January 13th, 2015

Dear Editor,
Please find enclosed the edited manuscript in Word format **(**file name: **NO: 15154 revised).**

Title: Altered distribution of regulatory lymphocytes by oral administration of soy-extracts exerts a hepatoprotective effect alleviating immune mediated liver injury, non-alcoholic steatohepatitis and insulin resistance

Name of Journal: *World Journal of Gastroenterology*

ESPS Manuscript NO: 15154.

The manuscript has been improved according to the suggestions of reviewers

Sincerely yours,

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First reviewer:

Khoury and colleagues tried to determine the immune-modulatory and the hepatoprotective effects of oral administration of two soy extracts in immune mediated liver injury and NASH. Oral administration of the combination of OS and M1 soy derived extracts exerted an adjuvant effect in the gut-immune system, altering the distribution of regulatory T cells, and alleviating immune mediated liver injury, hyperlipidemia and insulin resistance. They did good work. However, I have some comments: Major:

1- *While I mad reading the manuscript specially the Materials & Methods, Results and Figures parts, I feel that I am reading three separate manuscripts without any connection between them. Try to connect them.*

The authors accept the comment and the relevant sections were re-written to better connect the different sections of the manuscript.

2- *The beneficial effects of soy on ConA induced liver damage was discussed previously (see the papers with PMID: 18846580, 18602077, ...). In addition, the effects of soy on NASH was discussed in the following papers with PMID: 21333494, 17420940, ... What is the novelty of your work.*

The extracts describe in the present study are unique as they are not just crude soy extracts. In light of the comment we have revised the relevant section in the Material and Methods to further describe the two extracts used in the study. The 18846580, 18602077 describe the use of Letin and of beta glucosylceramide, 21333494, 17420940 describe the use of soy protein and isoflavons. While all are soy derived, they represent different fractions of the soy than the fractions used in the present study. In light of the comments we have revised the relevant sections in the discussion to include the relevant data on the use of soy derived glycolipids.

3- *You discussion depends mainly on illustration of the previous work and the importance of soy without discussing the results of your current work.*

We accept the comment and revised the relevant sections in the discussion section to elaborate on the results of the present study.

4- *This study heavily relied on biochemical markers for conA-induced hepatitis, such as serum ALT and AST levels. To make a solid conclusion, it is important for the authors to employ pathological changes as to show the H/E stained hepatic sections.*

We accept the comment, and as detailed description of the biopsies was not available we have toned down some of the conclusions made in the relevant sections.

Minor:

1- In the manuscript, you used both abbreviations: conA and con A. Please use one form. : Corrected

2- *In figures: - Too long figure legends. - No explanation for the abbreviations used in figure legends. - No explanation for the significant signs against what group.:* Corrected

3- *IN figures 2E and 3C: the H/E images are not clear. Please improve the resolution of your images and discuss your findings and use arrows*.: Corrected.

Second reviewer:

In the manuscript entitled "Altered distribution of regulatory lymphocytes by oral administration of soy-extracts exerts a hepatoprotective effect alleviating immune mediated liver injury, non-alcoholic steatohepatitis and insulin resistance", Khoury et al present evidence for the idea that soy extracts are hepatoprotective and immuno-modulatory in several different assays for both liver damage (ConA, HFD, MCD) and altered immune response. Overall, the evidence that there is something in the two extracts that ameliorates liver damage and alters the immune response is strong. The assays, however, bring us no clearer to understanding why or how.
What is(are) the active compound(s)?
Why combine the two extracts?

Major concerns:

1) *It is difficult to know exactly what is mediating the effects of these soy extracts. They are mystery combinations of many possible active compounds. There is little information on how or why these two mixtures should work well together.*

We accept the comment. In light of the critic we have toned down several of the statements in the Discussion section, and have elaborated on what is currently known from the literature on possible mecahnisms for the noted effect.

2) *There is also little evidence that there is any interbatch consistency in the extracts. I suspect that some of this may explain why the assays don't quite tell a consistent story. For example, it is hard to make much sense of the serum cholesterol and triglyceride level measurements. In one setting one drug-mixture dose/combination worked well at only one timepoint but when the other drug-mixture was added this efficacy was lost, and yet still if you then cut the doses tenfold and include both drug mixtures, that gave the best results (Fig 2A). It is hard to know what the ideal concentration (and combination) is based on most of the presented data. What is the best drug combination and why is there no consistency in it between assays. 0.3/3/6/9/30 ug???*

All studies were conducted using an identical batch from the same extract. The differences noted between dosages and models may depend on the inherited differences between the models used, which create different alterations in the immune background of the mice. The aim of the dosing studies was to assess a dose effect for each of the models. In light of the comment made by the reviewer we have elaborated on the differences in the relevant paragraphs of the Results and the Discussion sections.

Minor points:
"Compared with the dexamethasone treatment, 30 micrograms of OS and M, lowered the pro-inflammatory cytokines TNF-(alpha) and INF-(gamma)" is not actually true as far as this reviewer can assess based on the data. Dexamethasone appeared to be the MOST effective in lowering cytokine levels. Perhaps this was a misstatement and should have been compared with control. Corrected as suggested.

Third reviewer:

The present manuscript submitted by Khoury et al. deals with the effects of soy extracts in the progression of NAFLD/NASH in 3 mouse models inducing an autoimmune hepatitis and NAFLD. Despite some very interesting associations shown by the authors some issues should be further clarified:

1. *The effects on liver transaminases shown in Figure 1 (especially on the more liver specific ALT) are moderate and do not show a dose-dependency. Therefore the authors should rephrase their description of the results and mention the moderate effects observed and the absence of dose dependency.*

We accept the comment and corrected as suggested.

2. *Which dose of dexamethasone was used in the experiments?*

0.35 μg of dexamethasone was used. This was described in the revised version of the Methods section.

3. *Data on BMI as well as mean biochemical values (fasting glucose, cholesterol, triglycerides, transaminases) of the mice of every group should be illustrated in a separate table.*

We have revised the Results section and added a statement on body weight. Data on week 0 were included in the relevant figures.

4. *How do the authors explain the observed effects of the soy extracts when given in combination but not of each soy extract alone? Since b-glucosylceramide is considered as the effector second messenger mediating the immunomodulatory effects the authros should mention if GC is contained both in M1 and OS. Could this be a potentiall explanation of the observation of the synergistic effects despite an ineffective monotherapy?*

The actual content of GC in each of the extracts in not known. We anticipate that other soy-derived glycolipids, as well as other compounds may be involved in mediating the beneficial effect noted. In light of the comment we have revised the relevant sections in the Discussion to provide further explanation as to the possible mechanism of action.

5. *Figure 2F shows very moderate effects.*

We accept the comment and inserted an asterisks into the figure. The relevant section in the Results section was revised accordingly.

6. Figure 2A: how do the authors explain the observed effect with a dose of 0.3+0.3 but not with 3+3? 7. Which is the reason for the evaluation of spleen lymphocytes and not of serum lymphocytes via FACS-analysis? I believe the latter should be more representative for the alteration in the liver tissue and the whole organism.

It is anticipated that similar with other immunemodulatory agents a dose dependent effect is seen which may have a bell-shaped phenomenon. This may explain the noted effect in the lower compared with the higher dose. Spleen lymphocytes are somewhat

representative of serum lymphocytes due to the technical difficulties in obtaining a reasonable number of cells from the blood. Liver lymphocytes were not isolated in the present study. In light of the reviewer comments we have revised the relevant paragraphs in the Discussion section.

Minor comments*: "hepatic stellate cells" in the introduction, it is not common to show asterisks with variable P-values in each figure:* Corrected.