LETTER ANSWERING REVIEWERS

February 24, 2015

Dear Editor, Ya-Juan Ma,
Science Editor, Editorial Office
Baishideng Publishing Group Inc
World Journal of Gastroenterology

Please find enclosed the edited manuscript in Word format (15795-edited. doc).

Title: **Hepatoprotective effect of *Geranium schiedeanum* against ethanol toxicity during liver regeneration**

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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 1
Madrigal-Santillán et al. investigated the protective effect o an extract of Geranium schiedeanum (exGs) in regenerative livers of rats receiving ethanol as hepatotoxic. For this purpose, the authors measured ALT, AST, albumin, bilirrubin and lipid peroxidation in both plasma and liver. All these parameters were measured in partially hepatectomized rats at day 8 after the surgical procedure. Rats treated chronically with ethanol and exGs showed a significant decrease in mortality, oxidative stress and biochemical parameters of liver damage; compared with rats non treated with exGs. In addition, the treatment with exGs was associated with an increase in liver regeneration. The authors concluded that exGs modulate oxidoreduction processes and enhance liver regeneration in rats with liver damage induced caused by ethanol consumption. The experimental design is appropriate and the subject is clinically relevant. I only have some observations concerning the study.

1)How do the authors explain the regenerative effect caused by exGs? Is it due to an enhacement in hepatocellular hypertrophy, hepatocellular hyperplasy or both? Oxidative stress may modify the activity of signaling pathways involve in both processes. Therefore, experiments assessing changes in the cell cycle (Ki67, cyclin D, PCNA…) or in hepatocellular cell area at days 0, 2 and 4 would improve the quality of this study.

1. Response:

According to the reports of Dr. Michalopoulos (2012), the liver regeneration induced by Partial hepatectomy (PH) is due to hyperplasia and compensatory hypertrophy. With the results of our study, we cannot ensure that the geranium extract is participating in the hypertrophy or in the hepatic hyperplasia. More studies are necessary, such as those suggested by the reviewer, and different stages of liver regeneration should be explored, such as initiation, maintenance, and termination. We are projecting these new studies for the near future to provide more extensive information on the protective effect of the geranium extract exerted on the damage caused by ethanol to liver regeneration and will report them in another paper. Thank you for the suggestion.

Kang, L.-I.; Mars, W.M.; Michalopoulos, G.K. Signals and Cells Involved in Regulating Liver Regeneration. *Cells* **2012**, *1*, 1261-1292.

2)It would be interesting to compare results of hepatocellular apoptosis and/or necrosis in ethanol-treated rats after partial hepatectomy (e.g.: 0, 2 and 4 days) with or without exGs treatment. These results would support more robustly the author's conclusions.

2. Response:

Different stages exist in the liver proliferation process (initiation, maintenance, and termination), and it is necessary to conduct studies on each of these, as the reviewer suggests. In this study, we report data that focus primarily on the termination stage (8 days). It is important to conduct studies on the data, as suggested by the reviewer, and we will surely perform them within a brief period of time and report them in another paper. Thank you for the suggestion.

3)The appropriate test for comparison and visualization of mortality is the method of Kaplan and Meier and the comparison of curves with the log-rang test. This statistic strategy provides more information than Student t test and/or ANOVA.

3. Response:

We are grateful for the reviewer's suggestions to utilize these statistical tests, which are specific for evaluating survival when employing a new test. Upon reviewing these tests, we noted that they are used in clinical studies or to examine cancer treatments, in which survival is the main indicator to be evaluated. In our study, there are various indicators that evaluate the protective effect of the geranium with regard to the damage caused by ethanol to the regenerating liver. Mortality is indeed important, although it is not the principal objective of the study;

together with the other indicators, it provides us with data for evaluating the extract. On consulting an expert in Biostatistics (Dr. Tomás Fregoso-Aguilar, President, Academy of Physiology, ENCB-IPN, Mexico), we were informed that the tests utilized (Student's *t* test and ANOVA) are adequate for the objective of our study. Thank you for the suggestion.

