Dear editors and reviewers,

We sincerely appreciate your insightful comments and constructive advices regarding our manuscript entitled “Trimethylamine N-Oxide Attenuates High-Fat High-Cholesterol Diet-Induced Steatohepatitis by Reducing Hepatic Cholesterol Overload in Rats” (Manuscript No. 47797). We have made corresponding modifications which are highlighted in the revised manuscript. We hope these corrections would meet with your approvals and the point-by-point responses are addressed as follows.

Once again, thank you very much for your comments and suggestions.

Sincerely yours,

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Reviewer #1：

In general, this manuscript provides the useful information about TMAO modulate steatohepatitis in rats fed high-fat and high-cholesterol diet. However, there are some problems and flaws in presentation. Specifically, the discussion in this MS is inadequate. I hope that my comments are very useful for the improvement of this research.

Reply: We would like to thank Reviewer #1 for the insightful and constructive comments. We have now revised our manuscript according to these comments.

1. The authors should review the statistics. For this grouping, I think a two-way ANOVA is better.

Reply: Thanks for the insightful comments. Two-way analysis of variance (ANOVA) examines the influence of two different categorical independent variables on one continuous dependent variable and aims to assessing the main effect of each independent variable and if there is any interaction between them[1-3]. We have checked the statistical significance among more than two groups by analysis of two-way ANOVA and modified the descriptions in the materials and methods section of the revised manuscript.

2. It is necessary to consider whether the various effects obtained in this experiment are the effects of TMAO or TMA, that is metabolized from TMAO.

Reply: Thanks for the comments. Under physiological conditions, TMA is mainly produced by the gut commensal bacteria utilizing the dietary choline or L-carnitine as precursors. It has been shown that the bioavailability and absorption of orally consumed TMAO is near total without requiring

processing by gut microbiota[4]. Thus, the conversion of TMAO to TMA by the gut bacteria is minimal. The effect of TMA on the HFHC diet-induced steatohepatitis will be investigated in our further studies.

3. Authors showed that TMAO alter the gut microbial profile and restore the diversity of gut flora. But the relationship between the change of gut flora and NASH is not known in the current consideration. Please specifically discuss the relationship between this change in gut flora and NASH.

Reply: Thanks for the constructive comments. We have newly conducted the PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) analysis to predict functional alterations of the gut microbiota by HFHC diet and TMAO treatment. As shown in revised Fig. 6G, TMAO partially reversed the HFHC diet-induced alterations of pathways related to nutrient and energy metabolism, suggesting the improved microbial profile by TMAO may facilitate the restoration of energy homeostasis under nutrients overload conditions. Gut dysbiosis is a hallmark of NAFLD/NASH, in which reduced diversity of gut bacteria is an important characteristic[5, 6]. Gut dysbiosis leads to gut bacteria translocation due to the impaired intestinal barrier and increased production of harmful microbial metabolites such as LPS and endogenous ethanol, which contribute to the pathogenesis and progression of NAFLD/NASH[7]. The restoration of the gut microbiota diversity by TMAO may mediate its beneficial role in HFHC diet-induced steatohepatitis. Further studies concerning the modulatory effect of TMAO on gut microbiota and consequent metabolic benefits are needed. We have added these descriptions in the discussion section of the revised manuscript.

4. It would be better to measure the hepatic cholesterol level.

Reply: Thanks for the comments. We have measured the hepatic level of cholesterol. As shown in Fig. 4A, the hepatic level of cholesterol was greatly elevated in the HFHC diet-fed rats, and was significantly decreased with TMAO treatment.

5. Authors should measure the fecal cholesterol content. In this study, the intake of TMAO decreased the NPC1L1 and increased the ABCG5/8 levels in intestinal mucosa. These data do not indicate whether cholesterol absorption is inhibited. Cholesterol is absorbed not only by the route via NPC1L1 but also by passive transport in intestine.

