Peer Review File

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Reviewer A

This is an interesting animal model study that proposes to answer an important question relevant to cholecystectomy increasing the risk for later development of NAFLD.

In the introduction

1. An important mention/inclusion with references should be made in relevance to a lithogenic gallbladder bile with altered bile acid metabolism circulating in the enterohepatic circulation as important risk factors in conjunction with a dysbiotic intestinal microbiome that ensues because of it for the progression of NAFLD.

☞ As suggested by the reviewer, we have included the following information in the 'Introduction' section along with the necessary references (Page 4, line 97–102)**:**

The microbial diversity in patients with NAFLD is reported to be lower than that in healthy controls (1). Cholecystectomy impairs aging-related changes in gut microbial composition (2). Patients who have undergone cholecystectomy exhibit an increased abundance of bacterial species involved in bile acid metabolism (3). Bacterial dysbiosis-associated production of metabolites can be a feedback signal to the host (4). The contribution of the microbiome and other factors to the pathogenesis of NASH is unclear (5).

In the methods:

1. was the Hanyang University Institutional Animal Care and Use Committee also the approving animal ethics committee? Clarify

☞ Yes, the Hanyang University Institutional Animal Care and Use Committee approved the animal experiments. This information has been included in Page 7, Line 171–173 of the revised manuscript.

2. Did an independent researcher conduct the histological investigations? Clarify

☞ The following information has been included in the revised manuscript for improved clarity (Page 7, line 181–183):

Hematoxylin and eosin (H&E) staining was performed to evaluate NAFLD. All histological images were re-evaluated by an independent pathologist who was blinded to the experimental group.

3. The statistical section should be expanded to include more information relevant to the tests that were performed and on which set of data. Also describe the use of graphs employed for add further clarity on the data.

☞ As suggested by the reviewer, the following information has been included in the revised manuscript (Page 10, line 229–231):

The data for the standard diet-fed sham and cholecystectomy groups were compared using one-way analysis of variance, whereas the results of liver weight, liver/bodyweight ratio, serum biochemical, and qRT-PCR analyses of HF diet-fed mice were compared Normal diet using the unpaired t-test.

In the results:

1. Add labels to Figure 1 and 3 histology sections that show the reader what is being demonstrated

☞ As suggested by the reviewer, the legends of Figures 1 and 3A (histological sections) have been modified for improved clarity.

2. In Figure 2 F and G and also Figure 3 and 4 add labels to the Y-axis where necessary

☞ As suggested by the reviewer, Figures 2F–G, 3, and 4 have been modified in the revised manuscript as follows:

In Figure 2F and G, the unit μmol/L has been included in the Y-axis.

In Figure 3E, the units U/L (for ALT and AST levels) and mg/dL (for triglyceride and cholesterol levels) have been included in the Y-axis.

In Figure 4F and G, the unit μmol/L has been included in the Y-axis.

In the discussion

1. Cholecystectomy for gallstones presents a different clinical picture to that proposed by this study. Cholesterol gallstone development almost always shows an altered bile acid and lipid metabolic profile of the bile in the gallbladder therefore as such for the progression to NAFLD a semi or poorly functional gallbladder with gallstones and a lithogenic bile may be the requisite. Authors should comment on this and relate this to their study.

☞ The findings of this study cannot be directly extrapolated to humans. In humans, cholecystectomy is performed to alleviate gallstones or chronic inflammation. Altered metabolism in humans cannot be compared to cholecystectomy-induced metabolic changes in mice. The following information has been included in the discussion section as a limitation of the study (Page 16, line 371–374):

Several limitations were associated with this animal study. The GB resection condition in mice is different from that in humans. In humans, cholecystectomy is performed to alleviate gallstones or chronic inflammation. Thus, the pre-existing altered metabolism in humans cannot be directly compared to cholecystectomy-induced altered metabolism in mice.

