Dear Dr Lian-Sheng Ma,
Science Editor, Company Editor-in-Chief,

Please find enclosed the revised version of the manuscript Manuscript NO.: 69541: **"Dual therapy with zinc acetate and rifaximin prevents from ethanol-induced liver fibrosis by maintaining intestinal barrier integrity"** by Yuki Fujimoto, Kosuke Kaji, Norihisa Nishimura, Masahide Enomoto, Koji Murata, Soichi Takeda, Hiroaki Takaya, Hideto Kawaratani, Kei Moriya, Tadashi Namisaki, Takemi Akahane, Hitoshi Yoshiji for publication as an article in ***World Journal of Gastroenterology***.

We carefully evaluated the concerns raised by the Reviewer, performed the requested analyses, modified the text and added new data as suggested. Detailed responses to each of the Reviewers' comments are provided in the attached pages.

We would like to extend our thanks to the Reviewers for providing helpful and constructive comments on our work and to you for a chance to resubmit our manuscript.

I hope that we satisfactory addressed yours and Reviewers concerns and the revised manuscript is now acceptable for publication in ***World Journal of Gastroenterology***.

Sincerely,

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We thank the Reviewer for his/her positive evaluation of our work.

Reviewer #1:
**Scientific Quality:** Grade C (Good)
**Language Quality:** Grade B (Minor language polishing)
**Conclusion:** Major revision
**Specific Comments to Authors:** Overview of the manuscript The aim of this manuscript is to investigate the preventive effect of combined zinc supplementation and rifaximin from ALD-related liver fibrosis induced by ethanol+CCl4 in mice, which maintain intestinal barrier integrity and reduce hepatic LPS exposure, leading to Kupffer cell expansion and hepatic stellate cell via inhibiting the toll-like receptor 4 pathway. This is a well-written paper containing interesting idea which somewhat merit publication after several important issues to be addressed.

Details
1. Abstract section needs to use a structural style, that is, four parts for Aim, Method, Results, and Conclusion, limited to 250 words.
Answer
We rewrote Abstract according to the structural style, limited to 250 words (Page2,Line11 to Page3,Line12).

2. As described, rifaximin inhibited toxin-induced apoptosis and deprivation of tight junction proteins (TJPs) in human intestinal cells through pregnane X receptor (PXR)-dependent inhibition of the TLR4/ MyD88/ NF-κB pathway. Should the authors supplement PXR-related tests?
Answer
We appreciate for the reviewer's important comments.
In our original manuscript, we showed that ketoconazole (KCZ), a PXR inhibitor could cancel the rifaximin-induced increase of TJPs expression. However, KCZ did not specifically inhibit PXR. Thus, we additively investigated whether more specific PXR inhibitor, SPA70 (a potent and selective human PXR antagonist) could cancel the rifaximin-induced increase of TJPs expression as well as deactivation of NF-κB in EtOH or LPS-stimulated Caco-2 cells to elucidate the effect of rifaximin on restoration of EtOH or LPS-stimulated decreases of TJPs by PXR-mediated inhibition of TLR4/NF-κB signaling. As shown in the new Figure 6F, SPA70-mediated PXR inhibition apparently decreased the protein levels of ZO-1 and Occludin as compared to those in rifaximin-treated Caco-2 cells,

and these decreases by SPA70 were accompanied by increase of p65 phosphorylation. These results strongly support that rifaximin-mediated restoration of TJPs was dependent on the PXR-mediated inhibition of TLR4/NF-κB pathway. Moreover, we found that pharmacological inhibition of PI3K by LY294002 canceled the zinc acetate-induced increase of TJPs expression by inhibiting AKT phosphorylation in EtOH or LPS-stimulated Caco-2 cells (New Figure 6E).
We added these results in the revised manuscript (Page22/Line8 to 22).

3. Most of the tests are based on PCR. However, due to the influence of post transcriptional regulation etc., mRNA does not necessarily affect the phenotype. This is also the core bug of this research. They should use multiple detection methods to explain the same phenotypic change, rather than explain different phenotypic changes by the same detection method, which is easy to produce a certain degree of arbitrary conclusion. Has the author considered how to explain this issue?
Answer
We really agree with the reviewer's comments. As the reviewer mentioned, most of our results were based on the quantitative analysis of mRNA expressions which is not necessarily relevant to elucidate the molecular mechanisms related to the effects of both drugs in the current model.
Gratefully, the reviewer provided us quite important other suggestions including the requests of protein levels for several molecules, especially for tight junction proteins in other issues. Therefore, we additively assessed the measurement of protein levels by western blotting and/or immunohistochemistry according to other comments.