REVIEWER 2
Major points:
1.- The aim of this study is to investigate the hepatoprotective effect of the Gs extract on the toxicity induced by EtOH in partial post-hepatectomy liver regeneration in rat. However, since some of the assayed parameters are altered by partial hepatectomy (without ethanol administration), it is convenient to include a PH-Gs group of rats in this study to separately study the protective effects of Gs extract during liver regeneration in the absence of ethanol.
1. Response:
The study group suggested to us by the reviewer has been performed, and the explanation has now been included in the paper (page 7, paragraph 2). The result found in this latter group is that the geranium extract did not affect the indicators detected during liver regeneration, which was the reason why these had not been included. Thank you for the observation.

2.- The authors suggest a probable capacity of the Gs extract to preserve the normal structure of the liver, a histological study of the liver of the animals should be carried out in order to confirm this hypothesis.
2. Response:
Thank you for the suggestion. The histological data were added (page 8, paragraph 2**).**

3.- Why AST is decreased while ALT is increased in the of liver PH-EtOH rats?
3. Response:
Our investigation group previously reported the effect of ethanol on the AST enzyme levels in serum (Morales-González et al. 1999) and on the cytosol and mitochondria of the hepatocyte (Morales-González et al. 2004). As observed in Figure 2, there is an increase in the AST serum levels due to ethanol treatment, which can be explained as a selective release of the hepatic enzymes and is most likely due to a signaling mechanism for liver regeneration (Morales-González et al. 1999). Thus, AST serum enzyme elevation may be caused by an increase in the permeability of the mitochondria, which release AST into the cytosol; from there, AST moves into the intracellular space (serum) (Morales-González et al. 2004). In our study, we quantified AST activity in total liver homogenate, finding a decrease in its activity that may be caused by an increase in the mitochondrial permeability that releases this enzyme.

Morales-González JA, Gutiérrez-Salinas J, Yáñez L, Villagómez-Rico C, Badillo-Romero J, Hernández-Muñoz R. Morphological and biochemical effects of a low ethanol dose on rat liver regeneration. Role of the route and timing of administration. Digestive Diseases and Sciences 1999; 44: 1963-1974.
Morales-González JA, Gutiérrez-Salinas J, Piña E. Changes induced by ethanol in cytosol and mitochondrial enzymes activities during early times of liver regeneration after partial hepatectomy in rats. Archives of Medical Research 2004; 35: 263-270.

4.- Why is the total antioxidant status increased in liver after partial hepatectomy?

4. Response:

During liver regeneration, an increase in the lipoperoxidation of the remnant liver has been reported (Aguilar-Delfín et al. 1996). Therefore, we consider that the increase in the liver total antioxidant concentration is due to regulating a rise in the concentration of the free radicals produced during liver regeneration.

Aguilar-Delfín I, López-Barrera F, Hernández-Muñoz R. Selective enhancement of lipid peroxidation in plasma membrane in two experimental models of liver regeneration: partial hepatectomy and acute CC14 administration. Hepatology. 1996 Sep;24(3):657-62.

5.- In comparison with the PH and PH-EtOH groups, the total antioxidant status/capacity is increased in the serum but decreased in the liver of PH-Gs-EtOH rats. What is the explanation for this effect?

5. Response:

This question from the reviewer is highly interesting and comprises part of the novelty of this article. The hypothesis that we are investigating is the following: to determine whether ethanol is a generator of free radicals. These free radicals stimulate Nrf2 factor to increase the antioxidant systems in the hepatocyte antioxidant enzymes by increasing the total antioxidant capacity level of the regenerating liver. The novelty of this finding lies in that it appears that the geranium extract is a potent antioxidant that eliminates or deactivates the free radicals that form due to the metabolism of ethanol in this model. Thus, they do not activate factor Nrf2 and, consequently, do not increase the levels of the antioxidant systems in the hepatocytes, which was found in the PH-Gs-EtOH group. Currently, we are planning experiments to measure the different antioxidant systems in this liver regeneration model to determine the mechanism of the extract and/or its compounds.

