Dear Prof. Ke Chen,

Thank you very much for your letter and advice. Our manuscript entitled "FO alleviates liver injury induced by intestinal IR via AMPK/SIRT-1/Autophagy pathway" (Manuscript NO: 36732). We have revised the manuscript as your and the reviewers' comments, and would like to re-submit it for your consideration. The amendments are highlighted in red in the revised manuscript. Point by point responses to the reviewers' comments are listed as follows.

Replies to Reviewer #1:

Thank you for your helpful suggestion. We have carefully studied these comments, and the manuscript has been revised in depth according to these suggestions.

1. In the Introduction section, the authors should briefly introduce the circumstance of clinical treatment for liver injury induced by intestinal IR, and compare the therapeutic effect of FO to the drugs already in use.

There are seldom article directly describe the treatment for liver injury induced by intestinal IR in patients. However, The circumstance of parenteral nutrition associated liver disease in intestinal failure patient which always share the intestinal ischemia conditions have been reported in many studies [1-2]. And we have cited the article (ref 17.) in Paragraph 4 of introduction in the manuscript.

2. In the section of Results 3.1, paragraph 1, line 3, the authors said "the damage of liver was significantly improved by pretreating with FO emulsion, and the histopathological score was significantly reduced compared with the I/R group", which was inconsistent with the results exhibited in Fig. 1.

We have modified the sequence of photos which was inconsistent may because the disarrangement of the activation of word document.

3. The paper proved that FO can alleviate liver injury induced by intestinal IR. To prove this protective function was dependent on AMPK/SIRT-1/Autophagy pathway, the authors should exhibit more evidence to confirm it.

In vivo study, we demonstrated that intestinal I/R caused significant liver injury including histopathological changes and liver dysfunction. Further, We observed the decrease of p-AMPK/AMPK and SIRT-1 protein and mRNA associated with autophagy related proteins expression. FO restored the balance of the factors and alleviates liver injury effectively.

In vitro study, in LPS-stimulated HepG2 cells, RNA interfere has been performed, si-AMPK impaired the increase of p-AMPK, SIRT-1 and Beclin-1 but the decrease of TNF-α and MDA treated by FO. si-SIRT-1 impaired the increase of SIRT-1 and Beclin-1 but the decrease of TNF-α and MDA treated by FO.

These results have statistical significance using One-way analysis of variance (ANOVA) by SPSS 18.0 statistical software package (SPSS, Chicago, IL). This indicates FO alleviate liver injury induced by intestinal IR as least partially dependent on AMPK/SIRT-1/Autophagy pathway.

4. The authors should label each figure clearly, such as the results of histopathological analysis in Fig.1 and western blot analysis in Fig 3, Fig. 4 and Fig 5.

We have checked and label the figures clearly including Fig.1, Fig. 3, Fig. 4 and Fig. 5.

5. The quality of western bolt analysis in this paper should be improved.

We have improved the poor quality of western bolt pictures in this paper including beta-actin in Figure 3 and AMPKα1 in Figure 5.

6. There are some grammatical and spelling errors. Please modify.

We have modified the grammatical and spelling errors.

Replies to Reviewer #2:

Thank you for your helpful comments. We have carefully revised the manuscript in depth according to these suggestions.

However, there are also some important concerns.
1) It would be reasonable to include additional group of rats receiving FO but not subjected to I/R procedure. Intraperitoneally injected fish oil could have some effects on the signalling pathways of interest also in healthy rats.

We feel sorry that we didn't perform the FO control group. Our previous study have demonstrated that FO intraperitoneally injected induced the expression of AMPK and SIRT-1 in healthy rats[3], But there is no significance between the signalling pathways and the tissue injury as well as inflammatory reaction. We will try more reasonable research in future.

2) Why fish oil was administered intraperitoneally? Oral administration would be more reasonable if clinical/therapeutic implications are considered. In addition, it could not be excluded that ip. administered FO has some local irritating/proinflammatory effects. It would be convenient to compare the effects of FO with that of other lipid suspensions.

FO administered intraperitoneally has been widely used in animal studies due to its safety, convenient. It is also facilitated to be absorb and reach the s

teady plasma-drug concentration in rats [4-5]. Fish oil were also widely used via parenteral nutrition in patients who need fasting for solids and liquids [1-3]. Mor eover, FO may have some local irritating/proinflammatory effects occasionally, but we didn't find the significance in our and other laboratory previous studies [3,6]. For comparing the effects of FO with other lipid suspensions, soybean oil emulsion may have been employed mostly. But due to the adverse effect of pa renteral nutrition-associated liver injury [2], we didn't perform the soybean oil e mulsion control in present study.

3) FO was administered before I/R injury. It is unlikely to reproduce this schedule in patients. Protective effect of FO administered during/after I/R would be more promising.

Pretreatment is a widely used and accepted method for research. It has potential significance for disease prevention in hospital. Patients stayed in Intensive Critical Unit are always suffered potential intestinal ischemia and required fasting for solids and liquids conditions. In such cases, it is always routinely to give parenteral nutrition supplements[2]. This may prevent the intestinal ischemia induced-liver injury or enhance the body's ability to resist intestinal ischemia events. Our future study will explore the effect of FO administered during/after I/R.

4) Some details about MDA assays should be provided. What method) colorimetric?, HPLC?) was applied? How specific was the method for MDA itself vs. other lipid peroxidation products? Were any antioxidants added during tissue processing to prevent lipid peroxidation in vitro?

The colorimetric methods for MDA assays were used, and MDA has been a classical indicator to reflect lipid peroxidation in liver injury induced by intestinal I/R both in our and others' reports [3,6]. We didn't added any antioxidants during tissue processing.

