Author's response to reviews

Title: Fuzheng Huayu recipe alleviates hepatic fibrosis via inhibiting TNF-alpha induced hepatocyte apoptosis

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Author's response to reviews: see over
Dear Editor,

Thank you very much for the “9856739331263190 Decision Letter” on July 21, 2014. We hereby submit the revised manuscript (No. 9856739331263190, Fuzheng Huayu recipe alleviates hepatic fibrosis via inhibiting TNF-α induced hepatocyte apoptosis) to be considered for publication in BMC Complementary and Alternative Medicine.

We would like to express our heartfelt thanks to you and two reviewers for your great efforts in evaluating and reviewing our manuscript, and your constructive comments and suggestions will be very helpful for us to improve our manuscript.

We have carefully revised our original manuscript according to the reviewers' comments, some paragraphs and sentences are almost re-written, and we have responded point by point to them as listed below.

We hope our revised manuscript would meet the requirement of BMC Complementary and Alternative Medicine and thanks for your consideration!

Please do not hesitate to forward your queries and reviewers’ comments by e-mail [chenghailiu@hotmail.com].

Looking forward to hearing from you soon.

Yours Sincerely

Prof. Chenghai Liu

Institute of Liver Diseases, Shuguang Hospital affiliated to Shanghai University of Traditional Chinese Medicine
Reviewer #1 Weizhong Chang

The manuscript “Fuzheng Huayu recipe alleviates hepatic fibrosis via inhibiting TNF-α induced hepatocyte apoptosis” authored by Tao et al. presents the clear evidence that Fuzheng Huayu can significantly inhibit hepatocyte apoptosis and as the result it reduce the activation of HSC by the fragmented DNA from apoptotic heptocyte if inhibit the apoptosis of heptocyte by Fuzheng Huayu. The paper has provide some valuable molecular mechanism of the Fuzheng Huayu’a effect on apoptosis of heptocyte and activation of HSC. The paper fits the journal readers’ interest. It could be accepted after some reasonable revisions:

Moderate issues:
1. It’s not clear if the author was using the right control DNA. The control DNA for the activation of HSC should be DNA from heptocyte treated with vehicle.
Response:

The control DNA had not been clearly prescribed in the manuscript.
We used the right control DNA in this study. In Fig 5B &5E, the control was the DNA from heptocytes treated with phosphate-buffered saline (PBS), and the apoptotic DNA for the activation of HSC was the DNA from apoptotic hepatocytes treated with TNF-α and Act D. In the figure legend section (Figure 5), we described the control and apoptotic DNA.

2. As show in Fig 1E, there is no significant difference between the apoptotic index of different group at 8 weeks. It is difficult to attribute the effect of Fuzheng Huayu on the hepatic fibrosis to the inhibition of the heptoses of heptocyte which is significant at 18 hours based on data.

ANSWER: Yes, in this study we observed that there was no significant difference of apoptotic index among different groups at 8 weeks, but in there was a striking difference at 18h. Heptocyte apoptosis is an important early pathological changes of liver diseases, but also the basic conditions for cirrhosis and other advanced lesion formation [1]. As written in the introduction section, it is widely known that hepatocyte
apoptosis plays a pivotal role in hepatic fibrogenesis, and FZHY also shows a good
effect on hepatocyte apoptosis. Exactly, it is difficult to attribute the effect of Fuzheng
Huayu on the hepatic fibrosis to the inhibition of heptocyte apoptosis which is
significant at 18 hours based. Therefore, in vitro studies were carried out to explore the
effect of FZHY on hepatocyte apoptosis and HSC activation.

References:

1. Malhi H, Gores GJ, Lemasters JJ. Apoptosis and necrosis in the liver: A tale of two
deaths? Hepatology, 2006, 43(2 Suppl): 31~44.

3. The link between activation of the HSC and hepatic fibrosis needs to be
strengthened.

ANSWER: Thanks for your precious opinions. The link between HSC activation and
hepatic fibrosis has been described in the introduction.

Minor issue#

(1) Need more evidence from literature to support the design of the study.

ANSWER: More evidence supporting the design of the study has been added in
Materials and Methods as following. In vitro experiment was performed referred to
Watanabe [1].

References:

1. Watanabe A, Hashmi A, Gomes DA, Town T, Badou A, Flavell RA, Mehal WZ.
   Apoptotic hepatocyte DNA inhibits hepatic stellate cell chemotaxis via toll-like

(2) Some material in results section need move to background section.

ANSWER: The material in results section has been moved to background section.

(3) Need clarify controls.

ANSWER: In Fig 5B &5E, the control was the DNA of heptocyte treated with PBS,
and the apoptotic DNA for the activation of HSC was the DNA from apoptotic
hepatocytes treated with TNF-α and Act D. In the figure legend section (Figure 5), we described the control and apoptotic DNA.

