Author's response to reviews

Title: Fluvastatin attenuates hepatic steatosis-induced fibrogenesis in rats through inhibiting paracrine effect of hepatocyte on hepatic stellate cells

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Author's response to reviews: see over
Luca Valenti, MD.
Editor
BMC Gastroenterology

Dear Dr. Valenti,

We would like to submit the revised manuscript entitled “Fluvastatin attenuates hepatic steatosis-induced fibrogenesis in rats through inhibiting paracrine effect of hepatocyte on hepatic stellate cells” for your consideration for publication in BMC Gastroenterology.

In response to your request, we have revised our manuscript in light of the reviewers’ comments and made the required changes.

**Comment 1:** Language should be improved as suggested by all Reviewers, suggestions have been provided by Reviewer 3.

**Response 1:** The manuscript has been reviewed and edited by professional native English speaker in the medical field to ensure accuracy and fluency of English usage. In addition, suggestions provided by Reviewer 3 were also added into the revised manuscript accordingly.

**Comment 2:** Histological data presentation should be improved as required by Reviewer 1.

**Response 2:** In response to the Reviewer 1’s professional comments, H&E sections have been added to the revised manuscript (Fig. 4A) to correlate with the findings in Table 2. A scale bar has been added to each image of sections stained with H&E and Sirius Red for accurate comparison.

**Comment 3:** Gene expression analysis should be performed in a quantitative way as suggested by Reviewers 1 and 3.

**Response 3:** All of the experiments on mRNA expressions were repeated with quantitative PCR to establish accuracy and appropriateness of the results.

**Comment 4:** Additional controls should be provided for key experiments (as suggested by Reviewer 2).

**Response 4:** Additional controls for the key experiments have been added to the manuscript.
revised manuscript after repeating our experiments. (Figure 3C and Figure 3D of the revised manuscript).

**Comment 5:** Other points could be addressed by further discussion of the study limitations  
**Response 5:** Limitations of the present study were addressed in the discussion section.

Point-by-point responses to the reviewers’ comments were enclosed in the end of this letter.

Your kind consideration and editorial arrangement of our manuscript is deeply appreciated.

Thank you very much.

With my best regards,  
Yours sincerely,  
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Responses to Reviewer 1’s Comments

Please kindly note that relevant changes in the manuscript in response to the reviewer 1’s comments are highlighted in red.

Major comments:

**Comment 1:** In the paragraph "Histopathological examination," the authors described that liver sections were stained with hematoxylin-eosin (H&E) or Sirius Red to evaluate collagen distribution. The authors didn’t show representative images of liver sections stained with H&E. Moreover, it seems that the images of sections stained with Sirius Red have been acquired with different magnifications. Therefore it is difficult to compare the images of the different treatments at 4 and 8 weeks. In table 1, authors reported a steatosis score of 3.00 in rats maintained at CDAA diet for 4 and 8 weeks. However, the degree of steatosis seems greater in rats fed CDAA diet for 8 weeks. Similarly the amount of steatosis does not seem the same in rats treated with the low dose of Flu at 4 weeks compared to untreated rats. Moreover, it is difficult to compare the degree of fibrosis between rats treated with CDAA+Flu [5] and CDAA+Flu [10] at 8 weeks.

**Response 1:** In response to the Reviewer’s professional comments, H&E sections have been added to the revised manuscript (Fig. 4A) to correlate with the findings in Table 2. Regarding the apparently similar severity of steatosis as reflected in the steatosis score in Table 2 among the treated and untreated animals, it is mainly due to the definition of the scoring system that assigns a score of 3 to a severe degree of steatosis (i.e. Steatosis > 66%/HPF) that cannot be further increased even for more severe steatosis. The scoring system has been added to the Methodology section of the revised manuscript (Materials and Methods - eighth paragraph - Histological examination, highlighted in red) with the reference added (Brunt & Kleiner et al.) [1, 2] (Please refer to the table below).
After thorough checking, the images of sections stained with Sirius Red have been acquired with the same magnifications. Predominance of macrovesicular steatosis in CDAA sections at the 8th wk may lead to the false impression that the images were taken with different magnifications. A scale bar has been added to each figure for accurate comparison. As for fibrosis, the degree was assessed by pathologist blinded to the grouping in order to be unbiased. In compliance with the Reviewer’s insightful comments, results of statistical analysis on the difference in the degree of hepatic
fibrosis in animals being treated with Fluvastatin 5 and 10 mg/kg at the two time points (e.g. 4 weeks and 8 weeks) were added to Table 2 of the revised manuscript. The results showed that there were significant differences in the degree of fibrosis in rats being given different dosages at the 4th week but not at 8th week.

