World Journal of Gastroenterology Manuscript NO: 59136 – Notification on manuscript revision
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Reply all|
Today, 00:39
Wang, Guozheng; +9 more

Dear Dr. Wang,

We are pleased to inform you that, after preview by the Editorial Office and peer review, as well as CrossCheck and Google plagiarism detection, we believe that the academic quality, language quality, and ethics of your manuscript (Manuscript NO.: 59136, Basic Study) basically meet the publishing requirements of the World Journal of Gastroenterology. As such, we have made the preliminary decision that it is acceptable for publication after your appropriate revision. Upon our receipt of your revised manuscript, we will send it for re-review. We will then make a final decision on whether to accept the manuscript or not, based on the reviewers' comments, the quality of the revised manuscript, and the relevant documents. Please follow the steps outlined below to revise your manuscript to meet the requirements for final acceptance and publication.

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Please resolve all issues in the manuscript based on the peer review report and make a point-by-point response to the issues raised in the peer review report. Authors must resolve all issues in the manuscript that are raised in the peer-review report(s) and make point-by-point responses to the issues raised in the peer-review report(s), which are listed below:

Reviewer #1:
Scientific Quality: Grade C (Good)
Language Quality: Grade B (Minor language polishing)
Conclusion: Major revision

C1.
Specific Comments to Authors: In this manuscript, the authors provide evidence for histones released during hepatocyte injury as an important contributor to the pathogenesis of liver fibrosis and that neutralizing the circulating histones may have a beneficial effect. The idea

has novelty and the data are supportive of this notion. However, the data shown are minimal and additional experiments and data could make it an interesting paper.

R1.
Thank you for your constructive comments.

C2

The protocol for eliciting liver fibrosis and treatment with NAHP must give adequate details. Specifically, on page 11, for how long did the mice receive CCl4 for liver fibrosis induction? In mice receiving CCl4, for how long NAHP was administered? If it is every 12h, 4 weeks of CCl4 treatment (8 injections) was also accompanied by 56 injections of NAHP? (which is quite a lot of manipulation!) A schematic diagram representing CCl4 and NAHP administration is required to understand Fig 3C.

R2
The following diagrams have been included as a supplemental figure in the revised manuscript.