Reviewer #1 Ling Yang

In this study, Dr. Cheng-hai Liu and his colleagues explored the effects of FZHY on hepatocyte apoptosis in acute and chronic liver injury induced by CCl4 in vivo and in vitro. The effects of FZHY on the activation of hepatic stellate cells was also observed in the chronic liver injury induced by CCl4. Hepatocytes apoptosis was assayed by TUNEL staining, flow cytometry and DNA ladder. In vitro study, they creatively employed serum containing FZHY to stimulate hepatocytes and stellate cells, which excluded the toxin effect of the impure components of FZHY. From their study, they found that FZHY attenuated hepatocyte apoptosis with the down-regulation of TNFR1 and bax, alleviated liver injury and hepatic fibrosis induced by CCl4. In vivo study they found that the DNA of TNF-alpha/Act D treated hepatocytes could stimulate HSCs activation and the DNA of FZHY treated hepatocyte suppressed HSCs activation. This work showed a new method to study the mechanism of traditional Chinese medicine. The data control was well setup. The method is also well described in detail. Here are still some points needed to further prove:

1. From the acute liver injury study, FZHY attenuated hepatocyte apoptosis, while the ALT, AST level (the serum marker of liver injury and apoptosis) had no decrease, whether FZHY had effects on liver inflammation? Or how about other inflammation markers (TNFα, IL-1b, et al) expression? Or CCl4 induced necrosis and FZHY could not prevent necrosis induced by CCl4? Cell death as the initial step for liver inflammation is important liver fibrosis. In the discussion the author also wrote “excessive hepatocyte apoptosis is thought to lead to liver dysfunction and damage in a variety of liver diseases” and “FZHY could protect hepatocytes from apoptosis and necrosis in acute liver injury induced by LPS/D-GalN. So did FZHY have the same or different effect in the liver injury model induced by CCl4?
Serum ALT and AST activities have been measured again and the results were similar. The reasons were needed to further discussion.

Necroinflammatory activity in hepatic tissue was graded according to the Scheuer scoring system [1]. The semi-quantification of grading was added in the manuscript, and the statistics was made with ANOVA. A scatter diagram reflecting the average degree of necroinflammatory activity scores in liver tissue each group was presented in Figure 1F, and a corresponding revision was made in the Figure 1 legend. As shown in Fig.1B &1F, liver sections revealed less vacuolated and necrosis cells and significantly improved portal inflammation and necrosis in the FZHY or NAC treated mice compared to the control. The expression of TNF-α protein in liver tissue had been detected by ELISA. As shown in Fig. 1G, TNF α level was very low in normal liver. After a single dose of CCl4 treatment, TNF α protein expression was significantly increased, and FZHY or NAC treatment attenuated TNF α expression in CCl4-treated mice. These data suggested that FZHY could protect hepatocytes from apoptosis and necrosis in acute liver injury induced by CCl4.
apoptosis induced by CCl4. Mice were respectively treated orally with FZHY (4.0 g/kg) or NAC (0.1 g/kg) daily for 3 d. Then, mice were subcutaneously injected with 100 % CCl4 for 18 hours to develop acute liver injury (A). Liver sections were subjected to either hematoxylin-eosin staining to detect necrosis and inflammatory cell infiltration (B, ×100) or to the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay to detect cell apoptosis (C, ×200). (D) Serum ALT and AST levels in the four groups (Control group, n=10; CCl4 treated, n=11; FZHY treated, n=15; NAC treated, n=15). (E) The apoptotic index (AI) of five high-power fields at ×400 magnification in each tissue specimen. (F) Necrosis and inflammatory cell infiltration scores in the four groups (Control group, n=10; CCl4 treated, n=11; FZHY treated, n=15; NAC treated, n=15). (G) Quantitative ELISA analysis of hepatic TNF α expression in the four groups (Control group, n=10; CCl4 treated, n=11; FZHY treated, n=15; NAC treated, n=15). *P < 0.05, **P < 0.01 vs. control group; #P < 0.05, ##P < 0.01 vs. CCl4 group.

References:


2. In vitro study, did the author measure the level of LDH or ALT in supernatant? Did they have difference?

ANSWER: In vitro experiments were designed to observe the effect of Fuzheng Huayu recipe on hepatocyte apoptosis, unfortunately we didn’t pay attention to the level of LDH or ALT in supernatant.

3. Apoptosis has two pathways: the mitochondrial pathway and the death receptor pathway. In this study the author measured the expression of TNFR1, Bcl2, Bax. Whether FZHY has suppressed both pathways? Other markers such as FAS/FASL, caspase-3, cleaved-caspase3, caspase8 should be measured to clarify these question.

ANSWER:
In this study, hepatocyte apoptosis induced by Act D/TNF-α was assayed with immunofluorescence, flow cytometry analysis and DNA ladder. These results showed
FZHY treatment significantly suppressed hepatocyte apoptosis \((P < 0.01)\). Bcl-2 is specifically considered as an important anti-apoptotic protein and is thus classified as an oncogene. Bax is a proapoptotic member of the Bcl-2 family. At the same time, we observed the molecular mechanisms of Act D/TNF-\(\alpha\)-induced apoptosis in primary hepatocytes, especially focusing on the involvement of Bax/Bcl-2 and TNF-R1. In this study, Bax/Bcl-2 ratio and TNF-R1 expression were significantly increased in primary hepatocyte induced by ActD and TNF-\(\alpha\), and FZHY suppressed Bax/Bcl-2 ratio and the expression of TNF-R1. These molecular mechanisms were involved in hepatocyte apoptosis, and these effects might be part of the mechanisms of action of Fuzheng Huayu recipe.

Generally speaking, it is important and necessary to measure mitochondrial pathway, death receptor pathway and newly discovered other apoptosis signaling pathway. Unfortunately we didn't pay attention to other markers such as FAS/FASL, caspase-3, cleaved-caspase3, caspase8, and so on. We will measure the level of FAS/FASL, caspase-3, cleaved-caspase3, caspase8 in the in-depth mechanism study of Fuzheng Huayu recipe on hepatocyte apoptosis.

4. The legends did not match the figures: there are only five legends but 12 figures.

ANSWER: Thank you very much. We re-arranged the figures.