Author's response to reviews

Title: Protection of Early Phase Hepatic Ischemia-Reperfusion Injury by Cholinergic Agonists

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Author's response to reviews: see over
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Editor-in-Chief
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Dear Editor-in-Chief:

I would like to thank the reviewers for their constructive comments and critique of our manuscript titled “Protection of Early Phase Hepatic Ischemia-Reperfusion Injury by Cholinergic Agonists” (2281154607841258). We have taken into consideration the Reviewer’s comments and have added additional information in response to the reviewers’ suggestions. Specific point-by-point responses to the comments are attached. We believe that as a result of this revision the manuscript is in a greater quality presenting valuable data and information for those with research interests in the area of inflammatory tissue injury.

Enclosed is a copy of the manuscript, which we wish to resubmit for consideration for publication in BMC Clinical Pathology.

Thank you for your consideration.

Sincerely yours,

Elahé Crockett, Ph.D.
RESPONSE TO REVIEWERS:

“Protection of Early Phase Hepatic Ischemia-Reperfusion Injury by Cholinergic Agonists” – Crockett, et al. (2281154607841258)

Reviewer, Kevin J. Tracy

Discretionary Revisions:

1. Additional doses of DMPP for figure 6. Response: The aim of this experiment was to investigate whether the DMPP effect on the cytokine/chemokine production was at the message or posttranscriptional levels. The optimal dose of DMPP was selected based on its significant inhibitory effect on the cytokine/chemokine protein production. There was no effect of DMPP at the mRNA level (also supported by previously published data, Ref. 5 and 7). Thus, we feel that the data generated by DMPP optimal dose is sufficient for this aim of the study.
RESPONSE TO REVIEWERS:

“Protection of Early Phase Hepatic Ischemia-Reperfusion Injury by Cholinergic Agonists” – Crockett, et al. (2281154607841258)

Reviewer, Ian Hines

Major Compulsory Revisions:

1. In immunohistochemical staining of the liver tissue, it appears that the majority of the necrotic tissue is also stained. The neutrophil infiltration by additional methodology such as the biochemical myeloperoxidase assay is suggested. **Response:** Our laboratory has very much experience using various techniques (MPO, H&E, and immunohistochemistry) for demonstration of neutrophil infiltration in the liver (Ref. 11 & 14). Although neutrophils are the major source of MPO, other cells such as macrophages (Kupffer cells), monocyte, and lymphocytes also contain MPO (Brown et al, Am J Pathol 2001). In contrast, the immunohistochemistry technique using a specific monoclonal antibody to mouse neutrophil is specific for the neutrophils, providing a reliable method. The light brown color in the necrotic area is not due to the staining of the necrotic tissue-it is merely lack of staining of the hepatocyte’s cytoplasm with hematoxylin counter-stain (blue color in intact cells) because the damaged cells have limited histostructural integrity. The neutrophils within the necrotic tissue are stained in dark brownish black, and the presence of a large number of them within the necrotic area has created the appearance of the tinted light brown color in the necrotic tissue. This can easily be appreciated in Figure 4.G that is shown in a higher magnification. Additionally, lack of staining in Figure 4.B, a section of liver from an IR mouse (containing necrotic tissue) stained with a control mouse antibody, suggests that the stained cells are neutrophils and not of the necrotic tissue. Further, H&E slides clearly show the presence of neutrophils (IR-24h). We routinely, use two liver sections on each slide (H&E and histochemistry, total of 4 different liver sections), scanned and examined thoroughly under the microscope. Our data interpretation is based on examination of all these 4 tissue sections. Based on our experience and comments by other previous reviewers, we strongly feel that the immunohistochemistry tech is the most appropriate methodology to show neutrophil infiltration. In our previous reports, other reviewers have suggested that application of two different techniques for the purpose of the same objective is redundant and unnecessary, and should be avoided. In this study, due to application of most of the liver samples for RNA preparation, and the scarcity of the liver tissue, the Immunohistochemistry technique was selected based on it’s specificity for the neutrophils. We believe that sufficient evidence is presented (H&E, and immunohistochemistry) to indicate that the infiltrated cells are neutrophils. We hope that Dr. Hines is in agreement with our reasoning and other reviewers’ view.

