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Biomarkers of mitotoxicity after acute liver injury: Further insights into the interpretation of glutamate dehydrogenase

Mitchell R. McGill, Hartmut Jaeschke

Corresponding author
Mitchell R. McGill
Department of Environmental Health Sciences & Department of Pharmacology and Toxicology, University of Arkansas for Medical Sciences, Little Rock, AR, USA

Handling editor:
Michal Heger
Department of Pharmaceutics, Utrecht University, the Netherlands
Department of Pharmaceutics, Jiaxing University Medical College, Zhejiang, China

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Ref.: Ms. No. JCTRes-D-20-00131
Biomarkers of Mitotoxicity after Acute Liver Injury: Further Insights into the Interpretation of Glutamate Dehydrogenase
Journal of Clinical and Translational Research

Dear author(s),

Reviewers have submitted their critical appraisal of your paper. The reviewers' comments are appended below. Based on their comments and evaluation by the editorial board, your work was FOUND SUITABLE FOR PUBLICATION AFTER MINOR REVISION.

If you decide to revise the work, please itemize the reviewers' comments and provide a point-by-point response to every comment. An exemplary rebuttal letter can be found on at http://www.jctres.com/en/author-guidelines/ under "Manuscript preparation." Also, please use the track changes function in the original document so that the reviewers can easily verify your responses.

Your revision is due by Jan 06, 2021.

To submit a revision, go to https://www.editorialmanager.com/jctres/ and log in as an Author. You will see a menu item call Submission Needing Revision. You will find your submission record there.

Yours sincerely,

Michal Heger
Editor-in-Chief
Journal of Clinical and Translational Research

Reviewers' comments:

Reviewer #1: Very well-written and interesting review addressing continued questions that arise surrounding the use and limitations of GLDH as a biomarker of mitochondrial damage. I ask the that authors consider the following questions/comments:
* The authors conducted the experiment presented here using mitochondria obtained from a healthy mouse. Presumably, mitochondria released during ongoing hepatotoxicity would not be healthy, even if intact, and could potentially have leaky permeability. Have you considered repeating this experiment in mice given a toxic dose of furosemide? Can you address how the results might be different in the setting of ongoing liver injury? It seems relevant to me that the Triton X-100 treated mitochondria were more susceptible to the effects of freezing and thawing which again might imply that unhealthy mitochondria do not behave the same as healthy mitochondria.
* How does this new evidence from your lab and that of Church et al. support/refute your previous hypothesis that interpretation of GLDH measurements requires that fresh samples be processed for removal of mitochondria?
* Given that, unlike with intentional APAP over dose, most hepatotoxicity in the clinic is often discovered when injury is well underway and therefore secondary mitochondrial damage is likely to have already occurred, do you envision GLDH being useful as a mechanistic biomarker in the clinic? Or do you see this mechanistic function being primarily useful in exploring mechanisms in pre-clinical and in vitro studies?

Reviewer #2: The authors discuss the mechanistic meaning of circulating glutamate dehydrogenase (GLDH) in the context of drug-induced liver injury. This is a controversial area and important as this biomarker is being increasingly used to guide decision making in drug development. For this marker to have value we need to understand what it means. The authors have a track record of high quality publication in this field and they present data which support their hypothesis that GLDH in the plasma/serum reflects mitochondrial toxicity. I would agree with their critical review of the Church et al paper, I also interpret the data as suggesting GLDH reflects mitochondrial pathology.

In this article I would like the authors to suggest where and how GLDH should be used? What value do they think it has over and above ALT, particularly in humans. This is the key question, why should a drug developer use it? Furthermore, GLDH is not as sens/spec in APAP toxicity in humans compared with K18 and miR-122. If mitochondrial toxicity is central to the pathogenesis, how do the authors answer this?

At times the authors are overly bullish.

Page 4 - 'These data indicated that mtDNA and GLDH can serve as mechanistic biomarkers

for mitochondrial damage in patients with APAP hepatotoxicity.' I think too strong as the data were correlation. Let's see the effect of mitochondrial protective treatment on GLDH in humans.....

Page 5 - indicating that mitochondria are drivers of the injury in humans. Again, these data are correlation not causation.

Reviewer #3: The data presented are very interesting and relevant in the context of the cellular biology of the hepatocyte damaged by acetaminophen. As well as the importance of finding non-invasive biomarkers that allow determining the damage caused by the drug.
I find it interesting that the author discusses the histological details of liver damage in rodents and whether it can be considered a biomarker of early or late damage in humans.

