Format for ANSWERING REVIEWERS

March 24, 2013

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 2505-review.doc).

Title: Glycyrrhizic Acid attenuates CCl_4-induced hepatocyte apoptosis in rats via p53-mediated pathway

Author: Xiao-ling Guo, Bo Liang, Xue-wei Wang, Fu-gang Fan, Jing Jin, Rui Lan, Jing-hui Yang, Xiao-chun Wang, Lei Jin, Qin Cao

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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

Reviewer 02462102

(1)I will demand from the authors to name in the discussion, other possible models that could be examined in their experimental model to prove the action of GA as an apoptosis hepatoprotector.

Thank you for your comments. These are actions of GA as an apoptosis hepatoprotector on other possible models: Glycyrrhizin attenuates HMGB1-induced hepatocyte apoptosis by inhibiting the p38-dependent mitochondrial pathway [25], and GA also inhibits the apoptosis of hepatic injury induced by injection of lipopolysaccharide / D-galactosamine in mice [26].

(2)In the discussion, the human liver disease model that could have GA therapeutic application can be speculated briefly.

GA, used in the treatment and control of chronic viral hepatitis, is now routinely used in Japan, due to its well-recognized transaminase-lowering effect in clinical applications [52-54]. Neominophagen C is a Japanese preparation containing 0.2% glycyrrhizin, 0.1% cysteine, and 2% glyceine, and mainly acts as an anti-inflammatory or cytoprotective drug rather than an antiviral. It can improve mortality in patients with subacute liver failure and ameliorate liver function in patients with subacute hepatic failure, chronic hepatitis, and cirrhosis [55].

(3)Serum CK18 fragment ELISA as a biomarker of apoptosis can be also briefly discussed, in order to show the horizons of the paper.

Thank you for your insightful comments. We have added the discussion of serum CK18 fragment ELISA as a biomarker of apoptosis, and would like to add it in our further experiment.

Reviewer 00011994

(1) Table 1. What does the superscript "a" mean at control value? It should be removed.

We have removed superscript "a" in control group, and the "a" in GA group means the value in the

GA treatment group compared with the CCl_4 group (p<0.05).

(2) ***Table 2. The score of five rats are missing in control. What does the superscript "a" mean at control value? The superscript "b" at CCl_4 group should be changed to "a".***

We have changed "b" to "a" in the paper.

(3) ***Figure 1C. There are so many lipid droplets in H&E staining of GA group. Is there any relation between GA addition and the lipid accumulation?***

Thank you for your helpful comments. Steatosis and ballooning of hepatocytes are the earliest, most frequent, and most striking pathological changes observed in CCl_4-induced liver injury [56,59,60], and we found this pathological change using H&E staining , and many droplets observed in low magnification HE stainings in the GA group and CCl_4 group. So we think there is no relation between GA addition and the lipid accumulation.

(4) ***Figure 2. The author should describe what white arrows indicate in the legend.***

We have added the description in the paper.

(5) ***Figure 5(b). What does the symbol "#" mean?***

We have added the description, and the symbol "#" means the value in the GA group compared to the CCl_4 group.

Reviewer 01489939

There is the need for a revision of English language and of some typographical mistakes.

Thank you for your suggestion, and we have polished the language by native speakers.

Reviewer 02444854

(1) ***From the HE stainings in Fig. 1, I am not convinced that GA reduces liver fibrosis. It is an accepted standard to stain liver sections with Sirius red to show the collagen fibers. It would also be appropriate to quantify and compare the Sirius-red stained area e.g. using Image J software (freeware) as published elsewhere. In addition, authors should also provide low magnification HE stainings of the same samples which would much better illustrate the actual tissue damage.***