**Comment 2:** Authors asserted that half of the animals from each group were sacrificed after 4 weeks, while the rest were sacrificed after 8 weeks. Does CDAA diet result in weight loss? During the first weeks rats fed CDAA lose weight (about 67 g) compared to controls. We should expect a further loss of weight at 8 weeks.

**Response 2:** According to our experience and that by others [3], weight loss in rats seems universal after being fed with CDAA diet in the first 4 weeks. In addition, according to our previous unpublished data (summarized in Table below), male adult Wistar rats fed with CDAA for 8 weeks showed a less prominent decrease in body weight compared with those fed with CDAA for 4 weeks. Furthermore, at the end of 12 weeks of CDAA feeding, the difference in body weight between the CDAA-treated rats and the normal controls was even less than that for 8 weeks. Our previous findings, therefore, are consistent with the observation in the present study that weight loss was more conspicuous after the first 4 weeks of CDAA diet consumption, followed by weight gain as in normal rats. This may explain the apparently small difference in body weight between the normal rats and their age-matched counterparts fed with CDAA for 8 weeks.

**Table 1. General profiles in 4-week, 8-week and 12-week control Wistar rats and CDAA rats (Unpublished data).**

<table>
<thead>
<tr>
<th>Groups</th>
<th>4-wk</th>
<th>8-wk</th>
<th>12-wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat number</td>
<td>Control</td>
<td>CDAA</td>
<td>Control</td>
</tr>
<tr>
<td>Fibrosis score</td>
<td>0 ± 0</td>
<td>0.88 ± 0.13*</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>BW (g)</td>
<td>448 ± 26</td>
<td>419 ± 26</td>
<td>522 ± 23</td>
</tr>
<tr>
<td>LW (g)</td>
<td>14.3 ± 0.4</td>
<td>26.3 ± 3.7*</td>
<td>16.4 ± 0.9</td>
</tr>
<tr>
<td>GOT (U/L)</td>
<td>94 ± 15</td>
<td>285 ± 86*</td>
<td>87 ± 3</td>
</tr>
<tr>
<td>GPT (U/L)</td>
<td>24 ± 6</td>
<td>106 ± 33*</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>Cre (mg/dl)</td>
<td>0.52 ± 0.05</td>
<td>0.60 ± 0.09</td>
<td>0.62 ± 0.03</td>
</tr>
<tr>
<td>Serum TG (mg/dl)</td>
<td>58.8 ± 10.4</td>
<td>28.9 ± 5.7*</td>
<td>75.5 ± 6.2</td>
</tr>
</tbody>
</table>

*P< 0.05, CDAA rats in 4-wk, 8-wk or 12-wk compared with its respective controls.

BW: body weight; LW: liver weight; Cre: creatinine; TG: triglyceride
**Comment 3:** Authors have conducted in vitro experiments treating primary rat hepatocytes with Flu at different concentrations. It would be interesting to isolate primary hepatocytes from rats fed CDAA diet plus Flu at 4 and 8 weeks.

**Response 3:** We agree completely with the Reviewer that isolating hepatocytes from rats fed with CDAA diet plus different dosages of Flu would be an excellent idea to investigate the effect of Flu on hepatocytes. Since the rats for the present study has been sacrificed, we would surely consider the idea to be the next step of our study.

**Comment 4:** Have the authors tested higher concentrations of Flu in the in vivo model as described in literature?

**Response 4:** In response to the Reviewer’s professional comments, we have seriously sought relevant literature, both clinical and experimental. The usual clinical dose of Flu for human subjects is 80 mg/day which is equivalent to less than 1.5 mg/kg/day. Another clinical study demonstrated that Fluvastatin can be safely used at doses of 8 mg/kg/day in pediatric patients with cancer [4]. Previous *in vivo* study has reported a Flu dosage of up to 200 mg/kg in rats for the study of prevention of carcinogenesis [5]. In the present study, we adopted a dosage of 5 and 10 mg/kg to better mimic the clinical scenarios in accordance with previous experimental studies [6, 7]. We completely agree with the Reviewer’s opinion that it would be interesting to see if higher dosage would produce different effects, although we did not perform dosage study in the current investigation.

