Reviewer's report

Title: Hyperammonia Induces Specific Liver Injury through an Intrinsic Ca2+-independent Apoptosis Pathway

Version: 3
Date: 9 July 2014
Reviewer: Jason Coombes

Reviewer's report:

This paper still contains many formatting flaws and the scientific flow is not presented in a very logical manner.

However, scientifically, most of the conclusions and interpretations of the data here are not supported by the evidence. Repeatedly, I find conclusions that are inaccurate and not well-suited to the data.

Minor Essential revisions:

1) In the previous review, I reported that there were problems with the figures. They were mislabelled, confused, and arranged in a strange order. These problems still exist, and make this paper very difficult to assess.

What should be 2C, is labelled as 2B in the figure legend, whereas what should be 2B is not described in the figure legend. Please make the text, figure legends, and figures consistent.

On the version I see, the page labelled Figure 4 is completely blank. However, data in page labelled figure 5 appears to correspond with what should be in figure 4. Therefore, the successive figures are all mislabelled.

The data for Figure 5 appears to be in both Figure 6 and Figure 7.

Data for figure 6 is in figure 8.

This lack of accurate figure preparation and description makes the act of assessing this paper more difficult and frustrating than it should be.

2) Additionally, the order that the figures are presented is not very logical or consistent with the presentation of results in the main text.

Major essential revisions:

3) ALT: ALT was relatively decreased by NH4Cl at 48 hr, whereas it was relatively increased at 12 hr, and even more at 24 hr. Each of these timepoints were reported as statistically significant. Therefore there is no consistent evidence that NH4Cl induced injury, as indicated by ALT, and in fact the opposite may even be true at the other timepoints.

4) Time-dependent dose: In the text, it is stated in section 3.3.2 that “A dramatic time-dependent loss was found in cell viability when exposure to 20 mM and 50 mM ammonia, however the viability of cells recovered when exposed to 5 mM
ammonia for 48 h”. However, in the figures, 50 uM clearly does not have a
time-dependent response because viability increases markedly at 48 hr. 5mM
shows little statistically significant evidence of recovery. 5 mM simply looks like a
low level of toxicity.

5) Cell viability: I have a few questions here. In figure 2C, why is cell viability
increasing with increasing concentration of NH4Cl? I thought the basic premise
of the article was that NH4Cl increased toxicity (as it did in 2A and 2B?) Is this
the opposite? What units are displayed on the y-axis (percentage?)? This should
be consistent in each MTT figure. Why was 100 mmol/L NH4Cl used for this
figure when 20 and 50 were used for the others? This must be consistent,
especially considering in 2B, 20 and 50 mmol/L doses had very different kinetics
regarding viability.

6) Section 3.2.1 Activity of Na-K ATPase and 3.2.2 Arginine level
- These data don’t appear to be presented in any figures or have any statistical
analysis?

7) Apoptosis signalling:
Calmodulin and iNOS mRNA: No control levels (0 mM NH4Cl) are shown in the
graphs, however they are shown for Caspase 3 and Cytochrome C. They need to
be shown.
Calmodulin Western: there is a sharp peak in calmodulin shown at 5 mM dose,
however this appears to be unlikely given there is also an increase in beta actin
at this dose.
Cleaved caspase 3: cleaved caspase 3 must be calculated and shown as a
percentage of total caspase 3. The quantitation figure needs to show this.
For all these apoptosis-related westerns, which time point is shown? It appears
to be only one timepoint. The figure legend seems to suggest it might be 48 hrs?
This is unclear.
Additionally, why is only 1 timepoint shown for western blots? Despite this, there
is a chart in the bottom-right of the figure showing “relative expression level of
protein vs time”. The timepoint data (western blots) used for this graph is not
shown. Additionally, there is nothing to indicate which dose of NH4Cl is
represented by this graph. These details need to be absolutely clear. Also, no
quantification is shown for time 0 hr.

8) Immunohistochemistry in In Figure 7: (Expression of caspase-3 and Cyt C in
hepatocytes) –there are no negative controls shown (i.e. sections not probed with
primary antibody), and no quantitation. The staining here also looks very
non-specific – can the authors demonstrate that is indeed specific?
I can’t find anywhere in the results that mentions the immunohistochemistry
results presented in Figure 7. (Additionally, no text is provided in the legend for
Figure 7).
No description of immunohistochemistry methods is provided in the methods section.  
(In line 336-337 It is stated that Figure 7 shows NO and iNOS levels. This is not the case.)

In general, given the confusion and lack of clarity of this apoptosis data, it is hard to argue that these conclusions are supported by the data.

In summary, many of the conclusions of this manuscript are not supported by the data. I'm trying really hard to contemplate this paper, but there are many problems that are very difficult to understand. It needs extensive reconsideration, simplification, and the authors should put a lot of effort into deeply considering their data and what it really means.

**Level of interest:** An article whose findings are important to those with closely related research interests

**Quality of written English:** Needs some language corrections before being published

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**

I declare that I have no competing interests.