Thank you for your helpful suggestion. We have completed the Sirius red stain to show the collagen fibers, we also used the Image J software to quantify the degree of fibrosis, and H&E is available in the paper. The results revealed that liver histopathology was significantly changed in the CCl_4 group after 8 weeks of CCl_4 administration. The livers, in the control group, showed an integrated lobular structure with central venous and hepatic cord radiation (Figure 1). The staging score was 0 (Table 2). The positive area of Sirius red staining in the control group was around the central vein rather than in the hepatic parenchyma. There were numerous ballooning cells in the GA and CCl_4 groups. In the CCl_4 group, the liver showed fibrous connective tissue proliferation, fiber interval formation which was associated with disorder of lobular structure in the portal area, and most rat livers appeared to have pseudo lobules (Figure 1). The score of hepatic fibrosis in the CCl_4 group increased to 3.53 ± 0.74 (Table 2). The positive areas of Sirius red staining in the CCl_4 group were in the boundaries of the hepatic lobules and the ratio of the hepatic fibrotic region was 7.87 ± 0.66%. In the GA group, livers appeared to have fibrous connective tissue proliferation, the formation of a few fiber intervals in the portal area, and the occasional pseudo lobule (Figure 1). The score was 3.00 ± 0.76 (P < 0.05) in the GA group (Table 2). The positive area of Sirius red staining in the GA group was decreased, and the ratio of the hepatic fibrotic region (3.68 ± 0.32%, P < 0.05) was reduced compared with the CCl_4 group (Figure 1).

(2) ***Liver issue analysis 48 h after the first CCl_4 injection – as performed by many colleagues before –***

clearly demonstrates that CCl_4 injection predominantly induces necrotic liver injury and only minor apoptosis. This should be mentioned in the discussion. Of note, hepatocytes and non-parenchymal cells show massive cell proliferation after CCl_4 as published before.

Thank you for your comments. Apoptosis and necrosis contribute to the process of liver fibrosis [49,56]. Whether necrotic liver injury or apoptosis is dominant in CCl_4-induced liver injury models remains controversial. A previous study has showed that CCl_4 can induce acute hepatocellular damage which is characterized by necrotic cell death [57], while another study has indicated that a substantial number of hepatocytes undergo apoptosis in the acute stage after CCl_4 administration [56]. In the present study, we found both apoptosis and necrosis occurred in the CCl_4-induced chronic liver injury model. These results were consistent with other reports [48,49]. Discrepancies may be attributed to the time points of observation. Additionally, report has shown that apoptotic bodies were also frequently incorporated in the neighboring cells, such as ballooned hepatocytes, hepatocytes, Kupffer cells , stellate cells, and endothelial cells[56].

(3) Accordingly, with their TUNEL data authors cannot conclude that GA reduces specifically apoptosis. To proof this, authors should try to stain tissue sections with antibodies recognizing cleaved caspase-3 which would be really apoptosis-specific.

Thank you for your advice. To further examine the view that GA reduces specifically apoptosis, we have performed the experiment of immunohistochemistry to detect the expression level of cleaved caspase-3. The results indicated that the expression level of cleaved caspase-3 was high in the livers of rats in the CCl_4 group. Interestingly, this level was reduced in the GA-treated group (Figure 2A).

(4) Figure 3: p53 activation. The problem with this figure is that the IF staining is not supported by the immunoblot analyzes and the whole p53 data does not justify the conclusion that the GA effects are mediated via p53. P53 IHC staining in GA-treated liver is much stronger as suggested by the western blot analysis. Western blot suggests that GA reduces p53 to almost baseline levels. If this would be the main mechanism, liver histology and transaminases after GA treatment should be much more reduced compared to the actual data. Authors should confirm reduced p53 expression in more rats and also tone down their conclusion regarding p53.

Thank you for your comments. In the following study, we will knock down p53 gene in rats to observe the changes of relevant indicators, and explore the underlying mechanisms with small interfering technology of p53 in vitro.

(5) The majority of data in Fig. 4 looks scientifically sound. However, 2 aspects need revisions. Measuring Cytochrome C with regard to apoptosis does only make sense with the use of fractionated cell extracts, i.e. authors would have to isolate liver mitochondria and measure Cyt c in these extracts. Reduced Cyt C in mitochondria would then indicate increased Mitochondrial permeability transition and thus apoptosis.

Thank you for your insightful comments. We have separated the cytoplasmic and mitochondria fractions of lysates and estimated the expression levels of cytochrome C in the cytoplasm and mitochondria by Western blot. The result showed that the cytoplasmic fraction in the control group contained a negligible amount of cytochrome C. However, cytochrome C accumulated in the cytoplasm of liver tissue in the CCl_4 group, and GA inhibited the release of cytochrome C (Figure 4B).

3 References and typesetting were corrected

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

Sincerely yours,

Xiao-ling Guo

Xiao-ling Guo, MD
Putuo Hospital,
Shanghai University
of Traditional Chinese Medicine,
Shanghai 200062, China.
Fax: +86-21-52665957
Email:gxlshtcm@163.com