Body weights and plasma lipid levels for animals in the SED and EX groups are shown in
186 Table 1. Body weight did not differ between the groups at baseline. However, mice in the EX
187 group gained significantly less weight than SED mice during the intervention (6.7 ± 3.6 grams
188 vs. 12.9 ± 5.6 grams, $p < 0.01$), resulting in significant difference in body weights at sacrifice ($p <$
189 0.01). There were no significant differences in triglyceride levels between the groups at baseline
190 or final testing. Plasma cholesterol levels were slightly higher in the SED mice at baseline ($p <$
191 0.01), but did not differ between EX and SED at final testing.

192

193 **Gallstone development**

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

198

199 **Gene expression**

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

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

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

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DISCUSSION

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

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

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

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

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

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

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

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

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

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375 **Table I**376 **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

377 Values are means ± S.D.

378 **Figure Legends**

379

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

385

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

Figure 1

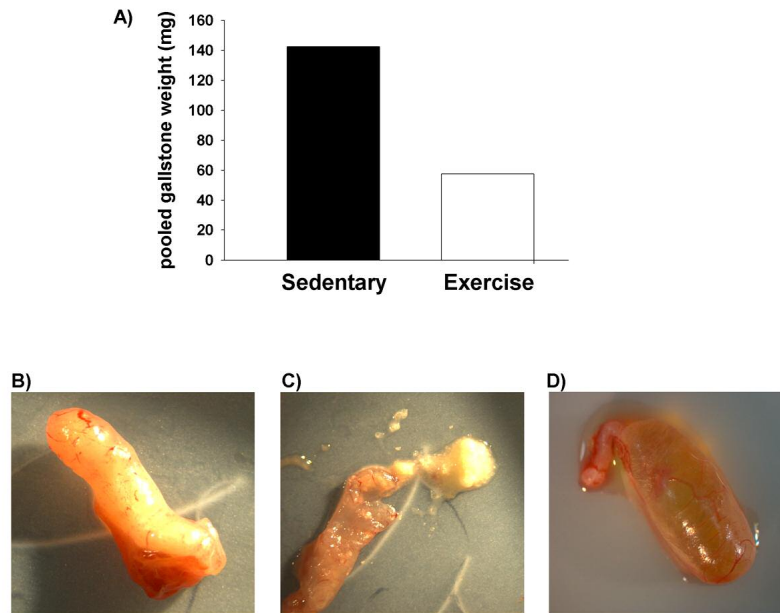


Figure 1. Pooled gallstone weights (a) and representative gallbladders (b-d) from SED and EX mice. Gallstones from all animals in each group (n = 25/group) were pooled and weighed at sacrifice. (b) Intact gallbladder from a mouse in the SED group. c) Gallbladder from the same SED mouse with gallstones 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.

254x177mm (150 x 150 DPI)

Figure 2A

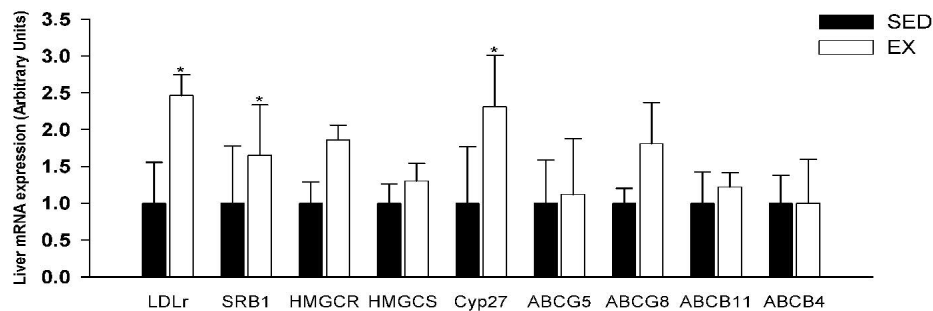


Figure 2B

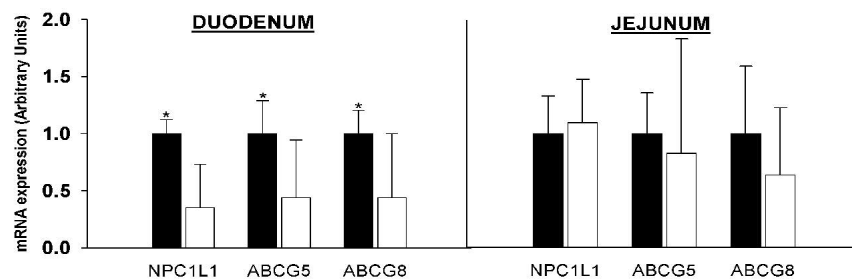


Figure 2. Quantitative RT-qPCR of liver (a) and intestine (b) RNA from SED and EX mice. Total RNA was isolated from the liver, duodenum and ileum of ten mice in each group at sacrifice. 2ug of RNA was reverse-transcribed into cDNA which was used for PCR analysis. Cyclophilin was used as the normalizer 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 \pm S.D. * $p \leq 0.05$.

254x177mm (150 x 150 DPI)