Nov 12, 2013

Dear Editor,

We are grateful to the editor for giving us the opportunity to revise our manuscript and judging our work as potentially important and within the scope of World Journal of Gastroenterology. Accordingly, we have addressed all concerns raised by the reviewers with changes in the form of presentation and more data, including additional new data generated by our new experiments. We are indebted for their valuable comments and feel that after incorporating the reviewers' advice, the revised manuscript has been greatly strengthened, the additional data support our original conclusions, and the significance of our findings is now more evident.

Please find enclosed the edited manuscript in Word format (file name: 5438-review.doc).

Title: Sophocarpine attenuates liver fibrosis through inhibiting TLR4 signaling pathwayin rats

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The manuscript has been improved according to the suggestions of reviewers:

1 Format has been updated

2 Revision has been made according to the suggestions of the reviewer

Review 1:

1. *Title and abstract: a) Title should indicate clearly that this study carried out in Rats. b) Abbreviations appear first in abstract before text without explanation (e.g. ECM, HSCS).*

We have revised our title and abstract as required.

2. *Materials and methods: a) Fibrosis model induced by dimethylnitrosamine (DMN) should be discussed briefly in the text. b) Why there was a difference between the number of rats in control (n=7) and treated group (n=12), statistically this will give wrong results. c) What are the doses used for DMN and Sophocarpine in the two models. d) Why the animals were sacrificed 3 weeks after BDL or 4 weeks after DMN administration. e) The author mention that three fields were selected randomly from each of two sections, and six rats from each group were examined. What is the relationship between the selected sections and rats in this paragraph? f) When ANOVA test is used it should be followed by multiple comparison tests.*

a) We have added discussion into the revised text.

b) This is an important point. In our study, the group contained 7 rats was named as “normal” or “sham” without DMN injection of BDL treatment. The control model group with DMN injection or BDL treatment had 12 rats the same amount as the treated sophocarpine group.

c) As per to the reviewer’s advice, the doses used for DMN and sophocarpiine were listed in the text: DMN injection (10 mg/kg, three injections per week for 4 weeks) and sophocarpine treatment (20 mg/kg sophocarpine dissolved in Ringer’s solution).

d) The two models were used in our labs for years. The main reasons for the listed sacrificed point were focused on the relative lower mortality and obviously ECM deposition in the livers comparing to other point.

e) We apologized for the error with this interpretation. In fact, all livers of sacrificed rats were tested by HE staining, Sirius red staining, Masson’s trichrome staining and Hydroxyprolinecontent which called for semi-quantitative or quantitative

measurement. We revised the interpretation in the text.

f) This is another obvious error. We did not use ANOVA test which is multiple comparison tests. Accordingly, we modified all the figure legends followed by the statistical analysis we used in the test.

3. *Results: a) Fig.2 there is no statistical data provided in the text although there is symbol (*) on some figures. B) Where are the scale bars for photos in fig. 3*

a) We have modified our data presentation in the original figures. As suggested by the reviewer, we modified all the figure legends followed by the statistical analysis we used in the test.

b) In figure 3, we used the presentation of "200×" instead of the scale bars in the title of photos.

4. *Discussion: a) the authors did not explain why they used two models for fibrosis and what is difference in mechanism of protection of Sophocarpine for each model. b) The author did not explain why the levels of liver parameters (ALT, AST……) are much higher in BDL model than in DMN model.*

a) The reviewer raised an important point. The two models we used were typical fibrotic models in rats. The main advantage of the two models was that the period of a single experiment was shorter than other models including TAA (6 months) and CCl4 (3 months) injection. We can duplicate our animal experiments for three times with lower consumption of time and money. More importantly, hepatic fibrosis in the two fibrotic models is inreversible relative to CCL4 model. Besides, the DMN and BDL models were progressed in different mechanism, but the fibrotic characters were obviously typical including diffuse hepatocytes necrosis and focal regeneration, inflammation infiltration, HSCs activation and ECM deposition. In our study, sophocarpine exhibited the potent ability in the inhibition of inflammation infiltration, HSCs activation and ECM deposition, which contributed to the alleviation of hepatic fibrosis in the two models. The possible functions of sophocarpine in the protection of hepatocyte and impaction on oxidation stress merits further study.

b) Although animal experimentswere repeated, we found the hepatic functional markers including ALT and AST were higher in BDL models than DMN models. The result reflects that hepatocytes necrosis is more severe in BDL models comparing to DMN models. The possible reason might be the cholestasis with BDL induced persistent insults to the hepatocytes. More importantly, we cut off the bile duct in case of recanalization and ensure the perseverativecholestasis in the liver. However, in DMN models, DMN was injected in rats for three times per week. Based on the potent regeneration of the hepatocytes, the incontinuous injury by DMN might induce weaker hepatocyte necrosis than continuous insult by BDL.