4. Figure 1. C57BL/6 mice were treated with ETOH + CCl4 for 8 weeks; however, the author is requested to provide the reasons for choosing 8 weeks as the treatment time, for instance, previous literature, previous research of their own or time-dependent gradient experiments, etc. Serum/Hepatic concentration of oxidative stress (CAT, MDA, and SOD), liver function (ALB, ALP, and GGT) and blood lipid (TG ,TC, HDL-C, and LDL-C) should be determined, meanwhile, the author should provide Oil Red O staining results in liver tissues and quantitative analysis.
Answer
We really appreciate the reviewer's comments.
We chose 8 weeks as the treatment time in reference to our previous report (Ishida K et al. *J Nutr Biochem*. 2021; 89: 108573). We added this information in the revised Materials and Methods (Page 7/Line 16 to 18).

According to the reviewer's comments, we added the results of hepatic concentrations of CAT, MDA and SOD, serum levels of ALB, ALP, and GGT and blood lipid TG ,TC, HDL-C, and LDL-C. As shown in the new Figure 2D-2F, hepatic MDA levels increased and oppositely hepatic antioxidant CAT and SOD levels decreased in the EtOH+$CCl_4$-treated mice indicating the accumulation of oxidative stress in these mice. These changes of oxidative markers coincide with the results from previous reports (Su et al. *Clin Exp Pharmacol Physiol*. 2014, 41:73-80, Han et al. *J Nutr Sci Vitaminol*. 2017, 63:35-43). Interestingly, these changes were attenuated by treatment with zinc or rifaximin which were augmented by both combination (New Figure 2D-2F). These findings suggest that both drugs suppress the oxidative stress in the liver of EtOH+$CCl_4$-treated mice. We added these results in the revised manuscript (Page16/Line14 to 21). As shown in the new Supplementary Figure 1C, serum Alb levels as well as ALP levels were not changed in EtOH+$CCl_4$-treated mice, and these levels were not altered by treatment with zinc and/or rifaximin. Serum GGT levels were elevated in EtOH+$CCl_4$-treated mice as compared with control mice and unchanged by treatments with zinc and/or rifaximin (New Supplementary Figure 1C). Serum TG levels were elevated in EtOH+$CCl_4$-treated mice which were attenuated by treatments with zinc and/or rifaximin, while there were no significant differences in serum T-cho, HDL-cho and LDL-cho levels among the experimental groups (New Supplementary Figure 1D and 1E). We added these results in the revised manuscript (Page15/Line8 to 18).
Moreover, we showed the representative pictures of liver sections stained by Oil Red O (New Figure 1F) and the results of semi-quantitative analysis (New Figure 1G). These results coincide with the changes of hepatic TG levels (New Figure 1H). We added these results in the revised manuscript (Page15/Line19 to 23).

5. Figure 2. What are the changes of metabolic enzymes related to alcohol, acetaldehyde and cytochrome（CYP2E1）in mice liver tissues when induced by Lieber -Decarli liquid diet and CCl4? Could rescue work after the dual-intervention with zinc acetate and rifaximin? Such experiment should be supplemented. There are multiple biomarkers of macrophages, such as F4/80, CD68, and RAM11, etc. The reasons for selecting F4/80 positive cells in this study should be stated. In addition, the author should consider to distinguish M1 and M2 subtypes macrophage after treated with Lieber -Decarli liquid diet and CCl4.

Answer

We fully agree with the reviewer's suggestions that the effects of zinc and rifaximin on

hepatic metabolization of ethanol and acetaldehyde is quite important in the present study. Thus, we newly evaluated the activities of alcohol dehydrogenase 1 (ADH1), aldehyde dehydrogenase 2 (ALDH2) and CYP2E1 in the liver tissues of experimental group. As shown in the new Figure 2A and 2B, EtOH and $CCl_4$ administration significantly decreased both ADH1 and ALDH2 activities in accordance with the results from recent report (Ren et al. Food Chem Toxicol. 2020 Feb;136:111070). Treatment with zinc acetate significantly suppressed the decline of ADH1 activity but did not affect ALDH2 in the EtOH+$CCl_4$-treated mice. On the other hand, neither ADH1 nor ALDH2 activities were changed by treatment with rifaximin. As shown in the new Figure 2C, CYP2E1 activity was increased in the EtOH+$CCl_4$-treated mice, and zinc acetate significantly suppressed the increase of CYP2E1 activity but rifaximin did not affected. These findings indicate that zinc acetate would attenuate MEOS-mediated ROS accumulation. We added these results in the revised manuscript (Page16/Line3 to 21).

Regarding to the choice of macrophage marker, I think F4/80 is good because there is a reliable source of antibodies and it stains well. I think that CD68 is not specific indicating that fibroblast and tumor cell lines, which are not blood cells, can also be positive (Ann Rheum Dis. 2005;64(2):342-3.) F4/80 can be positive for dendritic cells, but dendritic cells are also blood cells, so it is better than CD68. Since immunohistochemistry cannot be used to classify cells in detail, it is better to use a marker that can only be used for broad classification, and in that sense, markers that cannot separate blood cells from fibroblast and even tumor cells (CD68) that cannot separate blood cells from fibroblast and tumor cells is not very suitable.