Authors' response

Response to Reviewer Comments

Biomarkers of Mitotoxicity after Acute Liver Injury: Further Insights into the Interpretation of Glutamate Dehydrogenase

Mitchell R. McGill[1,2] and Hartmut Jaeschke[3]

[1]Dept. of Environmental Health Sciences, Fay W. Boozman College of Public Health, University of Arkansas for Medical Sciences, 4301 W. Markham St, Little Rock, AR, USA, 72205

[2]Dept. of Pharmacology and Toxicology, College of Medicine, University of Arkansas for Medical Sciences, 4301 W. Markham St., Little Rock, AR, USA, 72205

[3]Dept. of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, 3901 Rainbow Blvd., Kansas City, KS, USA, 66160

Running title: GLDH as a mitotoxicity biomarker

Address for Correspondence:

Mitchell R. McGill, PhD

Assistant Professor

University of Arkansas for Medical Sciences

Little Rock, AR

P. 501-526-6696

E. mmcgill@uams.edu

GENERAL RESPONSE TO THE EDITOR AND REVIEWERS

We thank the editor and reviewers for their constructive comments, which have helped improve the quality of the manuscript. Our responses to specific comments are below. **All changes to the manuscript are listed in bold text in this document and highlighted with bold text in the revised paper.**

REVIEWER #1

Comment 1: The authors conducted the experiment presented here using mitochondria obtained from a healthy mouse. Presumably, mitochondria released during ongoing hepatotoxicity would not be healthy, even if intact, and could potentially have leaky permeability. Have you considered repeating this experiment in mice given a toxic dose of furosemide? Can you address how the results might be different in the setting of ongoing liver injury? It seems relevant to me that the Triton X-100 treated mitochondria were more susceptible to the effects of freezing and thawing which again might imply that unhealthy mitochondria do not behave the same as healthy mitochondria.

Response: We thank the reviewer for raising this question. We agree that mitochondria from damaged liver may behave differently. Presumably, most leaky mitochondria would leak GLDH into the extra-mitochondrial space (serum *in vivo*, and reaction buffer in our experiment) where it would be detected as a reflection of the leakiness/damage, so it would be unlikely to have a large effect on the results from the centrifugation experiment. However, if membrane integrity is compromised in some other fashion (without leakiness), that could increase susceptibility to freezing and thawing as the reviewer astutely noted. **We have added a paragraph** to the manuscript in which we discuss this potential weakness of our work. (**Page 7, Paragraph 2**).

Comment 2: How does this new evidence from your lab and that of Church et al. support/refute your previous hypothesis that interpretation of GLDH measurements requires that fresh samples be processed for removal of mitochondria?

Response: This is an excellent and important question. Our data indicate that repeated freezing and thawing does not significantly increase measured GLDH activity, so it may be possible to store serum frozen before measuring GLDH as a biomarker of mitochondrial damage. **We have added a sentence** to explain that (**Page 7, Paragraph 1**): **"In addition, our results indicate that specimen processing to remove intact mitochondria may not be necessary before freezing even when using GLDH as a biomarker of mitotoxicity."**

Comment 3: Given that, unlike with intentional APAP over dose, most hepatotoxicity in the clinic is often discovered when injury is well underway and therefore secondary mitochondrial damage is likely to have already occurred, do you envision GLDH being useful as a mechanistic biomarker in the clinic? Or do you see this mechanistic function being primarily useful in exploring mechanisms in pre-clinical and in vitro studies?

Response: We envision using GLDH as a biomarker of mitochondrial damage in translational research, as we have done to determine if the mechanisms of APAP hepatotoxicity are similar in mice and humans (McGill et al. J Clin Invest. 2012). It may become a useful mechanistic biomarker in the clinic if new treatments designed to prevent or reduce mitochondrial damage

in liver injury are developed. If or when that happens, then the question of secondary mitochondrial damage will need to be directly addressed. For now, the major clinical utility of GLDH seems to be differentiating between liver and muscle as a source of elevated ALT (Schomaker et al. PLoS One. 2020). **We have added a paragraph** to the manuscript along these lines to explain how we envision GLDH can be used moving forward (**Page 8, paragraph 2; final paragraph of the manuscript**).

REVIEWER #2

Comment 1: In this article, I would like the authors to suggest where and how GLDH should be used? What value do they think it has over and above ALT, particularly in humans. This is the key question, why should a drug developer use it? Furthermore, GLDH is not as sens/spec in APAP toxicity in humans compared with K18 and miR-122. If mitochondrial toxicity is central to the pathogenesis, how do the authors answer this?