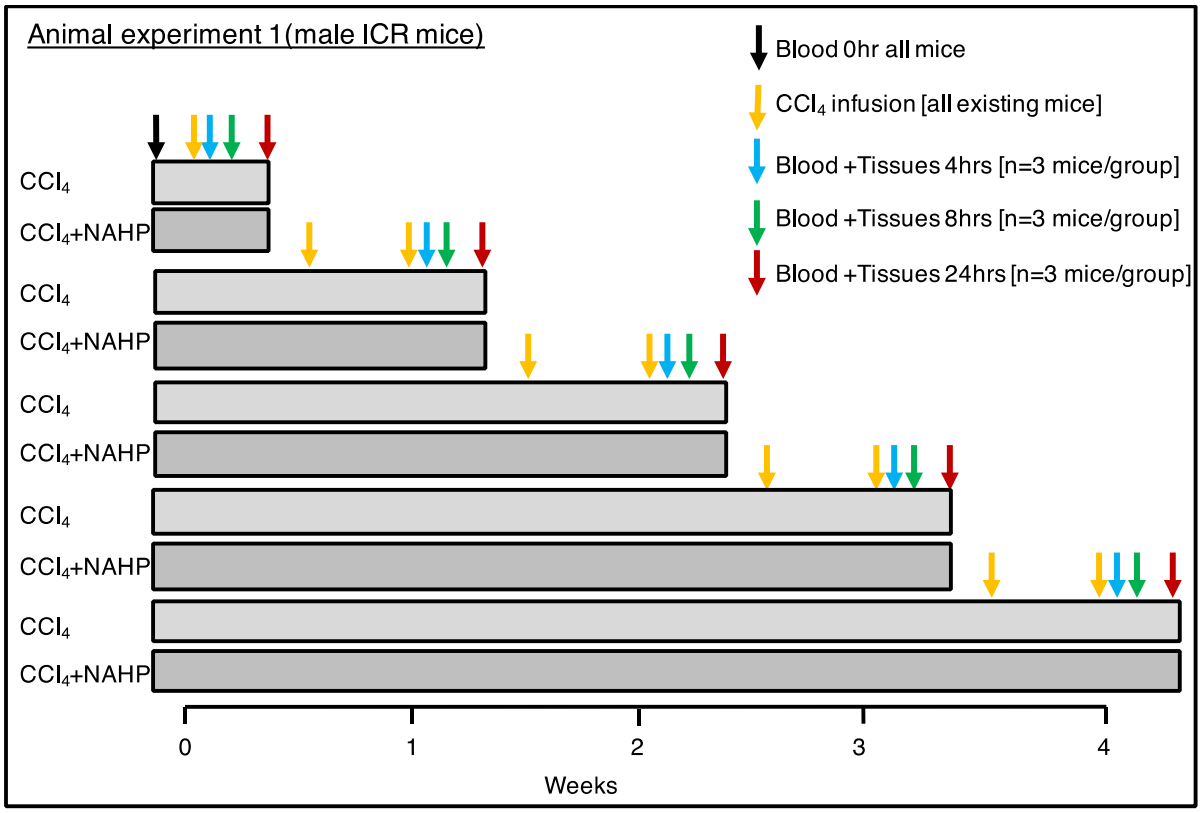

Figure 1 (Supplemental Figure S1). Diagrams of animal experiment 1. ICR mice were used, 3 mice per group per time point. Blood samples were taken from each mouse before the experiments (black arrow). CCl4, 5 µl/g of 25% (w/v) in olive, was injected intraperitoneally (i.p) 2 doses per week with interval of 84 hours (yellow arrow). NAHP: on the day of CCl_4 injection, 8 µl/g (4µg/ml in saline, filtered for sterility) were injected subcutaneously (s.c) every 8h. On the remaining days, 8 µl/g NAHP were injected s.c every 12hrs. Each group had

3 mice euthanized at each time point: 4h (blue arrow), 8h (green arrow), and 24h (red arrow) after first CCl_4 dose of each week and both blood and tissues were collected.

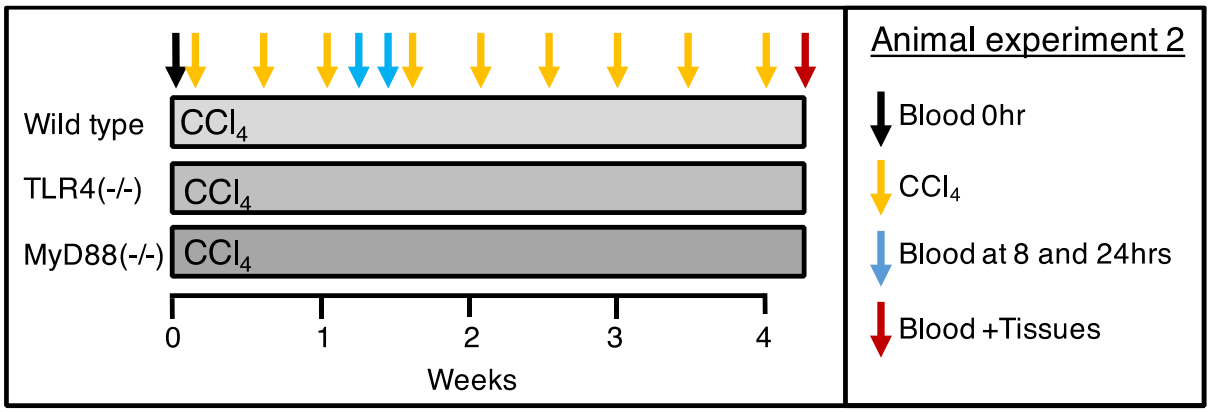

Figure 2 (Supplemental Figure S2). Diagram of experiment 2. C57BL/6 mice, wild type, TLR4 knockout and MyD88 knockout mice, 8 per group, were used. CCl_4 was injected intraperitoneally (i.p) 2 doses per week with interval of 84 hours (yellow arrow). Blood were taken before experiments as indicated by a black arrow and from 4 mice each group at 8h and 4 mice at 24h after first dose of CCl_4 of second week as indicated by blue arrows. By the end of 4 weeks, all mice were euthanized with blood and organs collected as indicated by a red arrow.