Reviewer 2:

1. *Title and Summary: Spelling error in "sophocarpine" (no sopnocarpine). There are some some abbreviations not explained.*

We have revised our title and abstract. The error in spelling was corrected. The explanations of ECM, DMN, BDL, PCNA and HSCs were interpreted in the abstract.

2. *Methods and results: a) Sample size is small, so it is difficult to assess these findings. Besides, there were 12 animals. Why only analyzed a half?. Technician is blinded, so they try to correct bias. They do not analyze all the samples. Why?b) Sometimes, as in human, liver damage it is not homogeneous. They analyzed different cytokines. But cytokines could be synthesized by other cells different from HSC. They did not control this. c) Why only LPS is used to stimulate and induce cytokine production? d) Sophocarpine administration in different dosage for 72 h. Did they try nother timing? Why 72 h? e) And sophocarpine dosage was the same in vivo than in vitro? f) Western blot is inespecific.*

a) As pointed out by the reviewer, animal experiment was most important in our study. According to the principles of animal experiment, we duplicated the animal experiment for three times (n=12, per group). Every experiment showed the similar effect of sophocarpine on the inhibition of hepatic fibrosis. Then, we apologize for the

error with the interpretation of ”a half”. In fact, all liver samples of sacrificed rats were tested by HE staining, Sirius red staining, Masson’s trichrome staining and Hydroxyprolinecontent which called for semi-quantitative or quantitative measurement. We revised the interpretation in the text.

b) The reviewer raised an interesting point. In hepatic fibrosis, cytokines are not only produced by HSCs but also by inflammatory cells including Kupffer cells. As the activation of proliferation of HSCs was the central event in hepatic fibrosis, the crosstalk between of HSCsactivation and inflammatory cellinfiltrationwas investigated by substantial researches. In our study, we used the HSCs for research in vitro mainly focusing on the activation and proliferation of HSCs which mainly contributed to the deposition of ECM. It would be of interest to determine whether inflammatory cell infiltration was reduced in sophocarpine treated livers and merit us for further investigation.

c) LPS elevation is universe in fibrotic patients and animals and correlated to the progression of hepatic fibrosis. LPS was a key stimuli for the activation of TLR4 pathway. Accordingly, we used the LPS tostimulate and induce cytokine production *in vitro*.

d) As pointed out by the reviewer,Sophocarpine was administrated in HSCs with different dosage at different time point (24h, 48h, 72h). At 48h and 72h, sophocarpine could inhibit the activation of HSCs relative to the control DMSO. As showed in Figure 4B, sophocarpine could significantly inhibit the activation of HSCs at 72h comparing to 48h. Accordingly, we showed the results of sophocarpine on HSCs mainly at 72h.

e) This is an important point. As the half-lethal dose of sophocarpine in rats is 200mg /kg per day, we used 20mg/kg and 40mg/kg per day for preliminary experiment in rats. There is no difference in the inhibition of hepatic fibrosis between the two groups. So we selected 20mg/kg for repeated experiments. In vitro, the dose of sophocarpine used in the treatment with HSCs is based on the gradient dose experiments.

f) According to the reviewer’s advice, we now provide the densitometry analysis

of immunoblots (figure 4D) and a new result of PCNA expression (Figure 5C).

3.*Discussion: authors could comment a little bit more about possible bias and limitation of the study. Besides, the downregualtion of cytokines could involve other signalling pathways.*

This is an important point. In our study, sophocarpine exhibited a potent ability on the control of liver inflammation, which could mainly contribute to the inhibition of hepatic fibrosis and HSCs activation. For decades, substantial researches have investigated that many stimuli, except for inflammatory cytokines, could drive the activation of HSCs including hepatocellular necrosis due to oxidant stress and apoptosis. The TLR4 and complement also plays an important role in oxidative stress and hepatotoxicity, especially initiating alcoholic steatohepatitis and fibrosis.[It is more likely that sophocarpine might have the impact on suppressing oxidant stress and subsequently protecting hepatocytes from necrosis or apoptosis, which merited investigation for us. Moreover, as a monomer derived from matrine, though sophocarpine could block the TLR4 pathway which was confirmed by our investigation, the direct target molecules of sophocarpine on the LPS-induced TLR4 pathway remain unknown, which called further study.