**Comment 5:** RT-PCR is a semiquantitative method. It would be better to assess gene expression by qRT-PCR.

**Response 5:** In compliance with the Reviewer’s professional comments, we performed quantitative PCR for assessing gene expressions. The results are shown in Figure 2B, 2C, 3B, 3D, 3F, 5B and 6 of the revised manuscript. Relevant descriptions have been added into the revised manuscript.

![Figure 2](image-url)
Figure 3

(A) Relative gene expression (Fold of control) for ICAM-1, IL6, and TNFα.

(B) Relative gene expression (Fold of control) for Collagen I, TIMP1, TGFβ1, and αSMA.

(C) Relative gene expression (Fold of control) for ICAM-1, IL6, and TNFα.

(D) Relative gene expression (Fold of control) for Collagen I, TIMP1, TGFβ1, and αSMA.
References


Responses to Reviewer 2’s comment
Please kindly note that relevant changes in the manuscript in response to the reviewer 2’s comments are highlighted in blue.

General comments:

Comment 1: It is not always easy to understand the text and a revision of the English is recommended to avoid some confounding data.
Response 1: In compliance with the comments of the Reviewer, the manuscript has been reviewed and edited by professional native English speaker in the medical field to ensure accuracy and fluency of English usage.

Comment 2: In the discussion too much space is dedicated mentioning the literature data, authors should write a little more about the use of Fluvastatin as potential treatment of NASH induced-fibrosis based on their results.
Response 2: In response to the Reviewer’s professional comments, general descriptions not directly related to the use of Fluvastatin as potential treatment of NASH induced-fibrosis have been deleted and replaced by descriptions based on the results of the current study.

Specific comments:
Comment 3: Authors show the effects of Fluvastatin on palmitate (PA)-treated hepatocytes, but no data regarding the effects of Fluvastatin alone on untreated hepatocytes are shown; this group is important to show the direct effects of Fluvastatin on hepatocytes.
Response 3: We would like to appreciate the Reviewer’s professional comments. We performed cytotoxicity assay and the result revealed that Fluvastatin of concentration 1-20 \(\mu\)M has no notable toxic effects on cultured HepG2 cells and PRH (Figures below).
Besides, in compliance with the comments of the Reviewer, we performed quantitative PCR on the expressions of three inflammation indicators (ICAM1, IL-6, TNF-alpha) in HepG2 cells and PRH using Fluvastatin alone (Fig. 2B and Fig. 2C of the revised manuscript). The results showed no significant differences in the expressions of these markers between the control group and the group treated with Fluvastatin alone (Second paragraph of the Results section, highlighted in blue).

Figure 2

![Image: Graph showing relative gene expression for ICAM1, IL-6, and TNF-alpha in HepG2 cells and PRH under various conditions.

Comment 4: Authors state that conditioned media from PA-treated hepatocytes induce activation of HSCs by showing the alpha-SMA protein expression, but no other data about their activation are shown (i.e. gene expression.)

Response 4: In compliance with the Reviewer’s insightful comments, pro-fibrogenic gene expressions including Collagen I, TGF-β1, TIMP-1 and α-SMA related to HSC activation have been assessed and analyzed (Figure 3B). The results have been added into our revised manuscript (Third paragraph of Results section, highlighted in blue).
Comment 5: When authors treat hepatocytes with Fluvastatin, they show gene expression, ROS production, NFkB nuclear expression, on the other hand, when they treat HSCs with Fluvastatin, they only focus their attention on alpha-SMA protein expression, I think more data should be shown to see the real effects of Fluvastatin on HSCs in absence and presence of Fluvastatin and in absence and presence of conditioned media. Moreover, as before, the group only Fluvastatin is missing.

Response 5: Considering the nature of HSCs as a downstream effector of HSC activation, we performed gene expression analyses for HSC activation-related molecules including Collagen I, TGF-β1, TIMP-1 and α-SMA as in our response to Comment 4 of Reviewer 2. In response to the Reviewer’s comments, we repeated our experiments in the presence or absence of conditioned media and in absence and presence of Fluvastatin treatment. The results are shown in Figure 3C and Figure 3D of our revised manuscript (Third paragraph of Results section, highlighted in blue).