6.- What is the difference between the total antioxidant status (TAS) measured in serum and total antioxidant capacity (TAC) measured in liver? What is measured in each one?

6. Response:

The antioxidant buffer system can be evaluated in different fluids, tissues, or cells because each conduit possesses different antioxidant systems and a different conjunction or integration. The technique developed for measuring the total antioxidant capacity of the biological samples assesses the ability of these antioxidant compounds, whether present in the fluid or in the cell, to reduce the

antioxidant species introduced into the assay system. In plasma, the total antioxidant capacity preferentially depends on the capacity and the amount of albumin and uric acid. When conducted in whole blood, blood total antioxidant capacity also evaluates antioxidant enzymes, glutathione, and NADPH. In the cells, it predominantly evaluates enzymatic antioxidant mechanisms, glutathione, NADPH, and endo- and exogenous antioxidant molecules (Quintanar-Escorza and Calderón-Salinas, 2009; Morales-González, 2013).

In general terms, this system is the same principle, only that it depends on the tissue or fluid to be studied. The commercial kit utilized to determine antioxidant systems in serum, i.e., stable systems such as TAS (Randox Laboratorios, Ltd., UK) and the commercial kit employed for determining antioxidant systems in the hepatocyte, establish these measurements, such as TAC (BioAssay Systems DTAC-100).

Quintanar Escorza, Martha Angélica; Calderón Salinas, José Víctor LA CAPACIDAD ANTIOXIDANTE TOTAL. BASES Y APLICACIONES Revista de Educación Bioquímica, vol. 28, núm. 3, septiembre, 2009, pp. 89-101 Universidad Nacional Autónoma de México, México.

Morales González, JA. Oxidative Stress and Chronic Degenerative Diseases- a Role for Antioxidants. (2013). Pp. Ed. Intech ISBN 980-953-307-650-3

7.- Minor points: - In the conclusion of the abstract: "diminution of free radicals and a greater presence of antioxidant agents in the samples analyzed." instead of "diminution of free radicals a greater presence of antioxidant agents in the samples analyzed."

7. Response:

Thank you. This observation has now been added to the Abstract.

REVIEWER 3

This manuscript entitled, "Biological evaluation of Geranium schiedeanum, a new hepatoprotective agent against the toxic action of ethanol during liver regeneration" was designed to evaluate the effect of an extract of Geranium schiedeanum as a potential hepatoprotector agent against the toxicity induced by alcohol in the partial post-hepatectomy liver regeneration model in rat. They showed that administration of the extract significantly reduced the unfavorable effect of ethanol on liver regeneration, the level of enzymes, and the metabolic processes that regulate glucose and lipid levels, as well as the mortality of the animals treated. And they concluded that the effect can be clearly related with the modulation of oxidoreduction processes by agents contained in the extract. The comments are as follows; 1. The hepatic protective function of geranium in livers treated by EtOH after PH is shown to be significant. However, in writing a manuscript, several mistakes are found.

2. Page 7, line 9: In "Total antioxidant concentration (TAC) was determined utilizing the Randox Kit (Randox Laboratories Ltd., U.K.) and is reported in

mmol/L.", "Total antioxidant concentration (TAC)' looks like a mistake of "total antioxidant status (TAS)".

2. Response:

Thank you for the observation, and this has been corrected (page 9, paragraph 1).

3. Page 9, line 2: In "while bilirubin values increased to 0.11 mg/dL in this group.", "increased" looks like a mistake of "decreased" to see the context.

3. Response:

Thank you for the observation. This has now been corrected (page 11, paragraph 2).

4. Page 9, line 24: In "Contrariwise, rats of the PH-Gs-EtOH group exhibited a significant diminution in serum as well as in ALT levels,", "a significant diminution in serum as well as in ALT levels" is not properly written.

4. Response:

Thank you for the observation, which has been corrected (page 12, paragraph 2).

5. Page 9, line 26: In "On comparing the ALT levels of this group with those of the group adminstered Et,", Et looks like a mistake of EtOH.

5. Response:

Thank you for the observation, which we have corrected (page 12, paragraph 2).