C3
I presume ICR ice were used in all experiments other than Fig. 4. Please indicate that in figure legends.

R4
Animal experiment 1 used ICR mice and animal experiment 2 used wt C57BL/6 and gene knock out mice, which are indicated in figure legends.

C5
The authors note that histones can activate TLR2, TLR4 and TLR9. They use only TLR2 neutralizing Ab (PAb-hTLR-2), and there is an error in the 'heading' of the methods section as well as in Fig. 4A, which indicate 'neutralization of TLR4'.

R5
The errors have been corrected in the revised manuscript.

C6
Do LX-2 cells express all three TLRs? If yes, what is the relative contribution of each to histone stimulation? Can it be blocked by a drug targeting a common downstream effector molecule/pathway?

R6

This is a good question. However, we have not examined the TLR2 and TLR9 so it is difficult to justify their contributions. This can be addressed in future studies.

C7

The LX2 activation by histones is related only to increased levels of collagen 1 on cells and in media (Fig. 2A, 3A, 4A). It is possible that the added histones may just interfere with the degradation of collagen. To determine if histones indeed elicit a pro-fibrogenic response in LX-2 cells, it is necessary to evaluate the expression of fibrogenic genes such as Acta1, col1a1, etc. by RT-PCR.

R7

Besides collagen I, α-SMA was detected using immunofluorescent staining (see Figure 2C). Semi-quantified PCR showed that histone-induced expression of Collagen I mRNA (see Figure 2 bellow).

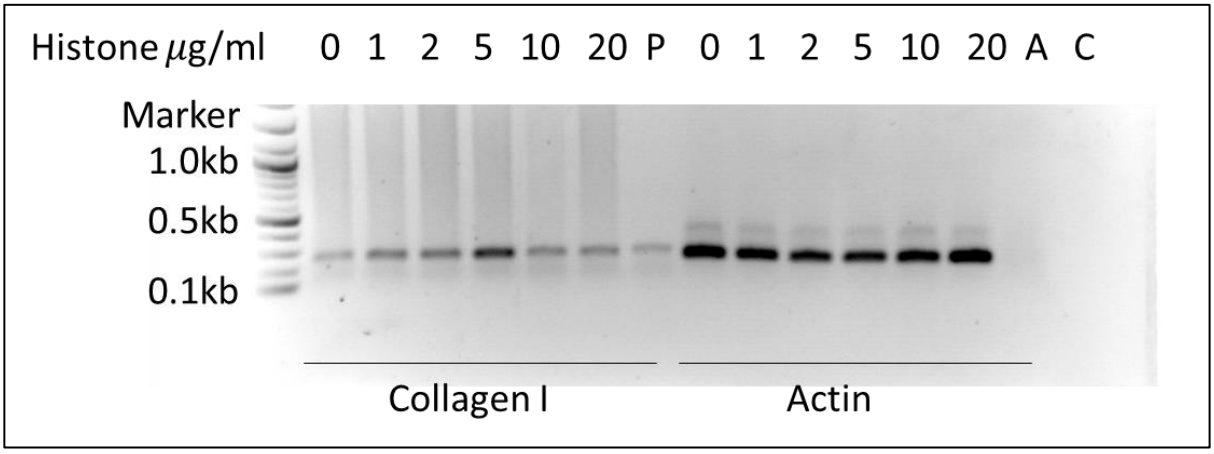

Figure 2. Semi-quantified PCR. LX2 cells were treated with different concentration of histones for 48h, and RNA was isolated using TRIzol™ Reagent (Thermos Fisher) and reverse transcribed using RT-PCR kit (Merck). The semi-quantification by limiting the cycle number (Actin = 18 cycles and collagen I = 21 cycles). P, positive control for Collagen I using a synthesized template. A, negative control for actin without templates. C, negative control for collagen I without template.