2. Demonstration of Real-time PCR by comparison of the sham and the test data. **Response:** We agree with Dr. Hines’ comment that the liver message expression by the real-time PCR should be relative to the sham operated control values. This is one method of data analysis and the one used in this manuscript is another method, depending on the question to be answered. Because the objective was to evaluate the
effect of acetylcholine receptor agonist vs. saline-treatment on mRNA cytokine expression and not of the IR vs. sham, thus, a comparison between the saline, nicotine and DMPP treated mice were made. We have performed comparison analysis (sham vs. IR) on multiple samples (from four different experiments) and there is a great difference between the sham and IR mRNA, as shown in Figure 6 (there is no or a faint band of TNF, IL-6, MIP-2 for the sham while a clear distinctive band is present for the IR (saline, DMPP, and nicotine). We then applied the real-time PCR to do a comparison between the saline, DMPP, and nicotine, because this technique is more sensitive and shows the mRNA products in several cycles (not limited to an end-cycle as in rtPCR). Therefore, we believe the comparison presented in the manuscript (Figure 7) is appropriate for the analysis of the study’s objective. We have consulted with a technical application specialist at Applied Biosystem and our Genomic Technology facility regarding the analysis of the data, and the validity of the method used in our study is confirmed.

3. The effect of cholinergic pathway stimulation on sinusoidal perfusion. Response: In our study we did not measure the blood reflow into sinusoids after hepatic IR since our laboratory is not tuned to this type of experimentation, and thus, we cannot answer this question. However, the report by Nashida et al (Am J Physiol 2000) and couple of others related studies on the effect of vagus nerve and acetylcholine agonist stimulation on microcirculation and endothelial cells in ischemia are interesting and comments regarding these observations are added into the discussion section of the manuscript (additional Ref. 45, 45, 46 are added).

Minor Essential Revisions:
1. The format of the references has been checked and corrected accordingly.
2. The legends to the figures were checked and appropriate changes were added.
Reviewer, Juan L. Contreras

Major Compulsory Revisions:

1- Discrepancy regarding ALT/TNF results with histopathology during “early” I/R injury: significant reduction in ALT at 3 and 6 hr of post-reperfusion, significant reduction in TNF but without histopathological correlation. **Response:** In this manuscript, we have reported histopathological changes in correlation with ALT/TNF after 3 hr of reperfusion as indicated in the 4th paragraph of the “Result” section. The histopathological changes in DMPP/nicotine treated liver sections were minor, “only minor patchy spots” as compared to the saline-treated mice. After 6 hours of reperfusion, the histopathological changes in DMPP-treated mice were slightly less than those of the nicotine treated mice. Additional note is added to the text to clarify this issue. The discussion related to the discrepancies between chemokine expression and PMN infiltration also includes liver injury (neutrophil-dependent liver injury). Additional information is added to the discussion section to clarify this issue (4th paragraph). In general the data of this study suggests that in the late-phase of hepatic IR injury the cytokines/chemokines role may be of limited relevance for hepatic neutrophil infiltration and liver injury, indicating participation of other more potent inflammatory mediators.

2- The number of samples used in liver histopathology and morphometric assessment of IR injury. **Response:** In our studies, H&E slides of the liver sections (ischemic and non-ischemic) are prepared for all the mice used in the experiments. The information regarding the sample numbers is added to the figure’s legend. The histopathology evaluation of the liver tissues is done by an expert veterinarian pathologist (J. H.) who is blinded to the experimental design. The histopathology is applied to get information regarding inflammatory changes (neutrophil infiltration, congestion, necrosis vs. apoptosis, and vacuolization) that can easily be done by an expert pathologist. We use a quantitative approach (plasma transaminase, ALT) to measure the hepatocellular injury. This is a standard approach utilized by many other investigators and we believe is a valid approach to evaluate our study.

3- In vivo implication of the cholinergic pathway: “Having demonstrated significant reduction in ALT and TNF-a in animals given DMPP vs controls without histopathological and PMN infiltration between treated and control groups, will be informative to know if pharmacological modulation of the cholinergic pathway have in vivo implications.” **Response:** In the study presented in this manuscript the data showed a clear correlation of ALT and histopathology between DMPP and saline (control) treated mice during the early- and late-phases of IR. Additionally, there was a correlation between the liver injury (ALT), histopathology, and the cytokine/chemokine production during the early phase of IR injury. However, during the late phase of injury, reduction of the cytokine/chemokine production did not correlate with
protection of liver injury (ALT, and histopathology), indicating that cytokines/chemokines was not a major player for neutrophil recruitment and liver injury. As noted in the discussion section, other studies (Dorman et al) support our observation. Therefore, application of this pharmacological modulation in vivo would be appreciated for cytokine regulation. The use of this pharmacological modulation in a lethal model of hepatic IR is unclear. In our laboratory, we have used the lethal hepatic IR (un-published data) and believe that a more complex mechanism is introduced due to the surgical excision of the non-ischemic liver lobe(s), which results in a massive inflammatory response to the trauma irrelevant to the IR. Therefore, in our studies, we do not use this model because investigation of the mechanism(s) of tissue injury in response to ischemia and reperfusion and not of the liver excision is of interest to us. However, our findings would be of interest to those investigators utilizing the lethal model of warm hepatic IR.