6. Page13, line 15: It is needed to show data on "Surprisingly, alcohol administration at early stages of liver regeneration in rats with PH diminishes the serum activity of these enzymes"

6. Response:

Thank you for the observation, which has been corrected (page 16, paragraph 3).

7. Page14, line 20: In "The increase in TAS (serum) and in TAC (liver) found in our study is in agreement with that previously cited, indicating that EtOH favors OS in this model, demonstrated by the high TBARS levels found (Figure 4).", looks like mistakes of "The decrease in TAS (serum) and increase in TAC (liver) found in our study is in agreement with that previously cited, indicating that EtOH favors OS in this model, demonstrated by the high TBARS levels found (Figure 5).

7. Response:

Thank you for the observation, which has been corrected (page 18, paragraph 2).

8. Why is AST level lowered in PH-EtOH group compared to others in Figure 2 (lower panel)? Discussion is needed.

8. Response:

The effect of ethanol on the levels of the AST enzyme in serum, cytosol, and mitochondria of the hepatocyte were previously reported by our investigation group (Morales-González et al. 1999; Morales-González et al. 2004). As can be observed in Figure 2, there is an increase in the AST serum levels due to ethanol

treatment, which can be explained as a selective release of hepatic enzymes as probable signaling mechanisms of liver regeneration (Morales-González et al. 1999). This increase in serum can be caused by an increase in the permeability of the mitochondria that release AST into the cytosol and from there into the extracellular space (serum) (Morales-González et al. 2004). In our study, we quantified AST activity in total homogenate, finding a decrease in its activity, which could be caused by the increase in permeability of the mitochondria.

Morales-González JA, Gutiérrez-Salinas J, Yáñez L, Villagómez-Rico C, Badillo-Romero J, Hernández-Muñoz R. Morphological and biochemical effects of a low ethanol dose on rat liver regeneration. Role of the route and timing of administration. Digestive Diseases and Sciences 1999; 44: 1963-1974.

Morales-González JA, Gutiérrez-Salinas J, Piña E. Changes induced by ethanol in cytosol and mitochondrial enzymes activities during early times of liver regeneration after partial hepatectomy in rats. Archives of Medical Research 2004; 35: 263-270.

9. If you have tissues of the experiment, I suggest you to show data on expression level of Ki-67 or PCNA that are markers of liver regeneration.

9. Response:

Thank you for your suggestion. With regard to our studies directed toward more deeply investigating the participation of the geranium, we are projecting these for the near future. Currently, we are obtaining the molecules found in the extraction order to determine a more adequate response to our investigation. We will conduct this study soon and report it in another paper.

REVIEWER 4

The work is a preliminary and phenomenologic study of the effect of a potential hepatoprotective extract from Geranium schiedeanum, against ethanol toxicity. Main points:

1. The definition of the extract is of Paramount importance

1. Response:

Thank you for the suggestion. More information has now been added concerning the manner of obtaining the extract (page 6, paragraph 3).

2. Authors need to provide additional inputs on the hepatoprotection: cyclins involved in cell proliferation, fibrosis and molecular signatures for this (MMPs, TIMs, etc)

2. Response:

We are deeply grateful for the reviewer's suggestions. At present, the objective of this work is to explore the protective effect of the geranium extract on the damage caused by ethanol to the regenerating liver, the effect of which we demonstrate in the results of our paper. We consider the reviewer's suggestions for the second stage of our study of utmost importance and will report them in another paper. Thank you for the suggestion.

3. Molecular markers of inflammation/toxicity due to ethanol. In the absence of these details, it is difficult to establish the scientific relevance of the experimental details given in the manuscript.

3. Response:

We are most grateful for the reviewer's suggestions. At this time, the objective of the work is to explore the protective effect of the geranium extract as a protector of damage caused by ethanol on the regenerating liver, the results of which are demonstrated in our paper. We consider the reviewer's suggestions to be of greatest import, and we will contemplate them in a second stage in our study, reporting the results in another paper. Thank you for your suggestion.

3 References and typesetting were corrected

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

Sincerely yours,

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