Reviewer B

In this manuscript by Kim et al, the authors investigated whether cholecystectomy (GBX) caused fatty liver development in mice on normal or high-fat diets. They found that mice that underwent GBX were not different in terms of various parameters from those with sham operation. While this study

provides important messages that the clinical association between the incidence of NAFLD and GBX cannot be directly recapitulated in animal models, there are a few concerns that needs to be addressed before this manuscript is in a publishable fashion. Specific comments are as follows:

Although the negative results provide information that there may not be a causal relationship between GBX and NAFLD, it is important to comprehensively discuss the differences between this study and other recent studies that showed different results. For example, a recent report from Wang Q et al. (2021) showed that although the two operations made little difference under standard diets, GBX resulted in a significant increase in fatty liver development and associated pathological parameters. Alexander C et al. (2021) discussed contribution of gender to the metabolic profile under normal diets. What may contribute to theses distinct outcomes?

☞ As suggested by the reviewer, the following information has been added in the 'Discussion' section along with the references (Page 15, line 345–355):

A recent study reported that NAFLD could be induced by altering the intestinal microbiome profile after cholecystectomy in the standard diet-fed and HF diet-fed groups (6). However, one study involving 32,428 human subjects reported that cholecystectomy may not be a risk factor for fatty liver disease (7). Additionally, the increased prevalence of NAFLD among patients undergoing cholecystectomy was attributed to the common risk factors. Thus, cholecystectomy has no causal relationship with the development and progression of NAFLD (8). Previous studies have reported increased levels of bile acid in the early stages after cholecystectomy. However, bile acid levels reach a steady-state at month 3 post-surgery. No marked changes in the amount and conversion rate of bile acids were observed among non-surgical patients (9). Therefore, the inconsistency of results between this study and previous studies can be attributed to different post-cholecystectomy analysis periods.

The sample size may be too small for a time course study. The sham operated group at 4 months only had 2 mice? How was the statistics performed with such small sample numbers?

☞ We agree with reviewer's comments that the sample size was small. Originally, we had planned to sacrifice four mice. However, two mice died during breeding. The following information has been added in the revised manuscript (Page 16, Line 374–377):

In study 1, the number of mice in the sham group was only 2 at month 4 post-surgery, which may affect the statistical power. However, this is a proof of study for hypothesis testing analyzing the possibility of serialization. Therefore, additional experiments are needed with a large number of animals.

The IHC quality (for M30) is inconsistent and so is the TUNEL assay. Comparing Figure 1E and 3G, it seems that cell death was prominent at one month in the

normal diet group but not nearly as evident in the HFD group. Do these results have physiological significance? In addition, Figure 3F and 3G were only cited but not described in the text.

☞ As suggested by the reviewer, the images containing the IHC and TUNEL assay data have been modified in the revised manuscript. We apologize for not describing Figures 3F and 3G in the text. The following information has been included in the 'Results' section of the revised manuscript (Page 14, line 322–325):

The results of the TUNEL assay revealed that apoptosis was not significantly different between the sham and cholecystectomy groups. The mRNA levels of *Bax* and *Bcl2* (apoptosis markers) were not significantly different between the sham and cholecystectomy groups (Figure 3G–H).

For the TUNEL assay, the authors described "intensity of TUNEL staining for DNA fragmentation was higher in cholecystectomy group than in the sham group of mice fed a normal diet". One cannot really tell the difference from the data in Figure 1F. Besides, there is a clear distinction between the TUNEL staining at 1 month and 2, 4 months. Does that mean the mice had significant liver cell death one month after surgery but recovered afterwards? These observations should be discussed.

☞ We agree with the reviewer's comments that the TUNEL staining intensity was not markedly different between the sham and cholecystectomy groups. However, the TUNEL staining intensity at month 1 post-surgery was different from that at months 2 and 4 post-surgery. This indicated the recovery of apoptosis. The following information has been included in the revised manuscript (Page 12, line 277–281):

Hepatic Krt18 expression and TUNEL staining intensity (indicates the degree of DNA fragmentation) were not significantly different between the sham and cholecystectomy groups. However, hepatic Krt18 expression and the TUNEL staining intensity at month 1 post-surgery were markedly different from those at months 2 and 4 post-surgery. This indicated that apoptosis at month 1 post-surgery was mitigated at months 2 and 4 post-surgery (Figure 1E–F).