Figure 3

Comment 6: In Figure 3B the use of TGF-beta as positive control is correct, but the groups Fluvastatin alone and TGF-beta+Fluvastatin are missing, please include these groups.
**Response 6:** In compliance with the Reviewer’s professional suggestion, the two groups have been added into the revised manuscript after repeating our experiments. (Third paragraph of Results section, Figure 3C and Figure 3D of the revised manuscript, highlighted in blue).

**Figure 3**

![Graph C](image)

![Graph D](image)

**Comment 7:** Before stating that PA has no effect on HSCs activation, did the authors check the expression of some gene at mRNA level?

**Response 7:** In response to the Reviewer’s comments, the mRNA expressions of Collagen I, TGF-β1, TIMP-1 and α-SMA have been compared between HSCs with and without direct PA stimulation, the results showed no significant differences in the expressions of pro-fibrogenic genes when HSC-T6 cells were treated with PA directly (Figure 3B of the revised manuscript, third paragraph of Results section, highlighted in blue).

**Figure 3**

![Graph B](image)

**Comment 8:** What happens if you stimulate the HSCs with the conditioned media from palmitate and Fluvastatin-treated hepatocytes? Does it also inhibit the HSC activation?
Response 8: We completely agree with the Reviewer that stimulation with the HSCs with conditioned media from palmitate and Fluvastatin-treated hepatocytes would shed considerable light on the upstream mechanism of HSC activation. After repeating our experiments, the results showed that both $\alpha$-SMA mRNA and protein expressions were significantly reduced in HSCs incubated with CM collected from PA-Flu-treated PRHs compared to that of CM collected from PA-treated PRHs. Furthermore, $\alpha$-SMA mRNA and protein expressions were also decreased in HSC-T6 cells when treated with the CM collected from Flu-treated PRHs compared to that of CM without Flu treatment. The results are shown in Figure 3E and 3F of our revised manuscript (Fourth paragraph of Results section, highlighted in blue).

Figure 3

Comment 9: For the in vivo model, how do authors choose the dose of Fluvastatin? And how do they know that the dose used is not hepatotoxic?
Response 9:
In response to the Reviewer’s professional comments, we have seriously sought relevant literature, both clinical and experimental. The usual clinical dose of Fluvastatin for human subjects is 80 mg/day which is equivalent to less than 1.5 mg/kg/day. Another clinical study demonstrated that Fluvastatin can be safely used at a dose of 8 mg/kg/day in pediatric patients with cancer [1]. Previous in vivo study has reported a Fluvastatin dosage of up to 200 mg/kg in rats for the study of prevention of carcinogenesis and no significant Fluvastatin-induced hepatotocity has been reported [2]. In the present study, we adopted a dosage of 5 and 10 mg/kg to better mimic the clinical scenarios in accordance with previous experimental studies [3, 4]. Hepatotoxicity has not been documented in other experimental studies adopting a Fluvastatin dosage between 5 to 10 mg/kg.

Comment 10: Animals with combined CDAA and Fluvastatin show a significant and important reduction in their weight, getting lower than control group. How do authors explain this effect?
Response 10: Sincere appreciation for the Reviewer’s keen observation. Although
body weight loss in rats after being fed with CDAA diet is a well-documented phenomenon [5], the reason for body weight loss in such rats after Fluvastatin treatment is not well studied. It is assumed that Fluvastatin, which is a lipid-lowering agent for human beings, may exert metabolic and immunomodulatory effects on CDAA diet-fed rodents that lead to body weight loss. Nevertheless, the exact cause remains to be elucidated.

Comment 11: It is not clear how the inflammation and steatosis scores have been assessed in the animals.

Comment 11: Sincere appreciation for the Reviewer’s comments. For the purpose of the present study that evaluated steatosis, lobular inflammation, hepatocellular ballooning, and fibrosis in a rodent NASH model, the NAFLD activity score (NAS) designed by the NASH Clinical Research Network (CRN) [6] was used to simulate the clinical picture. The scoring system has been added to the Methodology section of the revised manuscript with the reference added (Eighth paragraph of Materials and Methods section- Histological examination, reference 26 of the revised manuscript). (Please refer to the table below).

<table>
<thead>
<tr>
<th>Item</th>
<th>Definition</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steatosis</td>
<td>&lt; 5%</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>5%-33%</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>&gt; 33%-66%</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>&gt; 66%</td>
<td>3</td>
</tr>
<tr>
<td>Lobular inflammation</td>
<td>No foci</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>&lt; 2 foci per 200 x field</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2-4 foci per 200 x field</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>&gt; 4 foci per 200 x field</td>
<td>3</td>
</tr>
<tr>
<td>Ballooning</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Few balloon cells</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Many cells/prominent ballooning</td>
<td>2</td>
</tr>
</tbody>
</table>

Comment 12: Why do not authors use the Oil Red O staining to assess the steatosis in the rats?

