Author's response to reviews

Title: Pharmacologic cholinesterase inhibition improves survival in acetaminophen-induced acute liver failure in the mouse

Authors:

Niels Steinebrunner (niels.steinebrunner@med.uni-heidelberg.de)
Carolin Mogler (carolin.mogler@med.uni-heidelberg.de)
Spiros Vittas (spiridon.vittas@med.uni-heidelberg.de)
Birgit Hoyler (birgit.hoyler@med.uni-heidelberg.de)
Catharina Sandig (c.steinebrunner@med.uni-heidelberg.de)
Wolfgang Stremmel (wolfgang.stremmel@med.uni-heidelberg.de)
Christoph Eisenbach (christoph.eisenbach@med.uni-heidelberg.de)

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Author's response to reviews: see over
Dear Editor Professor Dr. Joel Lavine,

Thank you for the careful review and overall positive perception of our manuscript entitled "Pharmacologic cholinesterase inhibition improves survival in acetaminophen-induced acute liver failure in the mouse".

Our revised version of the manuscript now addresses issues raised by the reviewers and includes additional data. We appreciate the opportunity to re-submit a revised version and hope that these improvements may be sufficient to receive acceptance of the manuscript. Please find below our point-by-point reply to the reviewers’ comments.

Reply to reviewer Professor Dr. Mitchell Fink

Major Comments:

1. Although neostigmine pre-treatment prolonged survival, administration of this drug failed to change outcome. 100% of the animals died irrespective of whether or not they received neostigmine. The authors need to address this issue. Was their model too overwhelming? If so, additional studies using a model with lower mortality would be helpful. Was the dose of neostigmine inadequate? If so, additional, dose-response studies would strengthen the paper.

Since N-acetylcysteine (NAC) is an established and frequently effective treatment for patients intoxicated with acetaminophen (APAP) our intention was to evaluate an add-on therapeutic benefit of neostigmine for hard-to-treat patients with severe APAP-induced hepatotoxicity, for example due to ingestion of large amounts of APAP, for whom sole treatment with NAC frequently falls short.

In numerous publications APAP has been applied in various mouse models. Toxicity of APAP in each mouse model varies depending on the mouse strain, the sex of the mice or the fasting state [1, 2]. Therefore, lethal dosing of APAP in mouse models is reported to start in a range of 500 mg/kg – 750 mg/kg [3-9]. For male Balb/c mice, as used in our experiments, the LD$_{50}$ = 350 mg/kg applied intraperitoneally (i.p.) and lethal dosing with mice dying within 24 hours after application starts at 500 mg/kg i.p. [8].
With regard to the dosing of neostigmine, dose-finding studies were performed by our group in a previous murine model of cecal ligation and puncture (CLP)-induced sepsis [10]. A dosing of neostigmine with 80 µg/kg i.p., applied three times a day, comparable to the dosing in the current study, prolonged survival in this murine sepsis model. Additionally, according to data of the pharmaceutical supplier of neostigmine, the LD$_{50}$ of neostigmine in mice for a single dose subcutaneously is 0.66 (0.56 ± 0.80) mg/kg and 0.47 mg/kg for intravenous application.

We agree with the reviewer that dose-response studies would strengthen the manuscript, however ethical concerns limited further dose-finding experiments. As pointed out below in additional experiments now included in the revised manuscript, we could show that neostigmine treatment given in combination with NAC not only prolonged survival but also changed outcome.

2. N-acetylcysteine (NAC) is the current “gold standard” approach for managing acetaminophen overdoses in patients. Thus, it is appropriate to ask whether survival can be improved adding neostigmine to a treatment regimen based on administration of NAC.

As suggested by the reviewer we conducted additional experiments now included in the revised manuscript with a combination treatment of NAC and neostigmine. With a dosing of 750 mg/kg APAP i.p. and a therapeutical treatment with NAC 300 mg/kg i.p. after 2 hours all mice (4 of 4) survived a 48-hour observation period, consistent with previously published data [4, 6, 7]. To assess the value of a combinational treatment, NAC was applied at suboptimal dosing. Mice that were given 750 mg/kg APAP i.p. were treated with either NAC 75 mg/kg i.p. after 2 hours or with a combination of NAC 75 mg/kg i.p. after 2 hours and successive applications of neostigmine after 2, 7, 12 and 24 hours. In this experimental set-up neostigmine significantly (p < 0.05) prolonged survival and also changed outcome. Specifically, during a 48-hour observation period, mortality was 6 of 8 for APAP plus NAC and 2 of 8 for APAP plus NAC plus neostigmine (Fig. 6).

The findings of these experiments and its discussion have been introduced to the revised manuscript.
Minor Comment:
1. Neostigmine is a (reversible) acetylcholinesterase (not acetylcholine) inhibitor.

The mistake in the original manuscript has been corrected according to the reviewer’s suggestion.

Reply to reviewer Professor Dr. Ali Canbay
1. While the results shown depict an improving role of neostigmine in ALF-therapy it would be interesting how this cholinesterase inhibitor could probably support the common therapy with NAC. The authors should include this issue to their discussion.

As outlined in the reply to question # 2 of reviewer Professor Dr. Mitchell Fink we conducted additional experiments with a combination treatment of NAC and neostigmine in APAP-induced hepatotoxicity in mice. The combination treatment of NAC plus neostigmine significantly ($p < 0.05$) prolonged survival and changed outcome during a 48-hour observation period versus treatment with NAC alone in the experimental set-up described above.

These findings have been introduced to the revised manuscript according to the reviewer’s suggestion.

2. Furthermore the authors should include a section within the discussion that focuses more detailed on the role of cell death (apoptosis and overall cell death) in APAP-induced ALF with an indication of the publication of Bechmann et. al. (J Hepatol. 2010 Oct;53(4):639-47.) that describe the supporting role of cell death markers within ALF-therapy and as useful prediction markers.

Discussion of the publication by Bechmann has been introduced to the revised manuscript. In addition, we performed additional terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) stainings on liver sections. Mice that were administered neostigmine (80 µg/kg) 1 and 7 hours after intoxication with APAP (600 mg/kg) showed less extends of DNA fragmentation than control mice
(TUNEL-positive cells expressed as percentages of the total number of hepatocytes $19.5 \pm 3.8\%$ vs. $9.3 \pm 4.8\%$, $p < 0.05$) (Fig. 4 A-D).

The results of the additional experiments and their discussion have been introduced to the revised manuscript.

References