Reviewer C

The study by Kim et al. prospectively investigates the association between NAFLD and cholecystectomy in mice. The study largely finds no differences between mice receiving a cholecystectomy and sham operation on NAFLD parameters. I believe it is highly important to also publish these 'negative findings' which often does not happen. The experiments are performed well and provide valuable data. However, I have major concerns regarding the first experiment, the writing, the interpretation and discussion of the study. For the manuscript to meet the rigorous standards of peer-reviewed scientific publication, it needs a substantial overhaul. Therefore, I would advise against publication in its current form with the possibility of rereview for publication

after major revisions.

Major comments

The first experiment is designed to investigate whether cholecystectomy can lead to or increases the incidence of NAFLD in mice on a normal diet. However, this experiment is inherently flawed to answer this question as it is unlikely that mice on a normal diet will ever develop NAFLD. This does not mean that this experiment is not valuable but it should be very clear in all sections of the manuscript what this experiment demonstrates and what the limitations are (i.e. cholecystectomy does not increase the risk for developing NAFLD on a normal diet but this might not be relevant as NAFLD in mice never develops on a normal diet).

☞ The first experiment was designed to investigate whether cholecystectomy could induce the development of NAFLD in standard diet-fed mice. As the standard diet could not induce NAFLD, we conducted two experiments. In study 2, the effect of cholecystectomy on high-fat diet-induced NAFLD was examined in mice.

The English and scientific writing of the manuscript are poor. The manuscript would significantly improve by a multitude of minor changes to the spelling, sentence formulation and scientific correctness. Several examples are provided in the minor comment section but are not exhaustive. I would highly suggest to carefully go over the manuscript again and preferably also have a native English speaker review the writing.

☞ The revised manuscript has been edited by a professional English editing service.

The discussion is of poor scientific rigor and quality. While the authors acknowledge several studies demonstrating a various range of associations and effects of cholecystectomy on NAFLD, the interpretation and explanation of their own data in regards to these previous published studies is minimal and lacks the addressing of some important distinctions (such as the differences in bile acid metabolism between mice and humans).

☞ As suggested by the reviewer, the following information has been included in the 'Discussion' section of the revised manuscript (Page 16, line 377–381):

Furthermore, the bile acid composition in mice is significantly different from that in humans. The final product of bile acid metabolism in humans is CDCA. In contrast, CDCA is metabolized to MCA in mice. The human intestinal microbiota transforms primary bile acids (CA and CDCA) into secondary bile acids (DCA and LCA). In mice, hepatic 7α-hydroxylase transforms secondary bile acid into primary bile acid (10).

Additionally, the following information has been included as limitations in the 'Discussion' section of the revised manuscript:

The GB resection condition in mice is different from that in humans. In humans,

cholecystectomy is performed to alleviate gallstones or chronic inflammation. Thus, the pre-existing altered metabolism in humans cannot be directly compared to cholecystectomy-induced altered metabolism in mice. (Page 16, line 371-374).
In study 1, the number of mice in the sham group was only 2 at month 4 post-surgery, which may affect the statistical power. (Page 16, line 374-377).

Minor comments
Edit the figures to improve legibility and correctness (examples include the unit of measurement for TG and bile acids, change legends to include description of the figure rather than conclusions).
☞ As suggested by the reviewer, the figure size has been modified, and gene names have been included in the figures. The figure legends have been changed as follows:
Figure 1
(B) Histopathological analysis of liver steatosis, lobular inflammation, ballooning degeneration, and non-alcoholic fatty liver disease activity score (NAS).
(C) Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglycerides, and cholesterol.
(D) Representative images of hematoxylin and eosin-stained liver sections (magnification: 200×).
(E) Representative immunohistochemical images of apoptotic cells in the liver sections probed with M30 antibodies (magnification: 200×).
(F) Apoptosis was determined using terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining (green; magnification: 200×).
(G) M30-positive area was quantified using the image analysis system. mRNA levels of *Bax* and *Bcl2* were determined using quantitative real-time polymerase chain reaction analysis. (Page 18, Line 426–434).

