R1 version of the manuscript : "*N-acetylcysteine and Glycyrrhizin combination: benefit outcome in a murine model of acetaminophen-induced liver failure.* "

We would like to thank the reviewers for their comments and for the time they devoted evaluating our work. On the basis of their recommendations, we conducted additional experiments which, in our opinion, considerably improved the manuscript. We also hope that the answers provided will clarify the misunderstandings highlighted in the previous version of the manuscript. Please find below a point-by-point response.

Reviewer #1

Manuscript Title: N-acetylcysteine and Glycyrrhizin combination: benefit outcome in a murine model of acetaminophen-induced liver failure. Authors: Minsart et al. This paper presents interesting results concerning the potential use of a combination of N-acetylcysteine (NAC) and glycyrrhizin (GL) to treat acute liver failure induced by acetaminophen (APAP), expanding the therapeutic window and increasing survival of the patients. However, the paper needs extensive rewriting to properly explain the election of markers for liver failure or the figures themselves. Also the English needs revision at certain points of the text. Main comments:

1- ***What is the reason to choose the 500 mg/kg dose? Other authors have used lower doses to obtain the same outcome.***

We chose to work with a lethal dose, which is why a dose of 500mg/kg was administered in our murine model.

Indeed, it was important in our model to investigate both liver injury and acetaminophen-induced mortality. As demonstrated in the literature, a dose of 500 mg/kg was required to achieve these two parameters [1][2]. Lower doses of acetaminophen cause liver damage, as shown by increases in ALT, AST and necrosis score, but have no impact on mortality, as confirmed in the literature for the same murine model [3][4][5][6][7].

In addition, it was important in our model to observe extensive centrilobular necrosis resulting in the release of HMGB1 protein.

2- ***Why did you choose to work only with female mice? Wouldn't be interesting to compare effects in males vs females? Some effects may depend on gender and it is interesting to explore such a point in this particular case.***

We chose female mice for several reasons:
1) We wanted to model the clinical situation where APAP-induced acute liver failure frequently occurs in young women [8].
2) Other publications have used female mice for their model of APAP-induced liver injury [1-7].
3) We chose a model with low-grade inflammation to focus on the inflammation-independent HMGB1 effect (as suggested by our previous publication Minsart et. al,2020).

We agree with the reviewer that it would be interesting to replicate the experiment on male mice. However, ethically, we could not justify replicating all the experiments in the manuscript on male mice. But, we did receive the agreement of the ethics committee to compare the effect of NAC or NAC/GL administration in male mice on liver damage induced by an overdose of APAP.
Eight-week-old C57BL/6J wild-type male mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Upon arrival, mice were acclimatized to laboratory conditions (21 °C, humidity 50%) for 1 week prior to experimentation. Mice were maintained on 12-hour light-dark cycle with free access to food and water in accordance with *the Guide for the Care and Use of Laboratory Animals*. Animal protocols were approved by the local Ethic committee of the Université Libre de Bruxelles (Protocol Identifier: 734N). After 15h fasting with free access to water, mice received an intraperitoneal injection of APAP at the dose of 300 mg/kg body weight or/and N-acetylcysteine (150 mg/kg), combination of NAC/GL at various times. Mice were sacrificed by cervical dislocation under anesthesia; blood was collected, and the liver was removed. Blood samples were centrifuged at 13,523 x g for 5 min

and supernatants were stored at -20 °C. Upon removal, the biggest lobe of each liver was fixed in formaldehyde 4%.

As shown in Figure R1 and R2, combination of NAC/GL is also effective in male mice.