Since the student returned to clinical practice before he completed the Q-PCR work, these data have not reached the standard for publication and thereby are not included in the manuscript. However, there is no doubt that histones stimulate collagen I expression rather than slow down their degradation.

C8

The change in morphology of LX-2 after histone exposure (Fig. 2B) does not indicate strong activation. Therefore, it is necessary to use some other ligands (e.g., TGFb) for comparison

R8

We used TGF-β1 as a positive control for this assay. However, TGF-β1 stimulated Collagen I induction much more rapidly than histones. Specifically, peak Collagen I induced by TGF-β1 can be seen at around 24hrs, which then falls. In contrast, histones did not stimulate Collagen I at 24hrs, and became apparent after 6 days. In the manuscript, TGF-beta data were not included in 6-days induction as a comparison with histones due to the differences in dynamics and timings of the stimulation.

C9

The authors show reduced fibrosis in TLR4-/-, MyD88-/- mice (already known) as evidence for impaired histone mediated fibrogenesis. It is important to show if these mice present comparable amounts of histones in circulation following CCl4 treatment.

R9

The histone levels in the blood samples taken before CCl_4 treatment, after week1 and at 4 weeks (end of the experiment) are shown in supplemental figure 3. This has been discussed in discussion as following:

"All groups, including ICR mice treated with CCl_4 and CCl_4+NAHP, and C57BL/6, TLR4(-/-) and MyD88(-/-) treated with CCl_4, had comparable levels of peak circulating histones (Supplemental Figure S3).

