Journal of Clinical and Translational Research
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Mechanisms of acetaminophen hepatotoxicity and their translation to the human pathophysiology

Anup Ramachandran, Hartmut Jaeschke

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Handling editor:
Michal Heger, Rowan van Golen
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Ref.: Ms. No. JCTRes-D-16-00039

Intracellular Signaling Mechanisms of Acetaminophen-induced cell death and their translation to the human pathophysiology
Journal of Clinical and Translational Research

Dear Dr. Ramachandran,

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 10, 2017.

To submit a revision, go to http://jctres.edmgr.com/ 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

Rowan van Golen
Associate Editor
Journal of Clinical and Translational Research

Reviewers' comments:

Reviewer #1: The Review JCTRes-D-16-00039 "Intracellular signaling mechanisms of acetaminophen-induced cell death and their translation to the human pathophysiology" is a very well-written summary of all events triggered by APAP intoxication from a murine situation and the human translational view. This is essential in order to unveil novel biomarkers that can predict the course of liver injury after APAP overdose in humans.

MAJOR COMMENTS

L36 The authors need to elaborate on the sentence 'key mechanistic indices in injury'. Do they mean serum transaminases?
Maybe the authors should expand more the GSH depletion part after APAP intoxication, describing the pathophysiology of gluthathione depletion.
P7, L58 The authors should address this fact discussing recent publications such as Cubero (2016).
In addition, the authors should discuss other forms of cell death after APAP intoxication, e.g.: what happens to inflammasome? Ferroptosis? Pyroptosis?

P11, L31, please rephrase the sentence "JNK activation was also fleeting"

MINOR COMMENTS

The Abstract is missing some punctuations (commas) e.g L43…overdose, indicates…

Introduction: (L20, those in patients who took excessive…)

L46 ..in preventing APAP-induced ALF, it has to be administered early after APAP consumption to..(please check the construction of the sentence.

Reviewer #2: Reviewer's critiques:
JCTRes-D-16-00039

This is a well written and thorough review of the major signaling pathways and mechanisms in acetaminophen toxicity. However, the review is not exhaustive, some of the current segments need to be expanded upon and some additional segments need to be added to make is stronger.

1- In the entire text, instead of "programmed cell death" please use the phrase "regulated cell death". Please refer to Galluzi et al Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death 2012 Cell Death and Differentiation (2012) 19, 107-120.

"We suggest to preserve the adjective 'programmed' for those physiological instances of cell death - irrespective of the modality by which they are executed - that occur in the context of embryonic/post-embryonic development and tissue homeostasis. 'Regulated' should be used to indicate cases of cell death - be they programmed or not - whose initiation and/ or execution is mediated by a dedicated molecular machinery, implying that they can be inhibited by targeted pharmacological and/or genetic interventions"

2- Page 7-line 19: This activation of JNK can be mediated by the apoptosis signal inducing kinase 1… I suggest authors also include the role of MLK3 in the early phase of JNK activation (Sharma, Mol Pharmacol. 2012 REF [57]. While the authors have referenced this paper they have not elaborated on the early versus late phase of JNK activation. I suggest the authors expand on the MAPK cascade and discuss this in more detail.

3- The role of ER stress has been explored in acetaminophen toxicity. It is very feasible due to reactive adducts formed during overdose proteins malfold and clog the ER thereby causing stress. So ER stress probably does occur during toxicity, however this reviewer does not believe it is a major player in the cell death and toxicity from acetaminophen. Regardless, it should be referred to and discussed for completeness. For example, CHOP KO mice have been examined (Uzi D, J Hepatol. 2013 Sep;59(3):495-503.)

4- Page 9 line 56: However, chronic deletion of Parkin renders animals resistant to APAP.- Perhaps this is due to compensatory effects when you have an embryonic KO? If authors agree I would comment on that. It brings to mind the NNT mutation in J strain mice which renders them paradoxically more resistant to APAP due to compensatory mechanisms.

5- Page 13line 26. Genetic deletion of RIP3 delayed APAP induced cell death [65]. This is controversial and it should be commented that while it did delay toxicity in one study this was not observed in another study REF [66]. This is currently a controversial topic as most people agree that RIPK3 is not present in hepatocytes but may be induced in certain conditions. I suggest the authors only refer to what is definitely established in the field and agreed upon by most experts in the abstract. In the abstract the sentence starting with "this then amplifies the mitochondrial oxidant stress…" I would take out RIP3 and just discuss the controversy with RIP3 under the necrotic cell death.

6- Necrotic cell death in APAP overdose is the most ambiguous section. For example page 13 line 33, you refer to "cardiomyocyte necroptosis" REF[111] however nowhere in the text do the authors describe what necroptosis is, that it is receptor mediated cell death and in the context of caspase inhibition, who the players are etc. Most readers will not know what this is I suggest you discuss the forms of regulated necrosis in more detail and discuss what has and has not been investigated in the APAP model and the many controversies. This reviewer agrees that APAP cell death is a form of regulated necrosis. However, the authors should comment that the role of necroptosis which a particular mode of regulated necrotic cell death has been examined in APAP toxicity and two studies have found that MLKL knockout mice were not protected from APAP. REF [66] and Gunther et al, J Clin Invest. 2016 Nov. 1;126(11):4346-4360. So it is true that many have observed a role for RIPK1 in APAP this does not seem to be through the necroptosis pathway. RIPK1 and RIPK3 have other functions other than necroptosis execution which may explain some of the findings by these groups.

7- Page 16 line 12. At the end of the paragraph about biomarkers discussing HMGB1, can the authors comment on an intriguing recent report where HMGB1 neutralizing antibody was used to attenuate liver injury from APAP? And possibly comment on what possible mechanism are at play such as the role of sterile inflammation? Lundback Hepatology. 2016 Nov;64(5):1699-1710.

8- The role of connexins in acetaminophen: the role of gap junctions in acetaminophen is a very interesting topic has been examined lately and should be commented on. Du et al Toxicol Appl Pharmacol. 2013 Dec, Maes et al Biochim Biophys Acta. 2016 Jun;1862(6):1111-21.

Minor/stylistic critiques

1- Page 3 line 17 with intentional apap overdose (those in which patients took…

The authors are describing therapeutic misadventure but the sentence is a bit convoluted and the parenthesis is opened and never closed. Can you please simplify.

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cyclophilin D is generally…. Two sentences back to back start with "while" would change one.

5- Page 12 line 24, it has also been shown that DRP1 can influence release of … this sentence very long and is not structured properly and is missing a verb, please rewrite.

********Authors' rebuttal********

RESPONSE TO REVIEWER'S COMMENTS

Reviewer #1: The Review JCTRes-D-16-00039 "Intracellular signaling mechanisms of acetaminophen-induced cell death and their translation to the human pathophysiology" is a very well-written summary of all events triggered by APAP intoxication from a murine situation and the human translational view. This is essential in order to unveil novel biomarkers that can predict the course of liver injury after APAP overdose in humans.

MAJOR COMMENTS

Comment 1- L36 The authors need to elaborate on the sentence 'key mechanistic indices in injury'. Do they mean serum transaminases?

Response 1- The sentence dealt with key steps in the intracellular signaling subsequent to APAP overdose, such as mitochondrial dysfunction and this has now been made clear in the revised manuscript (Page 4).

Comment 2- Maybe the authors should expand more the GSH depletion part after APAP intoxication, describing the pathophysiology of gluthathione depletion.

Response 2- As suggested by the reviewer, the section on GSH depletion has been expanded as detailed below at the end of page 5 of the revised manuscript.

"While the initial depletion is similar in both GSH and GSSG, without affecting the GSSG:GSH ratio (1:200), the recovery rates are different, with GSSG content increasing faster than that of

GSH [19]. Recovery rate of GSH can significantly influence injury, since an induction of glutamate-cysteine ligase which correlated with faster recovery of GSH is one of the mechanisms by which female mice are protected against APAP-induced liver injury[20]. A differential metabolomics study suggests that the depletion of glutathione after low dose APAP (150mg/kg) is paralleled by elevation in the glutathione analogue ophthalmic acid, where the SH group of the cysteine residue of GSH is replaced with a CH3 group from 2-aminobutyrate [21]. Detection of ophthalmic acid in serum from APAP-induced acute liver failure patients was also more frequent in non-survivors [22]."

Comment 3- P7, L58 The authors should address this fact discussing recent publications such as Cubero (2016).

Response 3- The paper suggested by the reviewer has been discussed as detailed below on page 9 of the revised manuscript.

"While a few studies suggested that JNK was protective in acetaminophen toxicity [61, 62], the data from one of them [62] could be influenced by the differing susceptibility to APAP toxicity of mice sub-strains used in the study [63]. It is however unclear why the knocking down of both JNK1 & 2 in hepatocytes resulted in a paradoxical exacerbation of APAP-induced liver injury [61], and this requires further study."

Comment 4- In addition, the authors should discuss other forms of cell death after APAP intoxication, e.g.: what happens to inflammasome? Ferroptosis? Pyroptosis?

Response 4- The inflammasome has been discussed in a new section as detailed below on page 18 of the revised manuscript and ferroptosis has also been mentioned as described below at the end of page 17 in the revised manuscript.

"APAP-induced hepatocyte necrosis results in massive release of damage associated molecular patterns (DAMPs), which can then lead to recruitment of monocytes and neutrophils. [139]. While it is well established that inflammation is induced after APAP- induced liver injury, there has been some controversy in the literature regarding the biological role of this inflammation and whether it would be a useful therapeutic target. While a number of earlier studies suggested that inflammation played a role in hepatocyte necrosis with P2X7 receptor-mediated purinergic signaling thought to promote liver injury through the inflammasome [140, 141], the identity of the cytotoxic cell type involved in immune mediated injury is not clear and the effect of P2X7 was shown to be due to inhibition of P450 isoenzymes by the inhibitor of P2X7 and not through inflammasome activation[142]. A considerable amount of data, reviewed in Woolbright et al [139], exists that raises concerns about the role of sterile inflammation and the importance of inflammasome activation during APAP hepatotoxicity, and the majority of experimental evidence suggests that the extensive sterile inflammatory response during APAP hepatotoxicity is mainly beneficial by limiting the formation and the impact of pro-inflammatory mediators and

by promoting tissue repair [143]. The role of inflammation in APAP-induced liver injury is further discussed in detail in the Woolbright article in this special issue [144]."

"A recent report has identified an iron dependent non-apoptotic form of cell death termed ferroptosis [137] and studies on primary hepatocytes in culture seem to suggest that this form of cell death may play a role in APAP-induced hepatocyte injury [138]. However, the physiological significance of this form of cell death in APAP-induced liver injury in vivo needs further study."

As discussed in the section on inflammation in APAP, the preponderance of information on the role of inflammation in APAP induced liver injury suggests its role in repair and regeneration, rather than induction of cell death directly. Moreover, APAP induced cell death is necrotic, without activation of caspases. Since pyroptosis is a distinct form of programmed cell death induced by caspase 1 in the context of inflammation (1), it is unlikely to be involved in APAP hepatotoxicity.

Reference

1) Wree A, Eguchi A, McGeough MD, Pena CA, Johnson CD, Canbay A, Hoffman HM, Feldstein AE. NLRP3 inflammasome activation results in hepatocyte pyroptosis, liver inflammation, and fibrosis in mice. Hepatology. 2014 Mar;59(3):898-910.

Comment 5- P11, L31, please rephrase the sentence "JNK activation was also fleeting"

Response 5- The sentence has been corrected as requested on page 14 of the revised manucript

MINOR COMMENTS

Comment 6- The Abstract is missing some punctuations (commas) e.g L43…overdose, indicates…

Introduction: (L20, those in patients who took excessive…)

Response 6- Commas have been added as suggested in the abstract and introduction.

Comment 7- L46 ..in preventing APAP-induced ALF, it has to be administered early after APAP consumption to..(please check the construction of the sentence.

Response 7- The sentence has been modified as requested on page 4 of the revised manuscript.

Reviewer #2: Reviewer's critiques:

JCTRes-D-16-00039

This is a well written and thorough review of the major signaling pathways and mechanisms in acetaminophen toxicity. However, the review is not exhaustive, some of the current segments need to be expanded upon and some additional segments need to be added to make is stronger.

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"We suggest to preserve the adjective 'programmed' for those physiological instances of cell death - irrespective of the modality by which they are executed - that occur in the context of embryonic/post-embryonic development and tissue homeostasis. 'Regulated' should be used to indicate cases of cell death - be they programmed or not - whose initiation and/ or execution is mediated by a dedicated molecular machinery, implying that they can be inhibited by targeted pharmacological and/or genetic interventions"

Response 1- The term regulated necrosis has been substituted for programmed necrosis throughout the revised manuscript as requested.

Comment 2- Page 7-line 19: This activation of JNK can be mediated by the apoptosis signal inducing kinase 1… I suggest authors also include the role of MLK3 in the early phase of JNK activation (Sharma, Mol Pharmacol. 2012 REF [57]. While the authors have referenced this paper they have not elaborated on the early versus late phase of JNK activation. I suggest the authors expand on the MAPK cascade and discuss this in more detail.

Response 2- As suggested by the reviewer, the role of MLKL3 has been elaborated as detailed below on page 8 & 9 of the revised manuscript.

“JNK activation after APAP overdose occurs early after APAP overdose, and is then sustained during the signaling cascade inducing hepatocyte cell death. The apoptosis signal-regulating kinase 1 ASK1 is involved in APAP-induced activation of JNK, with ASK1 deficient mice being protected against the sustained JNK elevation [55] and a specific ASK1 inhibitor decreasing JNK activation at 1.5 h and preventing JNK translocation to mitochondria [56]. In addition, it was found that the mixed-lineage kinase 3 (MLK3) was activated by oxidative stress and was required for JNK activation in response to oxidative stress [57]. It was also seen that JNK phosphorylation at one, three and six hours after APAP treatment was significantly attenuated in MLK3-KO mice [57]. Since MLK3 has been suggested to be part of a feedback mechanism that regulates cellular responses to ROS [58] and the activation of JNK can be prevented by anti-oxidants [42], it is possible that APAP-induced, ROS mediated JNK activation occurs through multiple mechanisms with temporal changes in their interaction.”

Comment 3- The role of ER stress has been explored in acetaminophen toxicity. It is very feasible due to reactive adducts formed during overdose proteins malfold and clog the ER

thereby causing stress. So ER stress probably does occur during toxicity, however this reviewer does not believe it is a major player in the cell death and toxicity from acetaminophen. Regardless, it should be referred to and discussed for completeness. For example, CHOP KO mice have been examined (Uzi D, J Hepatol. 2013 Sep;59(3):495-503.)

Response 3- As suggested, a section on the role of ER stress in APAP hepatotoxicity as detailed below has been added in the revised manuscript on page 12.

“Protein folding in the endoplasmic reticulum (ER) is a critical cellular function and various cellular stresses such as ROS or alterations in cellular calcium can impair protein folding and initiate ER stress. Mice treated with 200mg/kg of acetaminophen showed activation of ER stress with upregulation of GADD153/CHOP by 6 hours after APAP administration, accompanied by a decrease in Grp78 levels [85]. Higher doses of APAP also induce markers of ER stress, with doses of 450mg/kg APAP inducing activation of ER stress-responsive transcription factor ATF6 and transcriptional activation and elevated expression of GADD153/CHOP [86]. CHOP deficient mice were also shown to be protected against APAP-induced liver injury, though interestingly the protection was only seen in animals given APAP by gavage and not in those given APAP as an intra-peritoneal injection [87]. Hence, while ER stress does seem to occur after APAP overdose, the mechanisms by which APAP induces ER stress is poorly understood [88] and need more study.”

Comment 4- Page 9 line 56: However, chronic deletion of Parkin renders animals resistant to APAP.-Perhaps this is due to compensatory effects when you have an embryonic KO? If authors agree I would comment on that. It brings to mind the NNT mutation in J strain mice which renders them paradoxically more resistant to APAP due to compensatory mechanisms.

Response 4- As suggested, the sentence has been modified to read “However, chronic deletion of Parkin renders animals resistant to APAP [82], possibly due to development of compensatory and adaptive mechanisms for the chronic loss of Parkin, which may contribute to the resistance to APAP-induced liver injury [82].” on page 11 of the revised manuscript.

Comment 5- Page 13line 26. Genetic deletion of RIP3 delayed APAP induced cell death [65]. This is controversial and it should be commented that while it did delay toxicity in one study this was not observed in another study REF [66]. This is currently a controversial topic as most people agree that RIPK3 is not present in hepatocytes but may be induced in certain conditions. I suggest the authors only refer to what is definitely established in the field and agreed upon by most experts in the abstract. In the abstract the sentence starting with "this then amplifies the mitochondrial oxidant stress…" I would take out RIP3 and just discuss the controversy with RIP3 under the necrotic cell death.

Response 5- The current literature regarding RIP3 and the fact that RIP3 could not be detected in hepatocytes by one study is now mentioned clearly over pages 15-17 of the revised manuscript. Also, RIP3 has been removed from the abstract as suggested.

Comment 6- Necrotic cell death in APAP overdose is the most ambiguous section. For example page 13 line 33, you refer to "cardiomyocyte necroptosis" REF[111] however nowhere in the text do the authors describe what necroptosis is, that it is receptor mediated cell death and in the context of caspase inhibition, who the players are etc. Most readers will not know what this is I suggest you discuss the forms of regulated necrosis in more detail and discuss what has and has not been investigated in the APAP model and the many controversies. This reviewer agrees that APAP cell death is a form of regulated necrosis. However, the authors should comment that the role of necroptosis which a particular mode of regulated necrotic cell death has been examined in APAP toxicity and two studies have found that MLKL knockout mice were not protected from APAP. REF [66] and Gunther et al, J Clin Invest. 2016 Nov. 1;126(11):4346-4360. So it is true that many have observed a role for RIPK1 in APAP this does not seem to be through the necroptosis pathway. RIPK1 and RIPK3 have other functions other than necroptosis execution which may explain some of the findings by these groups.

Response 6- Regulated necrosis has been introduced with more detail starting at the bottom of page 15 of the revised manuscript and the role of MLKL discussed as suggested.

Comment 7- Page 16 line 12. At the end of the paragraph about biomarkers discussing HMGB1, can the authors comment on an intriguing recent report where HMGB1 neutralizing antibody was used to attenuate liver injury from APAP? And possibly comment on what possible mechanism are at play such as the role of sterile inflammation? Lundback Hepatology. 2016 Nov;64(5):1699-1710.

Response 7- As suggested, the papers using anti-HMGB1 antibodies have been discussed on pages 21 of the revised manuscript.

Comment 8- The role of connexins in acetaminophen: the role of gap junctions in acetaminophen is a very interesting topic has been examined lately and should be commented on. Du et al Toxicol Appl Pharmacol. 2013 Dec, Maes et al Biochim Biophys Acta. 2016 Jun;1862(6):1111-21.

Response 8- A new section on the role of gap junctions in APAP hepatotoxicity has been added on page 19 of the revised manuscript and the papers discussed as suggested.

Minor/stylistic critiques

Comment 9- Page 3 line 17 with intentional apap overdose (those in which patients took…

The authors are describing therapeutic misadventure but the sentence is a bit convoluted and the parenthesis is opened and never closed. Can you please simplify.

Response 9- The sentence has been corrected to read "…,with unintentional APAP overdoses, (those in which patients took excessive medication over several days for ailments like pain or fever) being more common than intentional (suicidal) overdoses [2]."

Comment 10- Page 4 line 43, reaction of NAPQI to mitochondrial.. should it be instead: Interaction of NAPQI with mitochondrial…?

Response 10- The sentence has been corrected as suggested.

Comment 11- Page 5 line 12, "human liver slices" do you mean human liver sections, or biopsy samples?

Response 11- The study used precision cut liver slices and this has now been made clear on page 6 of the revised manuscript.

Comment 12- Page 10 line 43, while it has been suggested that Bax and Bak. And line 48 While cyclophilin D is generally…. Two sentences back to back start with "while" would change one.

Response 12- As suggested, the sentence has been modified on page 13 of the revised manuscript.

Comment 13- Page 12 line 24, it has also been shown that DRP1 can influence release of … this sentence very long and is not structured properly and is missing a verb, please rewrite.

Response 13- The sentence has been modified to read “It has also been shown that DRP1 can influence release of intermembrane proteins [104] implicating it in release of cytochrome c, Smac, AIF and endonuclease G which is evident after APAP [67, 107]”, on page 14 of the revised manuscript.

2nd editorial decision

Date: 16-Jan-2017

Ref.: Ms. No. JCTRes-D-16-00039R1
Intracellular Signaling Mechanisms of Acetaminophen-induced cell death and their translation to the human pathophysiology
Journal of Clinical and Translational Research

Dear Dr. Ramachandran,

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

Comments from the editor and reviewers can be found below.

Thank you for submitting your work to JCTR.

Kindest regards,

Michal Heger

Journal of Clinical and Translational Research
Review process file 03.2017S1.002

Editor-in-Chief
Journal of Clinical and Translational Research

Comments from the editors and reviewers:

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