We added these results in the supplementary section of the manuscript

Figure R1

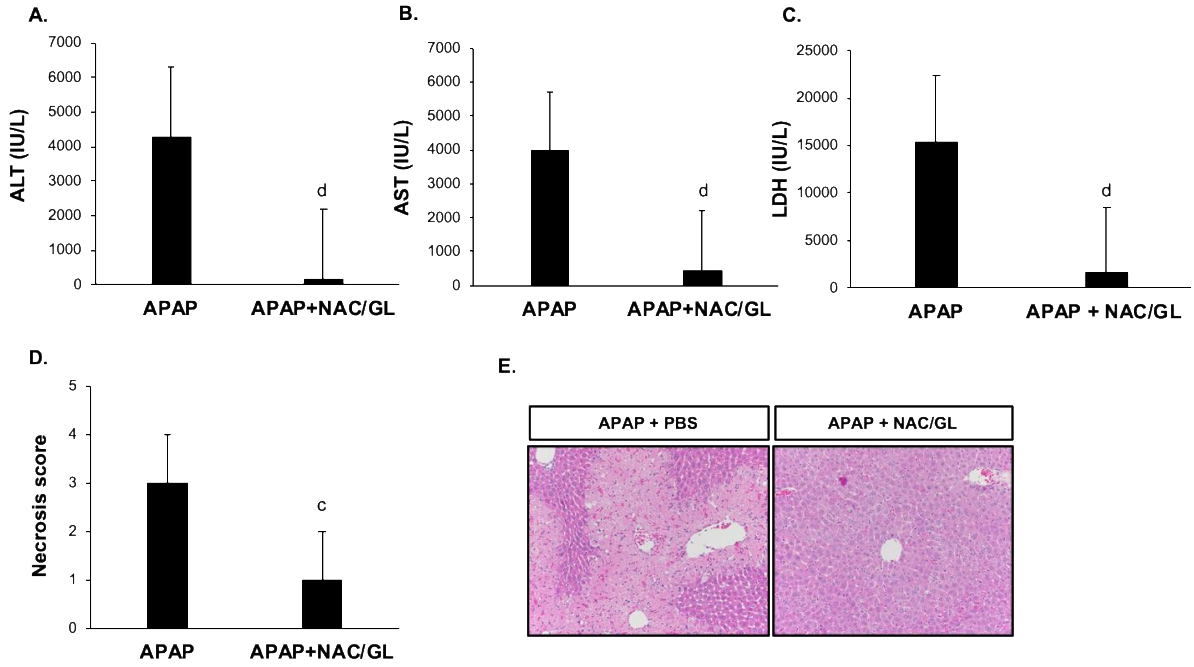

Figure R1 Concomitant administration of APAP and NAC/GL combination reduced the severity of liver injury. A: ALT levels were measured in vehicle-treated mice and in mice co-treated with NAC/GL and APAP at 12h (7 mice in each group); B: AST levels were measured in vehicle-treated mice and in mice co-treated with NAC/GL and APAP at 12h (7 mice in each group); C: LDH levels were measured in vehicle-treated mice and in mice co-treated with NAC/GL and APAP at 12h (7 mice in each group); D: Liver necrosis at 12h after APAP challenge was scored in the same group of mice; E: Representative H&E–stained images (magnification x200) of

murine liver 12h after APAP challenge. Results are expressed as mean ± SE. cP < 0.05, dP < 0.01, eP< 0,001 vs APAP.

Figure R2

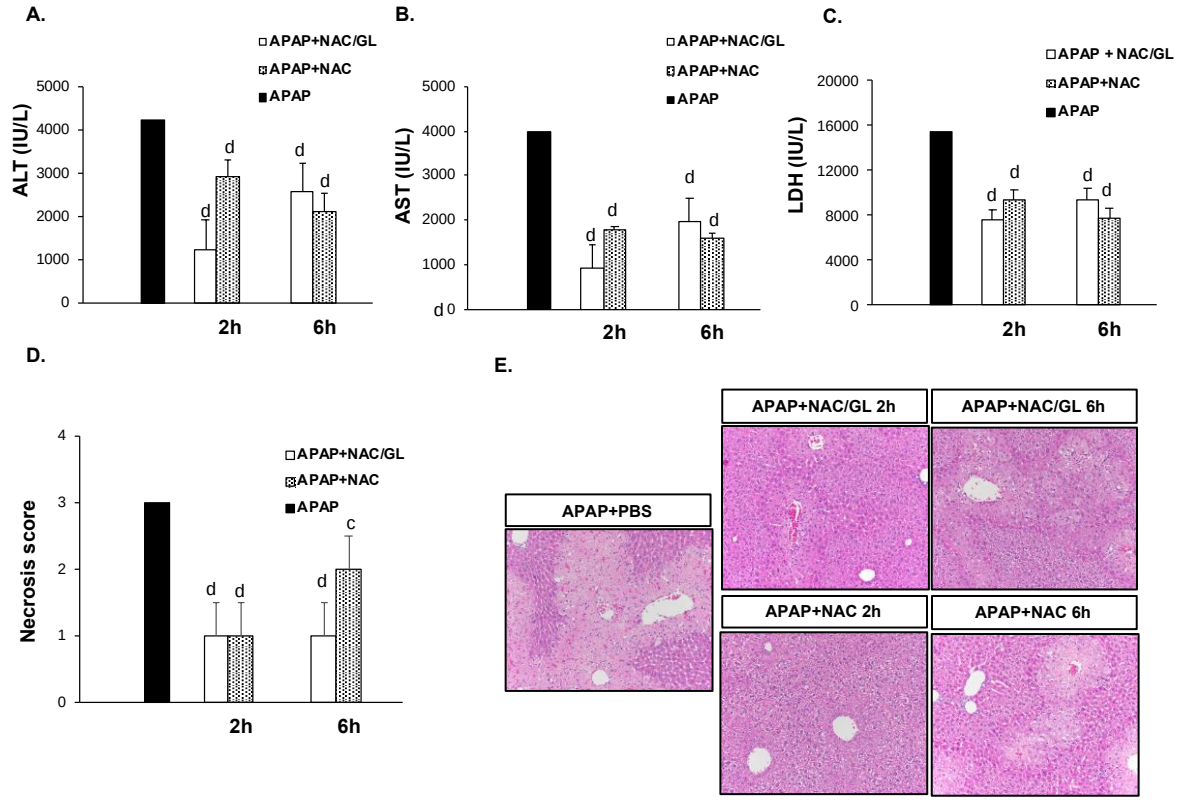

Figure R2 Delayed administration of NAC/GL combination reduced APAP-induced hepatocytes necrosis compare to GL or NAC alone. A: Plasma ALT levels were evaluated in vehicle-treated mice and in mice treated with NAC/GL, 2 or 6 hours after APAP injection (7 mice in each group). Mice were scarified 12h after APAP administration ; B: Plasma AST levels were evaluated in vehicle-treated mice and in mice treated with NAC/GL, 2 hours or 6 hours after APAP injection (7 mice in each group). Mice were scarified 12h after APAP administration; C: Plasma LDH levels were evaluated in vehicle-treated mice and in mice treated with NAC/GL, 2 hours or 6 hours after APAP injection (7 mice in each group). Mice were scarified 12h after APAP administration; D: Liver necrosis at 12h after APAP challenge was scored in the same group of mice; E: Representative H&E–stained images

(magnification x200) of murine liver 12h after vehicle or APAP challenge. Results are expressed as mean ± SE. cP < 0.05, dP < 0.01, eP< 0,001 vs APAP.

3- ***Are you measuring ALT concentration or activity? This is a very important point that needs clarification. It would be interesting to see also AST levels of LDH levels.***
In our experiments, ALT concentration was measured in plasma of mice.
As suggested by the reviewer, we evaluated the LDH and AST levels in our model.
As shown in Figure R3, AST and LDH levels were drastically decreased after treatment with NAC (150mg/kg), GL (200 mg/kg) and NAC/GL combination. These results are also observed when treatment was given later than acetaminophen. We have added these results in our manuscript (*RESULTS*, Figures 2, 3, 4 and 6).

Figure R3

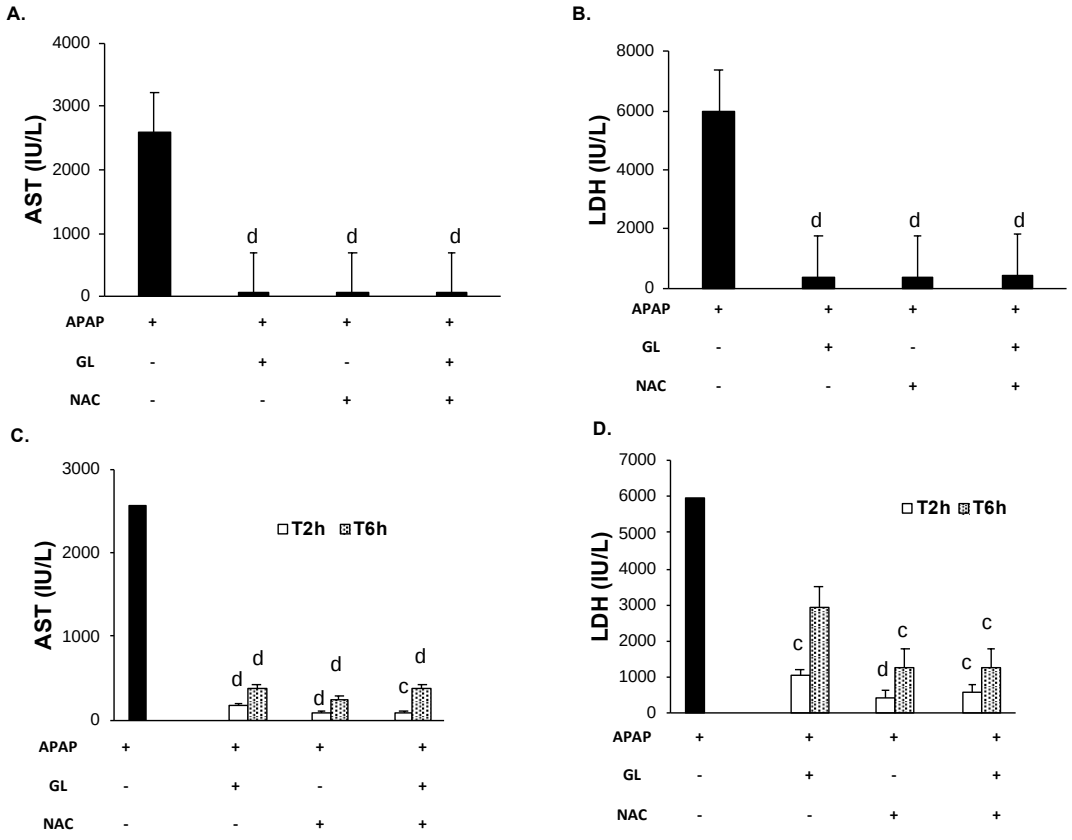

Figure R3 Evaluation of aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) concentration in a murine model of liver injury induced by acetaminophen. A: Plasma AST levels were evaluated in vehicle-treated mice and in mice treated with NAC (150 mg/kg), GL (200 mg/kg) or NAC/GL at same time of APAP (10 mice in each group). Mice were scarified 12h after APAP administration; B: Plasma LDH levels were evaluated in vehicle-treated mice and in mice treated with NAC (150 mg/kg), GL (200 mg/kg) or NAC/GL at same time of APAP (10 mice in each group). Mice were scarified 12h after APAP administration; C: Plasma AST levels were evaluated in vehicle-treated mice and in mice treated with NAC/GL, 2 hours or 6 hours after APAP injection (7 mice in each group). Mice were scarified 12h after APAP administration; D: Plasma LDH levels were evaluated in vehicle-treated mice and in mice treated with NAC/GL, 2 hours or 6 hours after APAP injection (7 mice in each group). Mice were scarified 12h after APAP administration;. Results are expressed as mean ± SE. cP < 0.05, dP < 0.01, eP< 0,001 vs APAP.

4- ***HMGB1 is a nucleocytoplasmic enzyme that changes its subcellular localization and interaction partners in disease. In my opinion, it will be important to know whether the nucleocytoplasmic distribution of HMGB1 is changed by the drugs you use, and immunoblotting would be the best way to follow such putative changes. Also, it would be nice to see if the interaction with some of its partners is altered.***

HMGB1 is defined as a nuclear component that acts as an architectural chromatin-binding factor involved in maintaining the structure of nucleosome and regulating gene transcription by binding DNA. This protein modifies its subcellular localization depending on the tissue, the role of the host cell (eg: immune cells) or other parameters [10].

We performed IHC on sections of mice liver, treated or not with APAP.

As shown in Figure R4, HMGB1 is mainly expressed in the nucleus of hepatocytes in PBS-treated mice. When APAP is administered, we observed the relocation of HMGB1 outside of the nucleus and finally hepatocytes necrosis induced the release of HMGB1.

Furthermore, we quantified the nuclear expression of HMGB1 (Figures 2G, 3G and 4G) and the results showed that the nuclear localization of HMGB1 is maintained regardless of the treatment administered.

About the investigation of the interaction between HMGB1 and its partners, we have not the expertise to carry out these experiments.

Figure R4

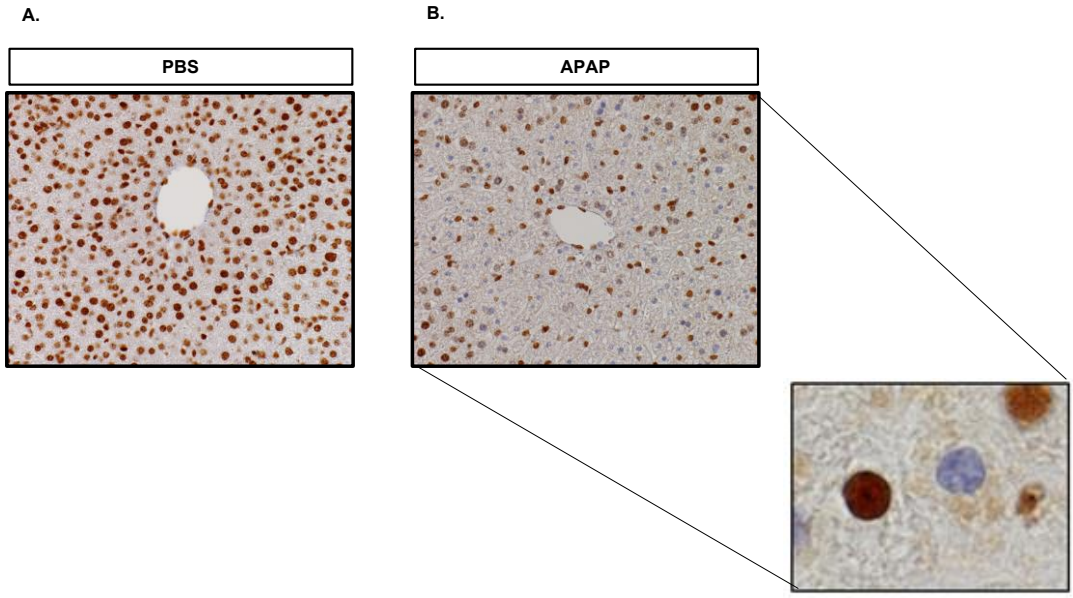

Figure R4. HMGB1-stained images of murine liver.

HMGB1-stained images (magnification x200) of murine liver treated (A) or un-treated (B) by APAP.

5- ***Is there any specific reason to used animals that are under fasting conditions? If so, please clarify.***

Yes. Fasting is necessary to induce APAP hepatotoxicity in mice as well documented in the literature [11][12]. In the same way in human, fasting increased the acetaminophen-induced liver injury compare to control group [13].

6- ***Please, remember that GSH (the reduced form of glutathione) is a metabolite, not an enzyme. Thus, what you are measuring is the levels of this metabolite. In addition, knowing whether the oxidized form GSSG is increased, and hence the GSH/GSSG ratio modified, is key to understand the impact that the drugs you use may have on the recovery of the normal function of metabolic pathways that are affected by APAP intoxication.***

We are agree with the reviewer.

Using GSH/GSSG Ratio Detection Assay Kit (Fluorometric - Green) and following the manufacturer's protocol (Abcam, Cambridge, England), this ratio was evaluated in our liver sample (*MATERIALS AND METHODS, Assessment of ratio GSH/GSSG*; page 8).

We have added these results in the manuscript (*RESULTS, Figures 5B and 7B*).

Figure R5

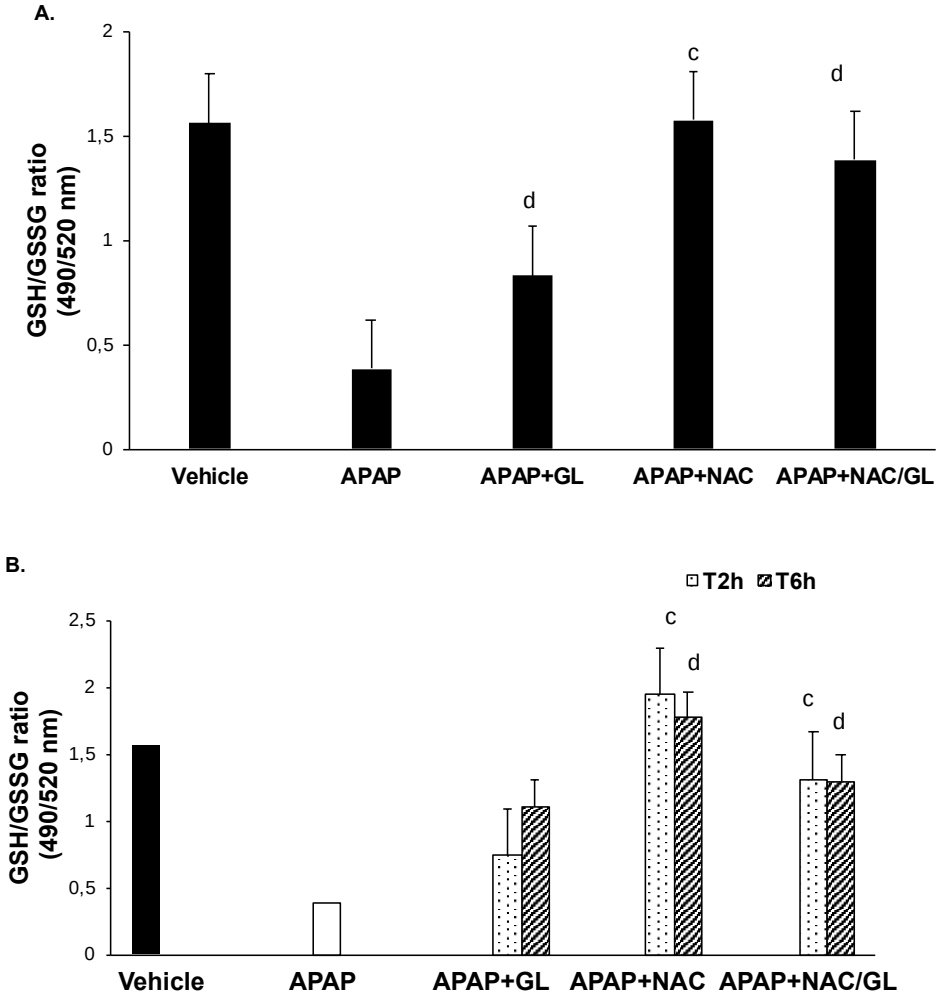

Figure R5 Assessment of GSH/GSSG ratio in our murine model of acetaminophen-induced liver injury. A: Hepatic GSH/GSSG ratio was evaluated using fluorometric assay kit in vehicle-treated mice and in mice treated with NAC (150 mg/kg), GL (200 mg/kg) or NAC/GL at same time of APAP (10 mice in each group). Mice were scarified 12h after APAP administration; B: Hepatic GSH/GSSG ratio was evaluated using fluorometric assay kit in mice treated with NAC (150 mg/kg), GL (200 mg/kg) or NAC/GL, 2 hours or 6 hours after APAP injection (10 mice in each group). Mice were scarified 12h after APAP administration.

Fluorescence measurement was performed at Ex/Em= 490/520nm. Results are expressed as mean ± SE. cP < 0.05, dP < 0.01, eP< 0,001 *vs* APAP.

7- How was the quality of the RNA analyzed? Please, explain in the Methods section.

The quality/purity of each sample was evaluated before RT-qPCR using Nanodrop. We evaluated the concentration of RNA in each sample, but we evaluated also the ratio 260/280 (to exclude the presence of protein, phenol and other contaminants) and the ratio 260/230 (to exclude the presence of co-purified contaminants). None of the samples used had a ratio less than 1.8.

We have added these details in the manuscript (*MATERIALS AND METHODS, RNA extraction and RT-qPCR;* page 9)

8- I understand that the sequence of the primers used for RT-qPCR might be the property of a company, but do they certify that the efficiency is 2? In my opinion, you should use more than just a reference gene in any RT-qPCR studies and use calculation methods that take into account possible variations in both the gene of interest and the reference gene during the treatments (e.g. Pfaffl method).

- We had received the confirmation by the company that the efficiency of the primers used is 2.
- RT-qPCR were performed with a second housekeeping gene: GAPDH
- As suggested by the reviewer, analysis were done with *Pfaffl* method *(14)*

We have added these results in the manuscript (RESULTS, Figure 10).

Figure R6

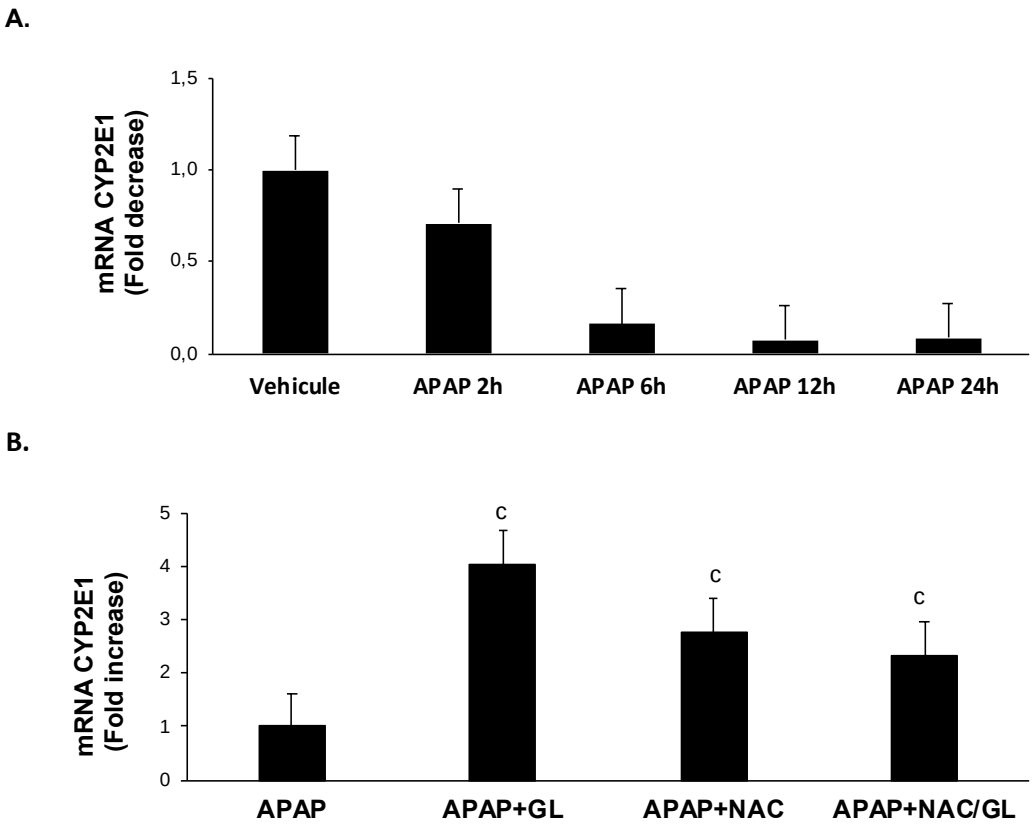

Figure R6 The protective effects of the NAC/GL combination do not result from inhibition of hepatic expression of CYP2E1. A: CYP2E1 mRNA expression was assessed by RT-qPCR in liver extract of vehicle-treated mice and mice sacrificed 2, 6, 12 or 24 h after acetaminophen (APAP; 500 mg/kg) injection (5 mice in each group); B: CYP2E1 mRNA expression was assessed by RT-qPCR in liver extract of mice treated with GL (200 mg/kg), NAC (150 mg/kg), or NAC/GL, at the same time of APAP (10 mice in each group). Mice were scarified 12h after APAP administration. The relative expression of the gene of interest was calculated using *Pfaffi Method*.

9- ***Along the text there are some sections where it is not clearly stated whether the treatment is carried out together with APAP or after APAP intoxication. Please, clarify in the text (e.g. Figure 3).***

We have clarified this point in the legend of each figure as well as in the manuscript.

10- ***I do not think that effects of the drugs on APAP metabolism are correctly reflected as "interference". Please, rewrite.***

Indeed, "interference" may not be the best word for it.

We have made the changes in the text.

11- ***Figure 7. NAC+GL lead to an increase in glutathione levels, but do not restore control levels.***

This point was added to the discussion (*DISCUSSION*; page 15).

12- ***At some point in page 10, the expression of human CYP2E1 is mentioned, when your samples are of mice. Please, correct accordingly, since RTqPCR primers should be from the mouse sequence.***

Indeed, it's a mistake. We made the corrections (*RESULTS, "The potential protective effects of NAC/GL combination were not result of effect on APAP metabolism"*; page 10).

13- ***It would be possible to measure NAPQI metabolite in your liver samples? What about the protein-adduct content? Those parameters would be of great interest and provide additional explanations for the combined effects of the drugs.***

We agree with the reviewer's comment. Unfortunately, we have neither the expertise nor the budget to carry out this experiment.

14- ***I do not think that rats can be defined as a bad model for APAP-induced liver failure, since these models has been proven useful by many authors. Please, remove such as statement of the Discussion section.***

We agree with the reviewer, we are too drastic in this sentence. We have adapted our words (*DISCUSSION*; page 15).

15- ***Figure legends, should be rewritten and improved in many cases. Also, I wonder why data regarding 2h are missing in Figure 1 panels and why GSH (a metabolite not an enzyme) levels are evaluated at different times than other parameters.***

As required by the reviewer, we tried to improve the legends of the figures.

We have explored GSH depletion at early time points because changes happen in the first hours after APAP administration.