Comment 12: For the purposes of the present study that evaluated steatosis, lobular inflammation, hepatocellular ballooning, and fibrosis, the NAFLD activity score
(NAS) designed by the NASH Clinical Research Network (CRN) was used [6]. We agree completely with the Reviewer’s opinion that Oil Red O staining can provides an excellent assessment for the degree of steatosis. On the other hand, to concomitantly evaluate inflammation and fibrosis, H&E stain was used for histological assessment with the NAFLD activity score (NAS) scoring system in the present study.

Comment 13: To propose Fluvastatin as a potential drug to be used as a treatment and not only as a prevention, would be very important to include other 2 groups in the animal studies: 1) the group treated only with Fluvastatin and regular diet, to look at the direct effects of Fluvastatin and study its hepatotoxicity and adverse effects (such weights loss, ALT, AST…); 2) induce the steatosis and fibrosis with your diet and then, when it is well established, treat the animals with the drug.

Response 13: We would like to express our appreciation for the Reviewer’s professional comments and suggestions. On the other hand, the requirement for additional groups of animals violates the regulation on the laboratory animal use at our institute that strictly restricts the number of animals used for each study. Despite our being unable to use more animals for the study the direct effects of Fluvastatin on rats receiving regular diet, we performed qPCR on the expressions of three inflammation indicators (ICAM1, IL-6, TNF-alpha) in HepG2 cells and PRHs using Fluvastatin alone in compliance with the professional comments of the Reviewer (Figure 2B and Figure 2C of the revised manuscript). The results showed no significant differences in the expressions of these markers between the control group and the group treated with Fluvastatin alone (Second paragraph of the Results section, highlighted in blue). Taking into consideration the lack of animal experiments to address this issue, we have included this as a limitation in the revised manuscript (Fourth paragraph of Discussion section, highlighted in blue).

References


**Responses to Reviewer 3’s Comments**

Please kindly note that relevant changes in the manuscript in response to the reviewer 3’s comments are highlighted in pink.

**Comment 1:**
- **ABSTRACT** - Results paragraph: “Flu-pretreated cells” instead of “Flu-treatment group”.

**Response 1:**
In compliance with the Reviewer’s professional comments, the term has been changed accordingly in the revised manuscript (Highlighted in pink).

**Comment 2:**
- **INTRODUCTION**: “nuclear factor kappa (NFkappaB) comprises a family of inducible transcription factors consisting among others of p65 and p50 subunit of Rel protein family”. Remember that NFkappaB is a dimeric transcription factor comprised of five family members RelA (p65), RelB, c-Rel, p50 and p52.

**Response 2:**
In response to the Reviewer’s comments, the sentence has been changed accordingly in the revised manuscript (Highlighted in pink).
Comment 3:
• MATERIALS AND METHODS – The title of Third paragraph must be “Measurement of reactive oxygen species (ROS) production”.

Response 3:
In accordance with the Reviewer’s comments, the subheading has been rewritten in the revised manuscript (Highlighted in pink).

Comment 4:
• MATERIALS AND METHODS – Third paragraph- “Performed at indicated time points by the excitation” instead of “Performed with”.

Response 4:
Sincere appreciation for the Reviewer’s comments, the mistake has been corrected in the revised manuscript (Highlighted in pink).

Comment 5:
• MATERIALS AND METHODS – The title of Fourth paragraph must be “Cytotoxicity assay”.

Response 5:
According to the Reviewer’s suggestions, the subheading has been rewritten in the revised manuscript (Highlighted in pink).

Comment 6:
• MATERIALS AND METHODS – Fourth paragraph- Cellular plating concentration must be indicated.

Response 6:
In response to the Reviewer’s comments, the concentration (1 x 10⁵ cells/well) has been indicated in the revised manuscript (Highlighted in pink).

Comment 7:
• MATERIALS AND METHODS – Fourth paragraph- “The absorption intensity at 540 nm” instead of “The A540 absorption intensity”.

Response 7:
In compliance with the Reviewer’s professional comments, the sentence has been corrected in the revised manuscript. (Highlighted in pink)

Comment 8:
• MATERIALS AND METHODS –Seventh paragraph- “levels were measured
following standard procedures using a colorimetric analyzer (Dri#Chem 3000).