Reply: Thanks for the comments. NPC1L1 plays a vital role in the regulation of intestinal cholesterol absorption. It has been demonstrated that NPC1L1 KO mice displayed a 97% reduction in intestinal cholesterol absorption compared to WT mice[8]. Our results show that TMAO treatment significantly decreased the expression levels of NPC1L1 in jejunum and ileum of HFHC diet-fed rats, which may facilitate the inhibition of intestinal cholesterol absorption by TMAO treatment under HFHC diet

feeding conditions. Furthermore, TMAO has been shown to decrease the intestinal cholesterol absorption by 26% in mice[9], which supports our results. The effect of TMAO on the passive cholesterol transportation will be further investigated in our future study.

6. Authors should describe the conditions of dissection including dissection time, anesthetic, etc.

Reply: Thanks for the constructive comments. At the end of the 16th week, the rats were fasted overnight and were euthanized by pentobarbital before the tissues were harvested in the morning[10]. We have added these descriptions in the materials and methods section of the revised manuscript.

7. There is no discussion as to whether hepatitis is induced by feeding HFHC diet. Authors should consider this point. I hope that my comments are very useful for the improvement of this manuscript.

Reply: Thanks for the insightful comments. In our study, the steatohepatitis was successfully induced by HFHC diet feeding for 16 weeks, as manifested by the presence of moderate to severe hepatic steatosis with lobular inflammation and hepatocyte ballooning (Fig. 1F). We have added these descriptions in the results section of the revised manuscript.

Reviewer #2：

Zhao Zh et al. investigated the effects of TMAO on the HFHC diet-induced steatohepatitis in rats. TMAO alleviated inflammation and hepatocyte ballooning, reduced ALT and AST, and decreased ER stress markers. Hepatic and serum cholesterol levels were reduced and the expression of NPC1L1 was reduced. TMAO altered the gun microbial profile and restored the diversity of gut flora.

1. The cholesterol markers, lathosterol and desmosterol, and cholesterol absorption markers, β-sitosterol and campesterol, should be measured to determine whether major target of TMAO is absorption of cholesterol

Reply: Thanks for the comments. In the present study, we found that TMAO treatment significantly decreased the expression of NPC1L1 and increased the expression of ABCG5/8 in the small intestines, which may facilitate the inhibition of intestinal cholesterol absorption by TMAO. Moreover, it has been shown that TMAO decreases intestinal cholesterol absorption by 26% using ^{14}C-labeled cholesterol, which supports our results and suggests that intestinal cholesterol absorption is a major target of TMAO[9].

2. Since the diversity changes in gut flora may induce the changes in short fatty acids in intestine, the authors should investigate the profile of fatty acids in the feces.

Reply: Thanks for the comments. Our results showed that TMAO restored the diversity of gut microbiota in HFHC diet-fed rats. However, LEfSe analysis demonstrated that the bacteria species regulated by TMAO are not short-chain fatty acids (SCFA)-producing bacteria (Fig. 6F). Therefore,

it is highly probable that the fecal profile of SCFA is not altered by TMAO treatment. The consequence of the improved gut microbiota diversity by TMAO needs to be further investigated in our future study.

3. Transplantation of feces from TMAO-treated animals ameliorate the steatohepatitis in rats?

Reply: Thanks for the comments. In the present study, we found that the attenuation of hepatic ER stress and cell death due to the hepatic cholesterol overload may mediate the protective role of TMAO in the HFHC diet-induced steatohepatitis. The restoration of gut microbiota diversity by TMAO may be a consequence of the altered bile acids metabolism, whose contribution to the beneficial effect of TMAO may not be a major one. Further investigations concerning the effect of TMAO on gut microbiota and metabolic consequences are needed. We have added discussions on the limitations concerning this point in the revised manuscript.