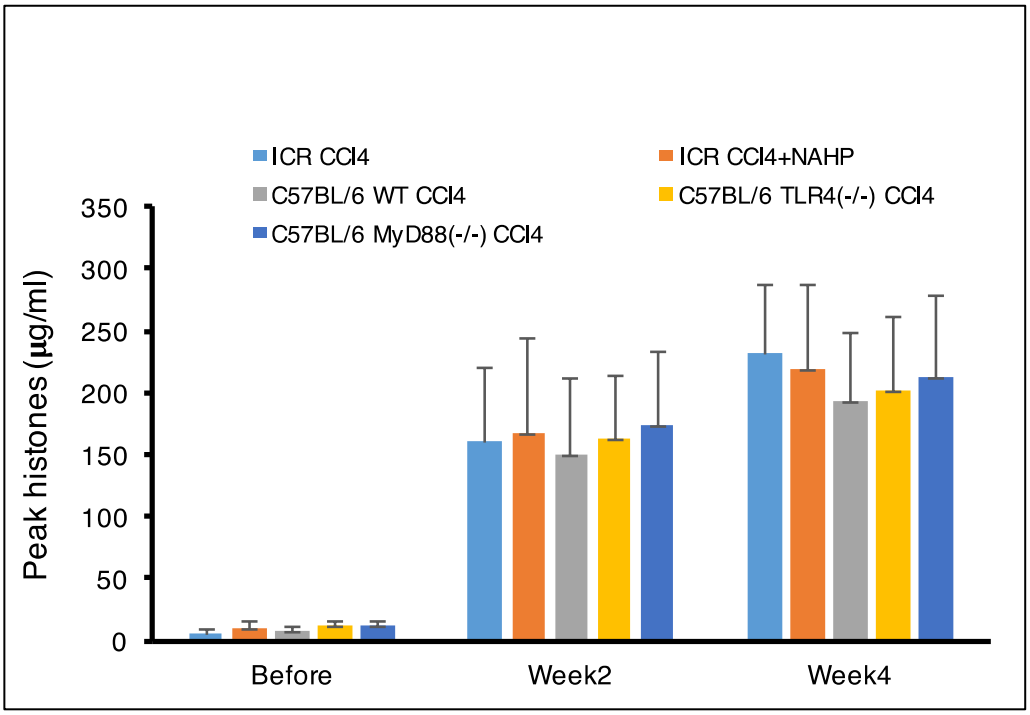

Figure 3 (Supplemental Figure S3). Comparable peak levels of circulating histones after CCl_4 treatment. Means±SD of histone levels in blood samples taken before experiment (Before), 8h and 24h after first dose of CCl_4 in week 2 (Week2) and 24hrs after the last dose in week 4 (Week4) are presented.

C10
Besides, it is important to show activation of downstream signaling molecules of TLR4 in CCl4 and CCl4+NAHP - treated mouse livers.

R10.

This is a very important point, but also a difficult experiment to do in a short period of time. The only possible experiment is to stain the liver sections using anti-NFkB components. We tried to use anti-P65 to do the staining and got some images (See Figure 4). However, it is not publishable until double confirmation has been performed in near future.

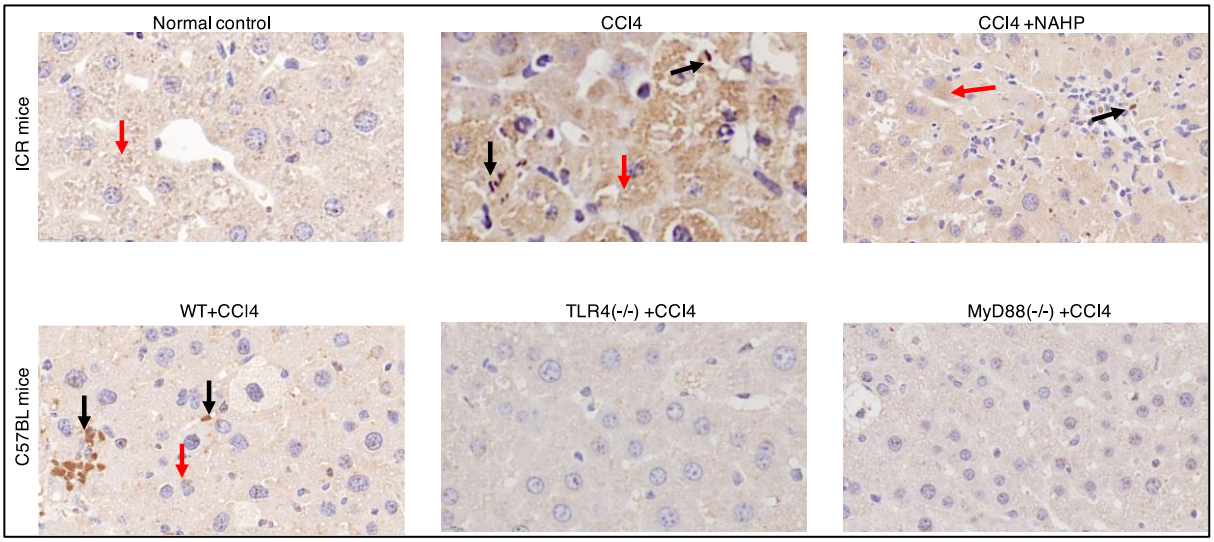

Figure 4. Immunohistochemical staining of liver sections with anti-P65 antibody. Liver sections from normal control (without CCl_4 treatment), ICR mice treated with CCl_4 and CCl_4+NAHP for 4 weeks. C57BL/6 Wild type (WT) mice, TLR4(-/-) and MyD88(-/-) mice treated with CCl_4 for 4 weeks were stained using anti-P65 antibody (1:100, Proteintech, China). Red arrows indicate positive staining in cytoplasm. Black arrows indicate positive staining in nucleus of certain cells.