Reviewer 3:

1) The Methods section notes that for both models there were 12 animals in each of the treatment and control groups. Yet only 6 animals per group were assessed for IHC and histology. Why were the livers of all animals not examined? Furthermore, were the liver lesions in both models diffuse or focal? If the latter, selection of only 3 fields per section will not be representative of the whole section. Could the authors comment?

As mentioned by reviewer 1 and reviewer 2, we apologize for the error with the interpretation of"*6 animals per group were assessed for IHC and histology*". In fact, all liver samples of sacrificed rats were tested by HE staining, Sirius red staining, Masson's trichrome staining and Hydroxyprolinecontent which called for

semi-quantitative or quantitative measurement. We revised the interpretation in the text. The liver lesions in the two models were diffuse, and the ECM deposition mainly focused on the periportal area. We selected the periportal area as the representative photo in all experimental livers.

2) For expression of inflammatory mediators, morphometric analysis of immunostained sections (Fig 3) is essential before any quantitative comparisons can be made between groups.

Thank you for the suggestion. Because the immunochemical results of the protein expressions were very obvious, we did not add the morphometric analysis. However, if you think it is essential, we would like to add it.

3) *Cytokines are not only produced by HSCs but also by inflammatory cells infiltrating the injured liver. It would be of interest to determine whether inflammatory cell infiltration was reduced in sophocarpine treated livers.*

The reviewer raises an interesting point. In hepatic fibrosis, cytokines are not only produced by HSCs but also by inflammatory cells including Kupffer cells. As the activation of proliferation of HSCs was the central event in hepatic fibrosis, the crosstalk between of HSCsactivation and inflammatory cellinfiltration was investigated by substantial researches. In our study, we used the HSCs for research in vitro mainly focusing on the activation and proliferation of HSCs which mainly contributed to the deposition of ECM. It would be of interest to determine whether inflammatory cell infiltration was reduced in sophocarpine treated livers and merit us for further investigation.

4) How do the doses of sophocarpine used in vitro relate to the dose used in vivo?

This is an important point. As the half-lethal dose of sophocarpine in rats is 200mg /kg per day, we used 20mg/kg and 40mg/kg per day for preliminary experiment in rats. There is no difference in the inhibition of hepatic fibrosis between the two groups. So we selected 20mg/kg for repeated experiments. In vitro, the dose of sophocarpine

used in the treatment with HSCs is based on the gradient dose experiments.

5) *For the in vitro data (Figs 4 and 5) no statistical analysis has been provided. What was the n and p value for the different comparisons? The mRNA data would be strengthened if western blotting was performed to assess whether changes at the transcriptional level were translated into changes at the protein level for the ECM proteins and inflammatory cytokines.*

We apologize for the carelessness in the interpretation of figure legend. Accordingly, we modified all the figure legends followed by the statistical analysis we used in the test.

This is a great suggestion. We provided the protein expression of α-SMA and Collagen Ⅰ(Figure 4B) to strengthenthe effect of sophocarpine on the inhibition of HSCs. The protein level of inflammatory cytokines should be measured by ELISA in our new-added plan. The reagents were purchased after manuscript revision and still on the way.

6) Was TLR4 expression in HSCs induced in LPS incubated cells? This is not apparent from Fig 4c since only relative mRNA expression has been provided. Fig 4d requires labels for various lanes of the immunoblot. Densitometry analysis of immunoblots is essential.

This is another mistake in the text we must apologized for. We added the labels in the figure now.

7) The PCNA western blot is entirely unconvincing. Densitometry data should be provided.

We provided a newly result of the PCNA western blot in the Figure 5C.

8) In general, while there may be some associated changes in TLR4 signalling in HSCs incubated with sophocarpine, this reviewer is not convinced that the effect of the compound is mediated via this pathway alone. It would be of interest to assess the

effects of sophocarpine on oxidant stress and apoptotic pathways.

This is a very good point.We also not believed the effect of the compound is mediated via this pathway alone. In our study, sophocarpine exhibited a potent ability on theprotection of liver function. It is more likely that sophocarpine might have the impact on suppressing oxidant stress and subsequently protecting hepatocytes from necrosis or apoptosis, which merited investigation for us. Moreover, as a monomer derived from matrine, though sophocarpine could block the TLR4 pathway which was confirmed by our investigation, the direct target molecules of sophocarpine on the LPS-induced TLR4 pathway remain unknown, which called further study.

9) *Minor: Grammar and spelling need attention. The title itself has a spelling mistake "Sopnocarpine" should be "Sophocarpine",*

Sorry for the mistake, we have corrected it.

3 References and typesetting were corrected

Thank you again for publishing our manuscript in the *World Journal of Gastroenterology.*

Sincerely yours,