4. The bile acids profiling by MS/MS analysis should be performed.

Reply: Thanks for the comments. It has been reported that TMAO alters the expression levels of the key bile acid synthetic enzymes Cyp7a1 and Cyp27a1 and causes smaller total bile acids pool size[9]. Our results also showed that the serum level of total bile acids was decreased by TMAO treatment in HFHC diet-fed rats (Fig.4E). The bile acids profiling may provide clues on the gut microbiota alterations but helps little to elucidate the protective role of TMAO in HFHC diet-induced steatohepatitis. The effect of TMAO on bile acids metabolism will be investigated in our further studies.

Reviewer #3：

In the present study authors have examined the effect of trimethylamine oxide (TMAO) administered orally for 8 weeks on the progression of high fat diet-induced non-alcoholic fatti liver disease. The results indicate that TMAO improved liver histology, reduced plasma transaminase activities, decreased hepatocyte ER stress and hepatocyte apoptosis, decreased the expression of cholesterol-absorbing protein, NPC1L1, and increased the expression of intestinal cholesterol exporters, ABCG5 and ABCG8. These effects correlate with the improvement of plasma lipid profile but are achieved without reduction of body weight or adiposity scores. Finally, TMAO restored diversity of intestinal flora which was restricted in high fat diet-fed rats. The results are of interest and the paper is well-written. Nevertheless, there are some concerns regarding experimental design and data interpretation.

Reply: We would like to thank Reviewer #3 for the positive comments. We have now revised our manuscript according to these comments.

1. The implications of the findings are unclear. TMA is well-known to be involved in the pathogenesis of cardiovascular diseases which often accompany NAFLD in patients with obesity/metabolic syndrome. Therefore, recommending TMAO therapy would be highly questionable.

Reply: Thanks for the insightful comments. We agree on the concern that TMAO may be involved in the pathogenesis of cardiovascular diseases (CVD). The potential harmful role of TMAO for the cardiovascular system limits the application value of our study. Our results highlighted the involvement of TMAO in the cholesterol metabolism and the beneficial role of TMAO in attenuating hepatic ER stress and cell death induced by hepatic cholesterol overload in the context of HFHC-diet induced steatohepatitis. The dose and delivery ways of TMAO need to be further defined and the effect of TMAO on the cardiovascular system will be further verified before TMAO is considered for therapeutic potential. We have toned down the clinical implications and added the discussions on the limitations of the present study in the revised manuscript.

2. Why this specific dose of TMAO was used?

Reply: Thanks for the constructive comments. The function of TMAO has been universally investigated using normal chow diet supplemented with TMAO (0.12% w/w)[11-13]. We transformed this mass fraction into a dose of 120 mg/kg for TMAO gavage based on the food intake of 10g/100g body weight/day by the rats. It has also been shown that TMAO supplement at the dose of 120 mg/kg induces a significant elevation of serum TMAO level[14]. Furthermore, TMAO treatment at lower dose may not exert negative effects on the circulatory system[15] and the minimal effective dose of TMAO in the NASH intervention will be defined in our further study. We have added these descriptions in the discussion section of the revised manuscript.

3. It would be of interest to measure TMA and TMAO levels in animals with NAFLD vs. control group as well as in TMAO-treated rats.

Reply: Thanks for the insightful comments. It has been shown that patients with NAFLD possess higher circulating TMAO level compared with healthy population[16]. Also, the HFD feeding causes an induction of circulating TMAO level in mice[17]. And TMAO treatment induces a striking elevation of serum TMAO but limited elevation of serum TMA[4]. The alterations of TMA and TMAO levels in rats need to be examined in our future study.

4. It would be of interest to compare the effect of TMAO with its precursor, TMA.

Reply: Thanks for the comments. Please see the same answer in Reviewer #1 comment 2.

5. What buffer was used for sample homogenization/lysis for Western blot? Were any protease inhibitors added during sample processing?

Reply: Thanks for the thoughtful comments. The lysis buffer recipe was as follows: 50 mM Tris-HCl,

pH 8.0, 1% (v/v) Nonidet P-40, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 μg/ml pepstatin. Phenylmethylsulfonyl fluoride, aprotinin, leupeptin, and pepstatin were used as protease inhibitors. We have added this information in the materials and methods section of the revised manuscript.