Reviewer #2:
Scientific Quality: Grade B (Very good)
Language Quality: Grade B (Minor language polishing)
Conclusion: Minor revision

C11
Specific Comments to Authors: In the manuscript the authors present the effect of histones in liver fibrogenesis, these are some of my comments. Title should include “in vitro and in mouse” and exclude “and potentially promote liver fibrogenesis”.

R11
This has been addressed. A new title as following:

Extracellular histones stimulate collagen expression in vitro and potentially promote liver fibrogenesis in mouse model via TLR4-MyD88 signalling pathway

C12
Please, be more precise with the P value. Although there are differences in 5 and 10 Ug/ml (in figure 2), the standard deviations are large to give a solid interpretation. Authors should be cautions about this.

R12
Yes. We treat them cautiously. The statistical analysis is repeated and the difference remain significant.

C13
This is an interesting paper with its limitations. As the same authors point out, the fibrinogenesis process is more complex than only the synthesis of collagen. They must indicate it in their manuscript

R13
This has been indicated specifically in discussion.

"In addition, fibrogenesis is not only collagen production but also involves many molecular and cellular mechanisms[55]. The co-operation of TLR4-MyD88 pathway with other signalling, such as TGF-β signalling and inflammatory response, also requires further investigation to determine their relative contributions to liver fibrosis."

Reviewer #3:
Scientific Quality: Grade C (Good)
Language Quality: Grade B (Minor language polishing)
Conclusion: Minor revision

C14
Specific Comments to Authors: This research is interesting through exploring the importance of histones in liver fibrosis via TLR4/MyD88. In vivo, how does the downstream signal pathway express through TLR4 or MyD88 knockout. Please check Fig 1 carefully.

R14
Since the student has gone back to clinical practice, he has no time to do complicated experiments. This is a big task to elucidate the downstream pathways both in vitro and in vivo. We expect initiate a new mechanistic project to perform this comprehensively in the future. He did the immunohistochemical staining of liver sections using anti-P65 (see R10, Figure 4). These data suggest that NFkB signalling may be involved and activated. However, this is not publishable at this time, until further confirmation has been performed in complementary experiments.

We have carefully checked Figure 1 and original data. We found that original Figure 1D using the means of histone levels in blood taken at 4h, 8h, and 24h after first dose of CCl_4 of each week is not the best presentation because histone levels at 4h were much lower than that

at 8h or 24h and thereby created huge error bars and sometimes misleading. Therefore, we excluded 4h data and used 8h and 24h data to represent the peak values of histones in each cycle. This change makes the graph more meaningful. The new graph has replaced original graph in the revised version.