Response: We thank the reviewer for these important questions. **We have added a new paragraph** to address this comment and a similar one from another reviewer (**Page 7, paragraph 2**). Briefly, regarding the utility of GLDH as a biomarker, our primary interest is using it as a marker of mitochondrial damage in translational research, as we have done to translate the mechanisms of APAP hepatotoxicity from mice to humans (McGill et al. J Clin Invest. 2012). We suggest that others could use it for similar studies under well-controlled conditions. Clinically and for regulatory purposes, it appears that GLDH may be more useful as a biomarker to differentiate liver and muscle as a source of elevated serum ALT in patients with skeleto-muscular diseases who are taking drugs with known liver-related liability (Schomaker et al. PLoS One. 2020), though we believe additional work is needed to fully test that.

Regarding the issue of low sensitivity and specificity despite the central role of mitochondria in the pathogenesis of APAP hepatotoxicity, few studies have actually addressed the sensitivity, specificity and post-test probabilities (or predictive values) of GLDH for either diagnosis or prognosis specifically in APAP hepatotoxicity in humans. To our knowledge, only three major studies have been done (Antoine et al., 2013; McGill et al., 2014; Dear et al., 2018). Nevertheless, data from these studies do indicate that GLDH has lower sensitivity and somewhat lower specificity than other biomarkers, as the reviewer noted. There are many possible factors that could explain the poorer performance characteristics of GLDH. For example, GLDH normally exists in a large, 318 kD hexamer, which may not easily leak from mitochondria even after some damage. Another possible explanation is that GLDH may be less stable under certain collection or storage conditions. To our knowledge, the effects of such pre-analytical variables on measured GLDH values have not been thoroughly explored. In any case, any such explanation would be speculative at this point. In our view, a better test of the role of mitochondrial damage in APAP toxicity is comparing serum GLDH values between survivors and non-survivors of APAP-induced acute liver failure, which we have done. We found that GLDH is higher in non-survivors (McGill et al. Hepatology. 2014). Those data are consistent with the hypothesis that mitochondrial damage is critical for APAP hepatotoxicity, and we mentioned that in the manuscript **(Page 4). In addition, we have added a new reference** to Dear et al. Lancet Gastroenterol Hepatol. 2018, which is the best

study to-date comparing the performance of GLDH to other biomarkers in order to direct readers with further interest.

Comment 2: At times, the authors are overly bullish. Page 4 - 'These data indicated that mtDNA and GLDH can serve as mechanistic biomarkers for mitochondrial damage in patients with APAP hepatotoxicity.' I think too strong as the data were correlation. Let's see the effect of mitochondrial protective treatment on GLDH in humans.....

Response: We agree with the reviewer and we thank him/her for this comment. We have attempted to soften the language by revising the statement "These data indicated that mtDNA and GLDH can serve as mechanistic biomarkers for mitochondrial damage in patients with APAP hepatotoxicity," to "These data **are consistent with the idea** that mtDNA and GLDH can serve as mechanistic biomarkers for mitochondrial damage in patients with APAP hepatotoxicity."

Comment 3: Page 5 - indicating that mitochondria are drivers of the injury in humans. Again, these data are correlation not causation.

Response: We agree with the reviewer. We have attempted to soften the language again by revising this statement to read "Indicating that mitochondria **could be** drivers of the injury in humans."

REVIEWER #3

Comments: The data presented are very interesting and relevant in the context of the cellular biology of the hepatocyte damaged by acetaminophen. As well as the importance of finding non-invasive biomarkers that allow determining the damage caused by the drug. I find it interesting that the author discusses the histological details of liver damage in rodents and whether it can be considered a biomarker of early or late damage in humans.

Response: We thank the reviewer for his/her kind words. We apologize if we misinterpreted anything, but we have not identified any specific criticisms from reviewer 3 to which we can respond.

2nd Editorial decision
10-Dec-2020

Ref.: Ms. No. JCTRes-D-20-00131R1
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Dear authors,

I am pleased to inform you that your manuscript has been accepted for publication in the Journal of Clinical and Translational Research.

You will receive the proofs of your article shortly, which we kindly ask you to thoroughly

review for any errors.

Thank you for submitting your work to JCTR.

Kindest regards,

Michal Heger
Editor-in-Chief
Journal of Clinical and Translational Research

Comments from the editors and reviewers: