Journal of Clinical and Translational Research
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Lack of direct cytotoxicity of extracellular ATP against hepatocytes: role in the mechanism of acetaminophen hepatotoxicity

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Michal Heger
Academic Medical Center, University of Amsterdam, the Netherlands

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1st editorial decision

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Ref.: Ms. No. JCTRes-D-15-00006
Direct Cytotoxicity of Extracellular ATP against Hepatocytes: Role in the Mechanism of Acetaminophen Hepatotoxicity
Journal of Clinical and Translational Research

Dear Professor Jaeschke,

Reviewers have now commented on your paper. You will see that they are advising that you revise your manuscript. If you are prepared to undertake the work required, I would be pleased to reconsider my decision.

For your guidance, reviewers' comments are appended below.

If you decide to revise the work, please submit a list of changes or a rebuttal against each point which is being raised when you resubmit your work.

Your revision is due by Aug 15, 2015.

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

Editorial comments:

Dear authors, there is interest in your paper as the editorial board and the reviewers find your data useful and relevant. Collectively, we want to work with you towards an acceptable paper. However, both reviewers find it necessary to perform some additional experiments, which in essence do not have to be that elaborate. For instance, conducting the experiments as advised (i.e., positive controls) in combination with a WST-1 assay (mitochondrial redox state and activity) and sulforhodamine B (protein content), which can be done with a single batch of cells, should be sufficient to address the major concerns. If you wish, we can provide detailed protocols for both.

Please let us know whether you're willing to revise your paper accordingly and at which points we can be of assistance.

Kindest regards,

Michal.

Reviewers' comments:

Reviewer #1: This is an interesting paper that tests the hypothesis that extracellular ATP can cause cell death and/or aggravate APAP-induced cell injury in primary mouse or human hepatocytes and in two different hepatoma cell lines. The authors found that even high concentrations of ATP did not have any significant effects on the viability of untreated or APAP-treated cells.

Although the data are "negative data", the results are important for improving our mechanistic understanding of drug-induced cell injury. The paper is very well written, and the Results, Method section, and Discussion are flawless. However, the authors need to address a number of points that would make the paper even better.

1. The results clearly show that extracellular ATP is not cytotoxic and that it does not aggravate APAP-induced cell injury. Therefore, the title of the manuscript is a bit misleading. The title should clearly state that there is an apparent absence of ATP effects.

2. Was there any positive control to demonstrate the functional integrity of the purinergic

receptor on hepatocytes?

3. How could the discrepancy between this paper and the previous results by Amaral et al. (2013) be explained?

Minor points:

4. page 4, line 4 (and elsewhere): the term "metabolically deficient" should perhaps be clarified (poor expression of CYPs).

5. Is the stability (t1/2) of ATP in the extracellular medium in the presence of viable or dying cells known?

Reviewer #2: The authors provide a valuable contribution to the field be examining the effect of metabolites on primary human hepatocytes in particular. The conclusions are entirely negative with regard to an effect of ATP on cell toxicity of hepatocyte cell types. This result is strikingly different from the published work of Amaril et al in 2013. The major differences between the two works are that Amaril found ATP at 10-100 uM to be cytotoxic directly to hepatocytes and to sensitize to APAP cytotoxicity. Different from the current work, Amaril also included experiments with non-metabolizable ATP analogs in vitro and apyrase in vivo and in vitro and utilized MTT and acridine orange/ethidium bromide staining (as opposed to LDH release). Finally, Amaril confirmed APAP mediated release of ATP by HPLC in vitro.

Major Comments:
1. Please consider additional experiments to support your conclusion- either use of apyrase in APAP or use of non-metabolizable ATP analogs.
2. Please consider use of a control to confirm that your ATP is biologically active (as all of the ATP data is negative) such as ATP mediated cell death of macrophages.

Minor Comments:
1. Please assess cell death by an additional modality- such as vital dye exclusion or MTT assay, etc

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Authors' rebuttal:

Thank you for the comments and the chance to revise the manuscript. We feel we have answered the question brought up by the reviewers. We would like to re-emphasize the purpose of the paper was a repeat of a study (Amaral et al., 2013) that yielded very different results. We have tried to faithfully reproduce that study and have added to the manuscript as follows.

Reviewers' comments:

Reviewer #1: This is an interesting paper that tests the hypothesis that extracellular ATP can cause cell death and/or aggravate APAP-induced cell injury in primary mouse or human hepatocytes and in two different hepatoma cell lines. The authors found that even high concentrations of ATP did not have any significant effects on the viability of untreated or APAP-treated cells.

Although the data are "negative data", the results are important for improving our mechanistic understanding of drug-induced cell injury. The paper is very well written, and the Results, Method section, and Discussion are flawless. However, the authors need to address a number of points that would make the paper even better.
1. The results clearly show that extracellular ATP is not cytotoxic and that it does not aggravate APAP-induced cell injury. Therefore, the title of the manuscript is a bit misleading. The title should clearly state that there is an apparent absence of ATP effects.

This has been updated. Thank you for the comment.

2. Was there any positive control to demonstrate the functional integrity of the purinergic receptor on hepatocytes?

While there was no positive control performed in this study, the purpose of the study was to repeat the results of Amaral et al. We did treat cells very quickly after isolation, which typically limits dedifferentiation (See Rippin et al., Hepatology 1999). While we cannot definitively say that the purinergic receptor is active, we can say that ATP does not exacerbate toxicity, and is not toxic alone. This was performed under identical conditions with both cell lines that Amaral et al used, as well as two other cell lines that better represent a human patient's response to APAP.

3. How could the discrepancy between this paper and the previous results by Amaral et al. (2013) be explained?

It would be difficult to fully comment on this without a side by side comparison of the procedures for the two studies. What can definitively be said about our study is that multiple types of ATP (ATP and ATγP) from the same commercial source (Sigma) failed to increase cell death using multiple assays in multiple hepatocyte cell lines or primary hepatocytes. In addition we have tested ATP in macrophage cell lines (RAW cells) where we could find limited toxicity at extremely high concentrations. We have chosen to use different cell death assays. We feel that LDH release is a better quantitative measure of cell death and PI/DAPI staining is a gold standard of cell necrosis.

Thus, overall our conclusions are based on 5 different cell types using 3 different cell death assays.

Minor points:
4. page 4, line 4 (and elsewhere): the term "metabolically deficient" should perhaps be clarified (poor expression of CYPs).

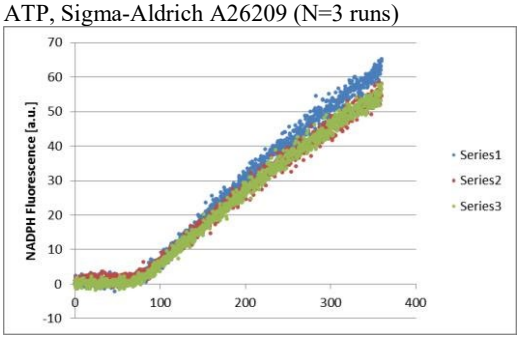
We have deleted "metabolically deficient" and replaced it with "….which does not express cytochrome P450 and other drug metabolizing enzymes and transporters (Wilkening et al., 2007)."

5. Is the stability (t1/2) of ATP in the extracellular medium in the presence of viable or dying cells known?

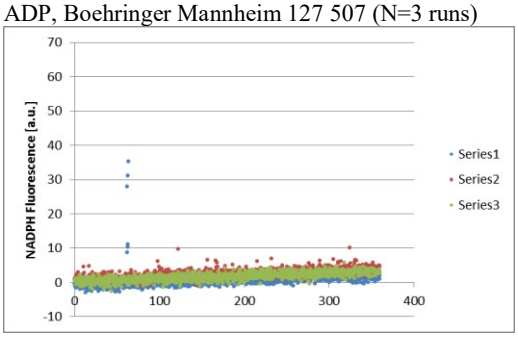
The half-life of ATP in solution is fairly stable as per Heger et al. (2010). Our own experience with ATP standard curves suggests that ATP is stable in solution for many hours. In addition, a hexokinase assay was performed that demonstrated the biological activity of ATP used in these studies and stability of several hours (see below, performed 3 hours after preparation for ATP, ADP, and AMP as per Heger et al. (2010)). One would assume that the stability of the nonhydrolyzable form would be much higher, and yet this form also did not cause cytotoxicity.

Regardless of this, experiments paralleling the experimental design of Amaral et al yielded dissimilar results.

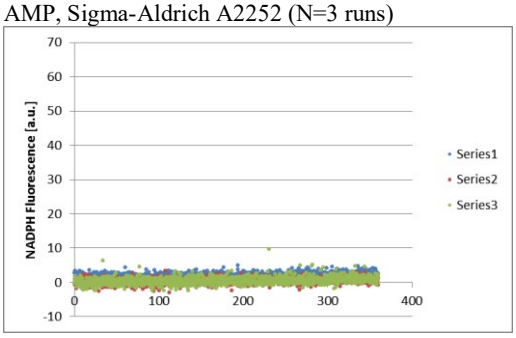
ATP, Sigma-Aldrich A26209 (N=3 runs)



ADP, Boehringer Mannheim 127 507 (N=3 runs)



AMP, Sigma-Aldrich A2252 (N=3 runs)



Reviewer #2: The authors provide a valuable contribution to the field be examining the effect of metabolites on primary human hepatocytes in particular. The conclusions are entirely negative with regard to an effect of ATP on cell toxicity of hepatocyte cell types. This result is strikingly different from the published work of Amaril et al in 2013. The major differences between the two works are that Amaril found ATP at 10-100 uM to be cytotoxic directly to hepatocytes and to sensitize to APAP cytotoxicity. Different from the current work, Amaril also included experiments with non-metabolizable ATP analogs in vitro and apyrase in vivo and in vitro and utilized MTT and acridine orange/ethidium bromide staining (as opposed to LDH release). Finally, Amaril confirmed APAP mediated release of ATP by HPLC in vitro.

Major Comments:
1. Please consider additional experiments to support your conclusion- either use of apyrase in APAP or use of non-metabolizable ATP analogs.

Thank you, we have included experiments with a non-metabolizable ATP analogue (new Figure 3).
This yielded identical results to the normal form of ATP.

2. Please consider use of a control to confirm that your ATP is biologically active (as all of the ATP data is negative) such as ATP mediated cell death of macrophages.

We have included data on ATP-mediated cell death of macrophages. This was done using ATP from the same company (Sigma-Aldrich). The data indicate that ATP is both stable and active in culture and kills RAW macrophages, although only at extremely high concentrations that may have little pathophysiological relevance. See New Figure 6.

Minor Comments:
1. Please assess cell death by an additional modality- such as vital dye exclusion or MTT assay, etc

We have included a PI/DAPI stain (cell necrosis) and JC-1 fluorescence (mitochondrial membrane potential) as additional metrics (Figure 1B and Figure 2). Thank you for the comment.

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2nd Editoral Decision

Date: 10-September-2015

Ref.: Ms. No. JCTRes-D-15-00006R1
Lack of Direct Cytotoxicity of Extracellular ATP against Hepatocytes: Role in the Mechanism of Acetaminophen Hepatotoxicity
Journal of Clinical and Translational Research

Dear Professor Jaeschke,

I am pleased to inform you that your manuscript has been accepted for publication in the

Journal of Clinical and Translational Research.

Thank you for submitting your work to JCTR.

You will receive the proofs of your article shortly.

Kindest regards,

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