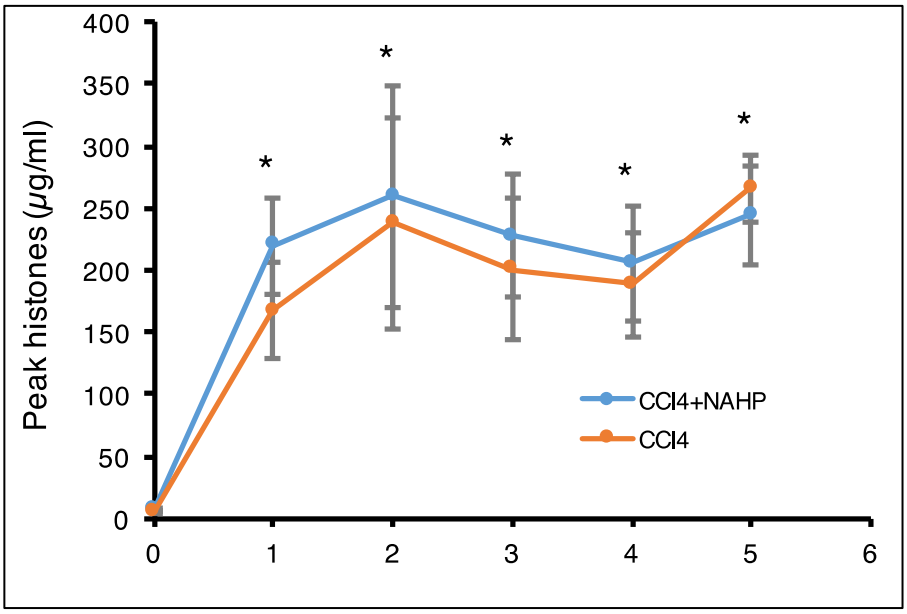

Figure 5 (Figure 1E). Peak circulating histones in each week. Means ±SD of 8 and 24hrs after first CCl_4 injection of each week are presented to represent peak histones during the model generation. *ANOVA test, $P<0.05$ comparing to time 0 (before first injection).

Reviewer #4:
Scientific Quality: Grade C (Good)
Language Quality: Grade B (Minor language polishing)
Conclusion: Minor revision

C15
Specific Comments to Authors: This study demonstrates the important role of histones in liver fibrosis via TLR4-MyD88 signaling pathway. Figure 1 may be revised to add the results of Western blots in 4 weeks after CCl4 injection. The labels of CCl4 and CCl4+NAHP may be added in Fig1 A and B. The results of Student Newman-Keuls test may be added in figures. Please carefully proofread the manuscript.

R15

The week4 Western blots have been added as Figure 1C (See Figure 6 bellow) and blots in Figure 1A and 1B have been labelled with CCl_4 and CCl_4+NAHP. P values were added to E and F. Proofreading has been done by two native English speakers.

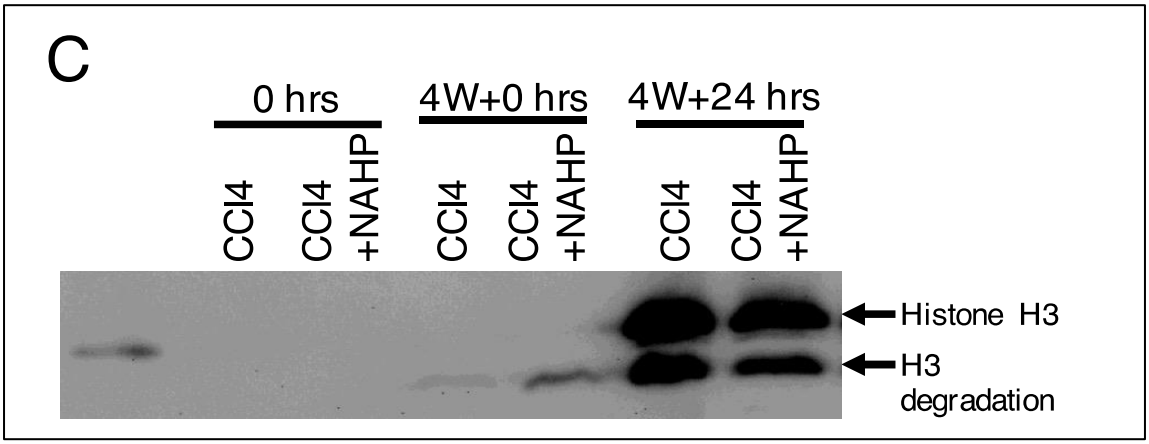

Figure 6 (Figure 1C). Western blot of histone H3 before experiment and at 4 weeks. The new blot has been added as Figure 1C. Blood samples from each group before first injection of CCl_4 (CCl4 0h and CCl4+NAHP 0h), before the last injection of CCl_4 (CCl4 4w 0h and CCl4+NAHP 4w 0h) and 24h after the last injection of CCl_4.

Reviewer #5:
Scientific Quality: Grade D (Fair)
Language Quality: Grade C (A great deal of language polishing)
Conclusion: Major revision

C16
Specific Comments to Authors: this paper report Extracellular histones stimulate collagen expression and potentially promote liver fibrogenesis via TLR4-MyD88 signaling pathway. it would bring some new information in this area.

R16
Thank you for the comments. This manuscript has been revised significantly and become much better now.