16- ***Figures 2, 3 and 4 should clearly state that there is coadministration of the drugs.***

We made the changes in the main text and in the legend of the figures.

17- ***Figure 3 do not shows quantification of nuclear HMGB1 labeling, neither serum values of that protein.***

As suggested by the reviewer, we added these results in the Figure 3.

18- ***Figure 4 lacks information regarding GSH levels, serum HMGB1 and quantification of nuclear HMGB1 staining.***

As suggested by the reviewer, we added these results in the Figure 4.

For the GSH levels, results are shown in Figure 5 and 8.

19- ***Figure 5 lacks GSH levels and nuclear HMGB1 staining, plus its quantification.***

As suggested by the reviewer, we added these results as shown in Figure 7 for HMGB1 staining and its quantification. For GSH levels, results are shown in Figure 5 and 8.

20- ***The legend of Figure 7 should be completely rewritten and the statistical analysis of panels A and B recalculated. In panel A, NAC + GL seem to induce a significant decrease in expression of CYP2E1. Panel B, shows a decrease in GSH levels by GL administration, as well by NAC+GL. Panels C and D should state clearly that they are showing effects of the drugs after APAP administration.***

Moreover, the presentation of all these results should be improved in the Results section and appropriately discussed under Discussion.

The elements of this figure have been split.

The results on GSH levels are shown in Figures 5 and 8.

For CYP2E1 mRNA expression, the results are shown in Figure 10.

As suggested by the reviewer, the legend of the figures have been revised and discussion section was adapted (*DISCUSSION*; page 14).

Reviewer #2

The manuscript by Charlotte et al. demonstrated that N-acetylcysteine/glycyrrhizin combination significantly increased decreased the liver necrosis score and improved the survival during acetaminophen-induced liver injury in mice. Overall, the results are interesting, but there is room for improvement.

1. ***The unit of "12000 rpm" should be changed into " ×g".***

We have made the changes (*MATERIALS AND METHODS, Animal model of APAP-induced liver injury and treatments*; page 6).

2. ***"-20°" should be "-20°C".***

We have made the changes (*MATERIALS AND METHODS, Animal model of APAP-induced liver injury and treatments*; page 6).

3. ***Abbreviations such as GSH , etc. should be given the full name when used for the first time.***

We have reviewed the entire manuscript and made the necessary changes as requested by the reviewer.

4. ***The English of your manuscript must be improved before resubmission. I suggest that you obtain assistance from someone whose native language is English.***

R1 version of the manuscript : ***"N-acetylcysteine and Glycyrrhizin combination: benefit outcome in a murine model of acetaminophen-induced liver failure. "***

As required by the reviewer we have obtained assistance from a second person to improve our manuscript.

5. ***If results are expressed as mean ± SEM, the bar in the figures shouldn't be so high. Statistical differences are not necessarily significant. Statistical expression "* vs. 0, aP<0.05; ** vs. 0, bP<0.01" are not standard.***

All statistical analysis have been reviewed and statistical expression rewritten.

6. ***The presentation and format of all figures are not standard enough. Please check and correct it thoroughly.***

As requested by the reviewer, we reviewed all the figures and we tried to standardize them as much as possible.

7. ***Why choose female mice? Please explain it?***

We chose female mice for several reasons:

1) We want to model the clinical situation where APAP-induced acute liver failure occurs frequently in young women [8].

2) Other publications used female mice for their model of APAP-induced liver injury [1-7].

3) We chose a model with low-grade inflammation to focus on HMGB1 action.

We performed the same experiments in male mice, and we confirmed our results (see response to Reviewer#1). Moreover, our in vitro experiments on hepatocyte cell line, excluded the possibility that this phenomenon is gender-dependent [9].

8. ***References are insufficient, and should be updated appropriately. Hepatotoxicity, liver injury, ELISA, and RT-qPCR should be further explained. Please refere to relevant literatures: e.g. Journal of Pharmacological Sciences, 2016, 130:94-100; British Journal of Pharmacology, 2017, 174:2818–2831; J. Agric. Food Chem. 2019, 67, 2856−2864.***

R1 version of the manuscript : "*N-acetylcysteine and Glycyrrhizin combination: benefit outcome in a murine model of acetaminophen-induced liver failure.* "

As required by reviewer, we have reviewed the "*MATERIALS AND METHODS*" section and further detailed the protocols listed above (*MATERIALS AND METHODS;* page 6-8). We have also updated the reference list.

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