Figure 2
mRNA levels of *Star*, *Abcg5*, and *Hnf4a* (A), *Scd1, Fas*, and *Srebf1c* (B), *Bsep*, *Slc10a1*, and *Cd36* (C), and *Cyp7a1, Cyp27a1, Cyp8b1, Cyp7b1, Cyp46a1*, and *Cyp39a1* (D) were determined using quantitative real-time polymerase chain reaction analysis. All data are presented as mean ± standard error of mean (*p < 0.05; one-way analysis of variance, followed by Šídák multiple comparisons test using GraphPad Prism8 (*p = 0.01–0.05; **p = 0.001–0.01; ***p = 0.001; ****p < 0.0001).
(E) The partial least-squares discriminant analysis (PLS-DA) score scatter plot (left panel) and the permutation test of the PLS-DA (right panel) results. (F) Taurine-conjugated ursodeoxycholic acid (T-UDCA), taurine-conjugated chenodeoxycholic acid (T-CDCA), and taurine-conjugated deoxycholic acid (T-DCA) were selectively represented from the metabolome profile data of PLS-DA and permutation test. (G) Total bile acids were represented from the metabolome profile data of PLS-DA of mice used in study 1. (Page 19, line 445–

454).

Figure 3
(D) Histopathological analysis of liver steatosis, lobular inflammation, ballooning degeneration, and non-alcoholic fatty liver disease activity score (NAS). (E) Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglycerides, and cholesterol. (F) Representative images of hematoxylin and eosin-stained liver sections (magnification: 200×). (G) Representative immunohistochemical images of apoptotic cells in the liver sections probed with M30 antibodies (magnification: 200×). Scale bar = 100 μm. (H) M30-positive area was quantified using the image analysis system. mRNA levels of *Bax* and *Bcl2* were determined using quantitative real-time polymerase chain reaction analysis. (Page 20, line 468–473)

Figure 4
mRNA levels of *Star*, *Abcg5,* and *Hnf4a* (A), *Scd1, Fas,* and *Srebf1c* (B), *Bsep, Slc10a1,* and *Cd36* (C), and *Cyp7a1, Cyp27a1, Cyp39a1, Cyp7b1, Cyp8b1*, and *Cyp46a1* (D) were determined using quantitative real-time polymerase chain reaction analysis. All data are presented as mean ± standard error of mean (*p < 0.05; one-way analysis of variance, followed by Šídák multiple comparisons test using GraphPad Prism8 (*p = 0.01–0.05; **p = 0.001–0.01; ***p = 0.001; ****p < 0.0001). (E) The partial least-squares discriminant analysis (PLS-DA) score scatter plot (left panel) and the permutation test of the PLS-DA (right panel) results. (F) Taurine-conjugated ursodeoxycholic acid (T-UDCA), taurine-conjugated chenodeoxycholic acid (T-CDCA), and taurine-conjugated deoxycholic acid (T-DCA) were selectively represented from the metabolome profile data of PLS-DA and permutation test. (G) Total bile acids were selectively represented from the metabolome profile data of PLS-DA of mice used in study 2. (Page 20, line 483–492)

Figure 2: make the expression graphs more legible, remove GAPDH and preferably put the gene name above the graph.
☞ The figure has been modified as per the reviewer's suggestions.