C17

4 LANGUAGE QUALITY
Please resolve all language issues in the manuscript based on the peer review report. Please be sure to have a native-English speaker edit the manuscript for grammar, sentence structure, word usage, spelling, capitalization, punctuation, format, and general readability, so that the manuscript's language will meet our direct publishing needs.

R17

Proofreading has been done by two native English speakers.

C18
5 EDITORIAL OFFICE'S COMMENTS

Authors must revise the manuscript according to the Editorial Office's comments and suggestions, which are listed below:

(1) Science editor: 1 Scientific quality: The manuscript describes a basic study of the extracellular histones promote liver fibrosis. The topic is within the scope of the WJG. (1) Classification: Grade B, Grade C, Grade C, Grade C and Grade D;

(2) Summary of the Peer-Review Report: This paper report Extracellular histones stimulate collagen expression and potentially promote liver fibrogenesis via TLR4-MyD88 signaling pathway. it would bring some new information in this area. However, there are some issues should be addressed. The data shown are minimal and additional experiments and data could make it an interesting paper. The fibrinogenesis process is more complex than only the synthesis of collagen. They must indicate it in their manuscript. The questions raised by the reviewers should be answered; and

(3) Format: There are 4 figures. A total of 60 references are cited, including 10 references published in the last 3 years. There are 10 self-citations. 2 Language evaluation: Classification: Grade B, Grade B, Grade B, Grade B and Grade C. 3 Academic norms and rules: The authors provided the Biostatistics Review Certificate and Institutional Animal Care and Use Committee Approval Form.

R18.

The forms have been attached.

C19

(4) The authors need to provide the signed Conflict-of-Interest Disclosure Form and Copyright License Agreement,

R19

The forms have been attached.

C20

(5) and fill out the ARRIVE Guidelines with page numbers. No academic misconduct was found in the Bing search. The authors have published preprint (https://www.biorxiv.org/content/10.1101/2020.09.17.302240v1).

R20

The document has been attached

(6) 4 Supplementary comments: This is an unsolicited manuscript. The study was performed with 10 financial supports. The topic has not previously been published in the WJG. The corresponding author has not published articles in the BPG.

C21

5 Issues raised: (1) I found the language classification was grade C. Please visit the following website for the professional English language editing companies we recommend: https://www.wjgnet.com/bpg/gerinfo/240;

R21

The language has been edited by 2 native English speakers.

C22

(7) I found no "Author contribution" section. Please provide the author contributions;

R22

This has been added to the revised manuscript.

"**Author Contribution:** Wang Z conceived the study; Cheng ZX assisted animal experiments and hydroxyproline measurement; Lin Z and Abrams ST assisted in performing in vitro experiments; Yates ED synthesized and characterised non-anticoagulant heparin. Abrams helped edit figures. Yu Q, Yu WP, Chen PS, Toh CH and Wang G supervised the work and were involved in data analysis and manuscript writing. All authors have read and agreed to the published version of the manuscript. "

C23

(3) I found the authors did not provide the approved grant application form(s). Please upload the approved grant application form(s) or funding agency copy of any approval document(s);

R23

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C24

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R24

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C25

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R25

This has been checked throughout the revised manuscript.

C26

Statistical significance is expressed as aP < 0.05, bP < 0.01 (P > 0.05 usually does not need to be denoted). If there are other series of P values, cP < 0.05 and dP < 0.01 are used, and a third series of P values is expressed as eP < 0.05 and fP < 0.01. 6 Re-Review: Required.

R26

We have checked this is consistent in the revised manuscript.

7 Recommendation: Conditionally accepted.

(2) Editorial office director: I have checked the comments written by the science editor.

(3) Company editor-in-chief: I have reviewed the Peer-Review Report, full text of the manuscript, and the relevant ethics documents, all of which have met the basic publishing requirements of the World Journal of Gastroenterology, and the manuscript is conditionally accepted. I have sent the manuscript to the author(s) for its revision according to the Peer-Review Report, Editorial Office’s comments and the Criteria for Manuscript Revision by Authors.

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