Finally, we thank for the reviewer's comment. We showed the mRNA levels of markers related to M1 macrophage, including IL-1β, IL-6, TNF-α and iNOS (New Figure 3D) and M2 macrophage, including IL-10, ARG1 and CD163 (New Figure 3E) in the experimental groups. The hepatic mRNA expressions related to both M1- and M2-polarized macrophages were increased in EtOH+CCl4 treated mice. The combination treatment significantly suppressed the increases of M1-polarized macrophages while it had little effect on M2-polarized macrophages in the liver of ethanol plus $CCl_4$-treated mice. We added these results in the revised manuscript (Page17/Line9 to 17).

6. Figure 3. The changes of NF-κB and IK-κB are opposite. Should the authors consider using IK-κB to verify the Western Blotting results. Besides, WB bands of p-p65 in E/RFX, p65 in E/Zn and E/RFX were quite different in only 2 samples. Better WB images should be provided. Experiment with western blotting and immunohistochemistry should be supplemented to evaluate protein changes of extracellular matrix accumulation in liver

fibrosis of E/V group.

Answer

We really appreciate for the reviewer's suggestion and apologize for the confusing data. In our understanding, LPS or CCl4 stimulates the phosphorylation of IKKα/β and in turn induces the degradation of IκBα leading to increase of NF-κB. Based on this pathway, our new analysis demonstrated that E/V group showed the increased levels of p-IKKα/β as well as decreased levels of IκBα and oppositely increased levels of NF-κB levels as compared with C/V group in response to increased expression of hepatic LBP. According to the decrease in hepatic LBP expressions by treatment with zinc and/or rifaximin, these changes were attenuated. We updated these results as the new Figure 3H and added the description about these findings (Page18/Line3 to 8).

Moreover, as the reviewer's suggestion, we added the data of protein levels of COL-1 evaluated by immunohistochemistry (the new Figure 4A and 4D) and western blotting (the new Figure 4E) (Page18, Line23 to Page 19, Line 4).

7. Figure 4. Western blotting for tight junction protein (such as Zo1 and Ocln) and immunofluorescence for Ocln protein should be added. High-magnification of HE staining and immunofluorescence images should also be provided.

Answer

According to the reviewer's comment, we added the results of WB for ZO-1 and Occludin (New Figure 5D) and immunofluorescence for Occuludin (New Figure 5A and 5C). Moreover, we replaced the pictures of HE and immunofluorescence by containing the pictures with high-magnification in their upper right (New Figure 5A). We added these results in the revised manuscript (Page20/Line7 to 12).

8. Figure 5. The authors should provide changes in cell electrical resistance values with line chart during cell culturing.

Concentration of EtOH and LPS need to detected at lower compartment after 5% EtOH or 2μg/ml LPS added to culture medium of upper compartment in Transwell plates. In the same way, protein levels of tight junction should be detected.

Answer

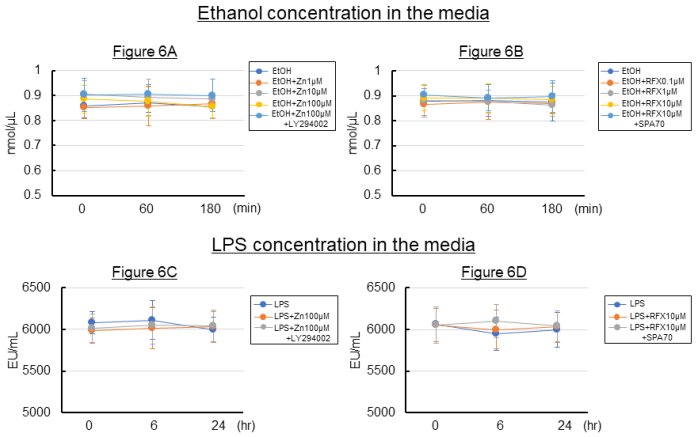
We appreciate the reviewer's comment. According to the reviewer's suggestion, the chronological changes in TEER during cell culturing are shown in the new Figure 6A-6D. Also, we measured the concentration of EtOH and LPS in the media at lower compartment by using Ethanol Colorimetric/Fluorometric Assay Kit (BioVision) and Limulus

Amebocyte Lysate Kinetic-QCL™ kit (Lonza), respectively.

As shown below, we did not observe the significant dispersion in the concentration of EtOH (detected approximately as 5%EtOH=0.86197 nmol/μl) in the media among each sample in each condition for new Figures 6A and 6B. Similarly, the LPS concentrations were unchanged by the culture condition (detected approximately as 2μg/ml=6000EU/ml) for new Figures 6C and 6D.

Moreover, protein levels of TJPs (ZO-1 and Occuludin) are detected by western blotting assays (the new Figure 6E and 6F).

Figure only for the reviewer 1 (Question 8)




9. Supplementary Figure 1. BUN should be detected together with serum creatinine for kidney function.

Answer

We agree with the reviewer's comment. We added the result of serum creatinine with BUN as the new Supplementary Figure 1B.

10. Besides, for the sake of data transparency, scatter chart or histogram should be used for the full text statistics.

Answer

We appreciate for the reviewer's suggestion. According to the reviewer's comment, we replaced all data by showing with scatter chart.