**Response 8:**
Sincere appreciation for the Reviewer’s comments, the sentence has been rewritten in the revised manuscript (Highlighted in pink).

**Comment 9:**
- **RESULTS – First paragraph, first sub-paragraph:** “after 24 hours of exposure” instead of culturing.

**Response 9:**
In response to the Reviewer’s comments, the sentence has been changed in the revised manuscript (Highlighted in pink).

**Comment 10:**
- **RESULTS – First paragraph, second sub-paragraph:** I disagree with the assertion “Flu (1-20 \( \mu \text{M} \)) concentration-dependently attenuated NFkappaB p65 nuclear translocation in both cell types (Figure 2A)”. In fact, even though it is visible that Pre-treatment with Flu for 2 hr reduced the NFkappaB p65 nuclear translocation in PA-treated HepG2 cells and PRHs at 6 hr after treatment, it is not evident a dose-dependent effect. The same assertion is found in the Discussion section.

**Response 10:**
Sincere appreciation for the Reviewer’s comments, the inappropriate term “concentration-dependently” has been deleted in both Results and Discussion sections in the revised manuscript.

**Comment 11:**
- **RESULTS – First paragraph, second sub-paragraph:** “Flu treatment inhibited the mRNA expression levels of pro-inflammatory gene transcripts (ICAM-1, IL-6, TNF-alpha) in both PA-treated HepG2 cells and PRHs”. It is not clearly visible in the electrophoresis the difference between the line 2 and the others especially for TNF-alpha (Figures 2B and 2C).

**Response 11:**
Sincere appreciation for the Reviewer’s comments, after repeating the experiments with quantitative PCR (qPCR) in compliance with your professional request, Figure 2B and Figure 2C that showed the results of RT-PCR in the original manuscript have been replaced by the results of qPCR that were consistent with our original findings but showed significant differences between PA-treated group and PA with Flu-treated groups instead of being equivocal in the original figures.

Figure 2
Comment 12:
• FIGURE 1 (A): On the ordinate axis of the HepG2 diagram “% of control group” must be use instead of “Fold of control group”.

Response 12:
In response to the Reviewer’s comments, the mistake has been corrected in the revised Figure 1 (A).

Comment 13:
• FIGURE 1(B), 1(C): the figure must be more similar for HepG2 and PRH cells: the
Response 13:
Sincere appreciation for the Reviewer’s comments, the two groups have been added in the revised Figure 1(B) and Figure 1(C).

Figure 1

Comment 14:
• FIGURE (2A): On the ordinate axis of the HepG2 diagram “P65/PCNA” must be used instead of “P65/beta-actin”.

Response 14:
We would like to thank the Reviewer for the keen observation. Actually, PCNA was used as internal control. Therefore, the term “beta-actin” was a mistake in manuscript editing that has been corrected in Figure 2A of the revised manuscript.

Figure 2
Comment 15:
• NOX could be added to abbreviations.

Response 15:
In response to the Reviewer’s comments, NOX has been added to abbreviations (Highlighted in pink).

Comment 16:
• Finally, I suggest the following Discretionary Revision:

The method used to evaluate mRNA expression is the Reverse Transcription and semi-quantitative PCR. I think that an approach of relative quantification using Reverse Transcription Real Time PCR could be more appropriate. It can provide better sensitivity and precision respect to the results shown in FIGURE 2B and 2C.

Response 16:
In compliance with the Reviewer’s professional comments, all of the experiments on mRNA expressions were repeated with quantitative PCR to be more accurate and appropriate. The results are shown in Figure 2B, 2C, 3B, 3D, 3F, 5B and 6 of the revised manuscript. Relevant descriptions have been added into the revised manuscript.

Figure 2
Figure 3

Figure 5
Figure 6

(B) mRNA expression (fold of control) for Collagen I, α-SMA, TIMP-1 at 4 weeks and 8 weeks.

4 weeks

- Collagen I
- α-SMA
- TIMP-1

8 weeks

- Collagen I
- α-SMA
- TIMP-1

Figure 6

MRNA expression (fold of control) for IL-6, iNOS, ICAM-1 at 4 weeks and 8 weeks.

4 weeks

- IL-6
- iNOS
- ICAM-1

8 weeks

- IL-6
- iNOS
- ICAM-1