Be consistent and correct in usage of gene/protein names. Make it clear whether the writing is about gene or protein in expression. When it is protein expression in mice the correct formulation is e.g. Cyp7a1 (italics, only first latter capital), when it is protein it is CYP7A1 (no italics, all capitals). Please change this in all applicable sections of the manuscript.
☞ The official mouse gene/protein symbols have been used in all figures.

Please make a more clear and coherent division of the figures and subsections of the results. For example, Figure 1E and F and Figure 2A are discussed together. Please combine these to the same Figure. This makes the section much easier to

follow. Add where necessary reasoning why certain parameters are presented. For example, the CK-18 and TUNEL staining, why where they assessed and what do changes imply.

☞ As suggested by the reviewer, Figure 2A has been moved to Figure 1G, while Figure 4A has been moved to Figure 3H in the revised manuscript.

Generally, some figures are large, consider splitting certain parts up if it makes scientific sense and improves legibility of the manuscript.

☞ Figure 2A has been moved to Figure 1, while Figure 4A has been moved to Figure 3 in the revised manuscript.

The results include several surprising findings such as Cyp7a1 only being decreased after 1 and 4 months. Such findings need to be discussed.

☞ The following information has been included in the 'Discussion' section along with the references (Page 17, line 399–404):

CYP7A1 is a rate-limiting enzyme in the classic bile acid synthesis pathway (11). One study reported that cholecystectomy significantly upregulated the expression of *Cyp7a1* in female mice without affecting the bodyweight (12). Nuclear receptors, such as PXR, PPARα, and LXRα regulate *Cyp7a1* expression (10). In this study, the expression levels of *Cyp7a1* and *Cyp27a1* were significantly downregulated at months 1 and 4 post-surgery. The underlying mechanism must be elucidated in the future.

In the material and methods, please define the diets and contents, include supplier.

☞ The details of the supplier for 60% high-fat (HF) diet (Research Diets Inc, NJ, USA) (Page 6, line 137, Page 7, line 164) and mice (Orient Animal Laboratory, Seoul, South Korea) (Page 7, line 162) have been included in the revised manuscript.

Specific example comments in regards to the writing:

Make the title more adequately reflect the actual study, change it from a question to the main conclusion or goal (also make sure it is clear that this is a murine study).

☞ The title of the revised manuscript is as follows:

Correlation between cholecystectomy and development of non-alcoholic liver disease in the mouse model (Page 1, line 3–4)

Line 31-32: the existence of an association does not imply a mechanism and therefore this sentence is incorrect and should be rephrased.

☞ As suggested by the reviewer, the word 'mechanism' has been changed to 'correlation' in the revised manuscript. (Page 2, line 36)

Line 36: 'was' should be 'were'. Full sentence should be rephrased, example:

‘Liver histology was evaluated after 2, 3, 4 and 6 months without sacrificing the mice’.

☞ We apologize for the error. The sentence has been modified in the revised manuscript as follows:

In study 1, 20 standard diet-fed C57BL/6N mice were sacrificed at months 1, 2, and 4 post-surgery. However, in study 2, 25 high-fat diet-induced NAFLD C57BL/6N mice were biopsied at months 2 and 3 post-surgery and sacrificed at month 6 post-surgery. (Page 2, line 40–43)

Line 36-38: change this based on what are the most important outcomes of the study. For example, one could argue that the degree of steatosis, triglyceride accumulation and bile acid metabolism are most important.

☞ The following information has been included in the ‘Results’ section of the revised manuscript (Page 2, line 52–56):

Cholecystectomy significantly downregulated *Cyp7a1* and *Cyp27a1* mRNA levels at months 1 and 4 post-surgery but did not affect the degree of steatosis and triglyceride levels. Analysis of bile acid metabolism revealed that taurine-conjugated bile acids were significantly downregulated in the standard diet-fed and high-fat diet-fed mice, but the histological and biochemical parameters were not markedly different.

Line 41: change ‘fat’ to ‘lipid’, ‘de novo’ should be in italics

☞ The change suggested by the reviewer was incorporated in the revised manuscript. (Page 2, line 48)

Line 46: conclusion should be reformulated to more adequately reflect the outcomes and future perspectives. For example, now the conclusion has no mention of NAFLD which is clearly what the authors studied (/intended to study).