6. More details about qRT-PCR should be presented according to MIQUE guidelines. Data such as primer sequence, amplification cycle conditions (duration and temperatures of consecutive phases), methods of assessing mRNA quality-quantity and results calculation should be included.

Reply: Thanks for the constructive comments. The following quantitative RT-PCR primer sequences were used: CCCCAAACTCCCTCATAAGCA (forward) and TATCCCCCAACAGCAAGGAAG (reverse) for rat NPC1L1; AAAAGGCTGCTGATTGCCC (forward) and GCAGGACAATCTGAGCAAAGAA (reverse) for rat ABCA1; TTGGCCCCTCACTTAATTGGA (forward) and GGACCATACCAAGCAGCACAAG (reverse) for rat ABCG5; ACTGCCATGGACCTGAACTCA (forward) and GCTGATGCCAATGACGATGA (reverse) for rat ABCG8; GGGCAGCCCAGAACATCAT (forward) and CCAGTGAGCTTCCCGTTCAG (reverse) for rat GAPDH. The procedure was as follows: the initial step was 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. The quantity and quality of extracted RNA was assessed using NanoDrop 2000 (Thermo Fisher Scientific, Wilmington, DE). The 260/280 and 260/230 ratios were used for RNA purity assessment[18]. Data were analyzed using the ΔΔCT threshold cycle method. The mRNA levels of genes were normalized to those of GAPDH and presented as relative levels to control. We have added these descriptions in the materials and methods section of revised manuscript.

7. The rate of apoptosis was estimated according to cleaved caspase-3 only. JNK phosphorylation may be but is not always associated with apoptosis; the role of this kinase in apoptosis depends on the experimental system. Overall, the experimental approach regarding apoptosis used in this study is insufficient.

Reply: Thanks for the insightful comments. It has been well known that JNK signaling pathways play important roles in the hepatocytes apoptosis of NASH[19-21]. However, we agree on the opinion that the downstream effects of JNK activation are very complicated. Besides apoptosis, JNK may also participate in regulation of necrosis, inflammation and mitochondria dysfunction[22-25]. Our results showed that TMAO reduced ER stress-induced hepatocyte death in the HFHC diet-induced steatohepatitis, in which the attenuation of JNK activation was involved. We have modified the descriptions concerning the implication of JNK phosphorylation in the results section of the revised

manuscript.

8. Page 10: serum level of triglycerides was NOT altered by TMAO; the text needs revision.

Reply: Thanks for the kind reminder. We have corrected this description in the results section of the revised manuscript.

9. Why the expression of intestinal cholesterol transporters was measured only at the mRNA but not at the protein level?

Reply: Thanks for the comments. It has been shown that the regulation of intestinal cholesterol transporters is mainly in transcriptional level. Several transcription factors are involved in the regulation of NPC1L1 expression, such as HNF4α, PPARα and SREBP2[26-28]. Similarly, the expression of ABCA1 is regulated by LXR/RXR[29] and the expression of ABCG5/8 are transcriptionally modulated by the nuclear orphan receptor liver receptor homologue-1 (LRH-1) and HNF4α[30, 31]. Therefore, we assessed the effect of TMAO treatment on the expression of intestinal cholesterol transporters at mRNA level. We will examine the effect of TMAO on the post-transcriptional regulation of intestinal cholesterol transporters in our future study.

10. It would be of interest to discuss how much of the effect of TMAO is exerted directly in the liver and how much results from the improvement of plasma lipids, insulin sensitivity, etc.

Reply: Thanks for the insightful comments. It has been reported that TMAO participates in the regulation of bile acids metabolism in the liver[9]. The direct effect of TMAO on the liver in the context of metabolic disorders is largely unknown. Ex vivo and in vitro approaches will be performed in our further studies to investigate the direct effect of TMAO on the metabolic regulations in the liver.

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