March 28, 2014

Dear Editor,

Please find enclosed the edited manuscript in word format (file name: 9570-review)

Title: the effects of acute doxorubicin treatment on hepatic proteome lysine acetylation status and the apoptotic environment

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

1. We have incorporated all of the suggestions made by reviewers #00109509 and #00397384 into the revised manuscript. A recently published study has validated the use of Ponceau staining as a reliable alternative to actin as a loading control (Romero-Calvo et al. (2010) Analytical Biochemistry 401 (2): 318-320). We have improved Figure 5 and added scans of Ponceau staining of the samples shown in the representative Western blots. We have included these scans in a new figure (Figure 9). We did not include each scan with the Western blots in each previous figure because we believe the appearance of the scans decreases the overall quality of the figures.

2. Regarding the evaluation of our manuscript by reviewer #00004176, we respectfully point out to the Editor that many of the comments were not constructive in nature which makes it difficult to improve the manuscript based on the feedback given. First, English is our first language and it is clear from reading the manuscript that the written language exceeds acceptable. It is unexplainable how the language can be evaluated as "reject." Secondly, the reviewer is incorrect in his/her understanding of the ethical use of multiple organs from the same animal. Drugs, including Dox, have different effects on various organs. Thus, the use of the same animal to study the effects of Dox on skeletal muscle and liver is ethically acceptable. Research Review Boards encourage researchers to maximize the use of animals. Furthermore, the results between the 2013 paper that the reviewer referenced and the current submitted manuscript are different. The 2013 paper in skeletal muscle concluded that Dox did not affect lysine acetylation status or caspases. The current manuscript shows that the effects in liver are different; Dox caused lysine deacetylation, alterations in procaspas-9 expression and caspase-12 activation, which opened the door for further exploration. We explored the possible mechanism of lysine deacetylation and included several other analysis which were not included in the 2013 paper. Additionally, the 2013 paper included data that was not included in this submitted manuscript. Thus, we disagree with the reviewer that we can be "accused" of any deviation from ethical research conduct.

The rationale for studying lysine acetylation status was introduced in the Introduction section and expanded in the first paragraph of the Discussion section. The cited papers concluded that alterations in lysine acetylation status is associated with alterations in apoptosis and cell survival. This conclusion is relevant in forming a rationale for the study of acetylation status in our study, in that we wanted to test

if this was also true in the case of Dox-induced toxicity; if lysine deacetylation was associated with an apoptotic environment. We believe that the cited studies in both the Introduction and Discussion support the rationale behind our aim.

The reviewer states that it is impossible to know what Figure 1 is showing and questions whether one can discern if it is a Western blot of acetylated lysine. We respectfully disagree. The figure legend title states "proteome lysine acetylation status" and the legend describes that it is a Western blot showing that Dox induced proteome lysine deacetylation. Furthermore, it is stated in the Methods section that protein lysine acetylation was determined by Western blot and lists the specific antibody that was used. To our understanding, the only way to determine proteome lysine acetylation via Western blot is by the use of an antibody for lysine acetylation.

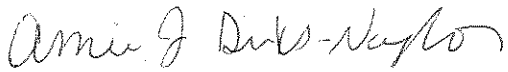
The reviewer questions why we did not include all of the HDAC enzymes in Figure 2. We did not pursue further analysis of all of the specific HDACs (which are numerous) because the data (general HDAC activity) suggested that an increase in HDAC activity is not the mechanism behind Dox-induced lysine deacetylation. There was not a strong rationale for pursuing this large amount of data, especially given a limited budget and tissue restraints.

We have added the word "conventional" to the Figure 3 legend.

The reviewer states that "the decrease in HDAC activity looks better than that of HAT activity" and questions our interpretation. The data show that there was a trend for a decrease in HDAC activity in the Dox group, but it was not statistically significant ($p=0.29$). However, there was a statistically significant decrease in HAT activity in the Dox group. Thus, we concluded that the Dox-induced deacetylation is likely due to a decrease in HAT activity rather than an increase in HDAC activity. We respectfully point out to the reviewer that a decrease in HDAC activity cannot explain the Dox-induced deacetylation. Thus, whether or not the decrease in HDAC activity in the Dox group was significant or not, it would not change the conclusion; that Dox-induced deacetylation is likely due to a decrease in HAT activity rather than an increase in HDAC activity. We believe that this is the only interpretation that can be supported by the data presented. When running the HDAC activity assay we ran each sample in triplicate. Samples were run in quadruplicate when doing the BCA protein concentration assay. Thus we are confident in the results. N =8 for the Dox group and N=7 for the control group. This is stated in the Animal and Experimental Design section. We added a second statement in the Statistical Analysis section that the N for all analysis is 8 and 7. It is stated in the Methods that the manufacturer's protocol was followed, thus the time course was 1 hr. Previously to testing all samples, we tested several samples over a longer time course, but concluded that 1 hr as suggested by the manufacturer was sufficient. This is the same for all enzyme assays included in the manuscript. We ran the assay for a longer time course, but ended up reporting the time point that was suggested by the manufacturer since the results at later time points were no different.

The reviewer states concerns regarding experimental details, reproducibility and data interpretation of the caspase data. The reviewer does not back up his concerns with specific examples or further explanation. Without constructive suggestions, we do not know with what specifically he/she has concerns. It is not clear if the concern is with the Western blots or the caspase activity. In both cases we believe we have included necessary experimental detail. There is no explanation of why the reviewer is concerned with the interpretation of results. We believe all results have been interpreted appropriately based on the data presented and the existing body of literature.

Thank you,



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