☞ The ‘Conclusion’ section was modified in the revised manuscript as follows (Page 3, line 58–62):

Cholecystectomy did not increase the incidence of NAFLD in standard diet-fed mice. Additionally, NAFLD incidence was not significantly different between the HF diet-fed sham and cholecystectomy groups. Furthermore, the histological parameters were not markedly different between the sham and cholecystectomy groups fed on standard or HF diet. These findings suggest that cholecystectomy does not induce NAFLD.

Line 53: be more specific

☞ This line was modified as follows in the revised manuscript (Page 4, line 90–91):

Glucose homeostasis is impaired in patients undergoing cholecystectomy due to increased postprandial glucose fluctuations and decreased postprandial duodenal bile acid concentration (13).

Line 56: be more specific, what happens to FXR activation?
☞ This line was modified in the revised manuscript as follows (Page 4, line 95–96): Activated FXR improves insulin sensitivity and downregulates the plasma glucose level by downregulating gluconeogenesis and upregulating glycogen synthesis.

Line 60-62: give a better description of NAFLD, include lifestyle, what is already known and where the gap of knowledge is (lack of comprehensive understanding of the pathophysiology).
☞ This section has been edited as follows in the revised manuscript (Page 4, lines 106–107): Globally, rapid changes in lifestyle and diet have contributed to an increased prevalence of obesity and NAFLD. However, the ability of cholecystectomy to induce NAFLD is unknown.

Line 64-66: what makes this study different?
☞ This (References 21) is a large-scale follow-up study that investigated the correlation between NAFLD and cholecystectomy among the Chinese population. In contrast, we aimed to determine whether cholecystectomy increases the incidence of NAFLD in mice fed on standard or high-fat diet.

Line 66-68: why is this study controversial? Why do the authors mention it?
☞ We apologize for the lack of clarity. Our intended meaning was to indicate that the correlation between NAFLD and cholecystectomy is unclear. The phrase 'which is still controversial' has been removed in the revised manuscript.

Line 69: please be consistent and correct in using words such as occurrence and incidence. They have very specific and different definitions. Make sure the wording used is in line with what can be inferred from the literature.
☞ As recommended by the reviewer, the word ‘incidence’ has been replaced with ‘occurrence’ (Page 5, line 118).

Result section: Generally, this section can still significantly improve from more precise writing. Example: please use ‘the’ before ‘sham group’ (it also lacks sometimes, but less when describing ‘the’ cholecystectomy group) when describing comparisons.
☞ As recommended by the reviewer, the article “the” has been included before the group names (Page 13, line 303).

Line 112-113: remove sentence ‘Only…group’
☞ As suggested by the reviewer, this sentence has been removed in the revised manuscript.

Line 140: I think(/hope) this is a typo as the study does not show a causal relationship. Please remove and rephrase.

☞ This line has been changed as follows (Page 15, line 337):
This study investigated whether cholecystectomy could increase the incidence of fatty liver in mice.

Line 141-143: please be more specific in defining the findings.
☞ This section has been modified as follows (Page 15, line 340–344):
Cholecystectomy did not induce fatty liver in standard diet-fed mice. Furthermore, cholecystectomy did not increase the risk of fatty liver or exacerbate fatty liver in HF diet-fed mice. The major strength of this study is the continuous monitoring of the development of fatty liver through liver biopsy without sacrificing mice after cholecystectomy.

Line 174: you do not show causality of taurine-conjugated bile acids resulting in decreased in anti-oxidative and anti-apoptotic effects. Please rephrase.
☞ This section has been modified as follows (Page 17, line 396–398):
Reduction of taurine-conjugated bile acids may be associated with anti-oxidative and anti-apoptotic effects. Further studies are needed to elucidate the underlying mechanisms.

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