5) In vivo studies were performed in the rat whereas in vitro experiments in human HepG2 cells. Were antibodies used for Western blotting species-specific or the same antibodies used in both sets of experiments? According to current description, only one antibody was used for each protein which raises concerns about their species specificity. The name of secondary antibodies should also be specified.

We have chosen the species-specific primary monoclonal antibody which recommended for rats, mouse and human universally. For second antibody, we chosen the species-specific antibody corresponding to the originate specificity of primary antibody.

6) Was total AMPK measured or only phospho-AMPK?

We have measured both total AMPK and phospho-AMPK. Ratio of phospho-AMPK/total AMPK was used for statistics analysis.

7) qRT-PCR: it is stated that RNA was isolated from lungs; why? How the results of qRT-PCR were calculated?

We feel sorry that we made mistake in writing, we have modified “lung” to “liver”. The quantitative RT-PCR results were calculated by $^{\triangle}$CT method.

8) According to Fig. 5 SIRT-1 siRNA and AMPK siRNA have similar effect on SIRT-1 and Beclin expression but a SIRT-1 siRNA had greater effect on TNF and MDA. This suggests that the mechanism of the effects of AMPK and SIRT-1 only partially overlap, and that autophagy itself does not mediate all the protective effects of SIRT-1. This issue should be discussed.

We didn’t compare the significance on TNF and MDA between SIRT-1 siRNA and AMPK siRNA. Generally, AMPK is located upstream of SIRT-1 and autophagy is the downstream of SIRT-1 in many pathogenesis process[7]. However, As the complicated circumstance in vivo, there may have other potential pathways crosstalk with this pathway which cause to the aberrant phenomenon. So we concluded that FO alleviates liver injury induced by intestinal I/R at least partially though AMPK/SIRT-1/autophagy pathway after the statistic analysis.

9) Both SIRT-1 and AMPK siRNAs abolish injury-induced up-regulation of the respective proteins but have no effect on their baseline levels (in the absence of injury). This observation is surprising; gene knockdown should reduce baseline level of the respective protein.

All hepG2 cells were stimulated by LPS and accept the RNA interfere and FO treatmentin this study. We only perform the control group (in the absence of injury) in preliminary experiments and get a 47% and 61% gene knock down of SIRT1 and AMPK mRNA expression (Figure 1).

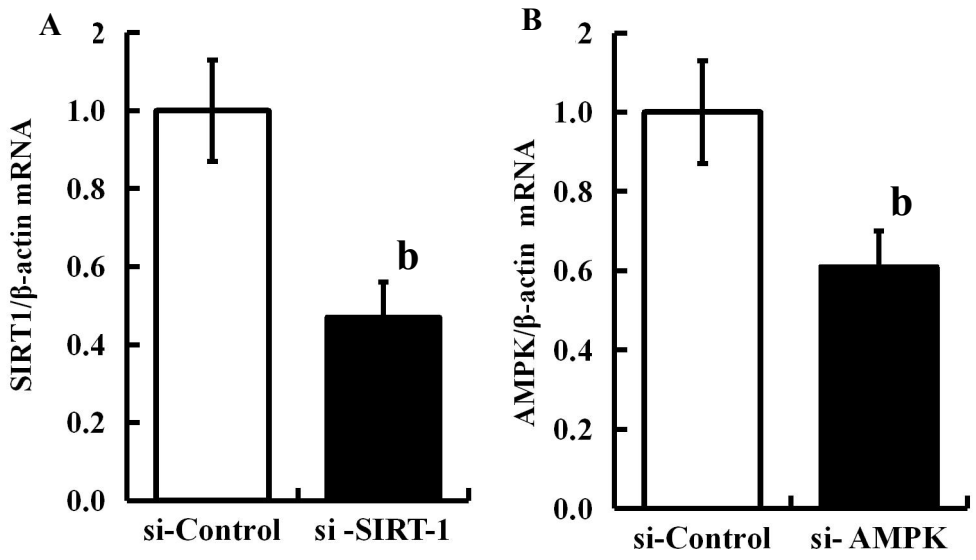

Figure 1. SIRT-1 and AMPK expression in the HepG2 cells. HepG2 cells transfected with the nontargeting control or SIRT1 small interfering RNA (siRNA). A, mRNA expression of SIRT1. B, mRNA expression of AMPK. si = si RNA. $^{b}P<0.01$ compared with si-control.

10) Protective effect on the liver could be secondary to reduced injury of the intestine as evidenced by histology results. Thus, the effect of FO on the liver could be the indirect one.

The protective effect of FO on the liver may because it prevented the initial organ intestine injury according to the presumably that the vasculature of liver is associated with intestine [3]. Further, Protective effect of FO on the liver also could be directly because its pharmacokinetics that it can be accumulated rapidly in liver and its induction role on liver drug enzymes. [4-5]. In present study, we focus on the mechanism of AMPK/SIRT-1/autophagy pathway mediated the protective effects of FO on liver injury.

Minor comments:

1) The abbreviation “FO” should be replaced by full name in the title.

FO have been replaced by full name in the title.

2) “MDA activity” should be changed to “MDA concentration”.

“MDA activity” have been changed to “MDA concentration”

3) It is unclear what is actually presented on figure 4 left bottom panel. One cannot

measure “p-AMPK mRNA”. AMPK phosphorylation is the post-translational modification; mRNA encodes AMPK irrespectively of its later phosphorylation.

We have changed the left bottom panel that it should be “AMPK/β-actin mRNA”.

Reference

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