Dr. Daniela Foti

Academic Editor

*PeerJ*

 November 15, 2017

Dear Dr. Daniela Foti:

Enclosed is our revised manuscript (Article ID: 19899) entitled "**Formononetin inhibits lipopolysaccharide-induced release of high mobility group box 1 by upregulating SIRT1 in a PPARδ-dependent manner**". I would appreciate it very much if you would kindly re-examine the manuscript for publication in *PeerJ.*

Following the reviewers’ suggestions, we carried out additional experiments and included the data in the manuscript, or in the figures attached to this letter.

We have made other corrections and changes in the text as indicated by the reviewers.

The authors hope this manuscript is now acceptable to your journal. I truly thank you and the reviewers for the time and effort.

I would greatly appreciate it if you could let me know your decision at your earliest convenience by e-mail (hgseo@konkuk.ac.kr).

 Sincerely yours

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**Responses to questions raised by Review:**

**Editor’s Comments**

*1. Please answer to all of the issues raised by both reviewers. In particular, reviewer 1 suggests to use primary macrophages to confirm results.*

🡪 As your suggestion, we responded to questions raised by reviewers as follow. And we also confirmed the effects of formononetin on the LPS-induced HMGB1 release in human primary macrophages. The results are now included in Figure 1D and commented in RESULTS (page 10, lines 198-199 and 211-214, underlined in RED). Accordingly, Figure 1 is rearranged to integrate these findings and new LEGEND for Figure 1D is added (page 25, line 536-541, underlined in RED). MATERIALS & METHODS is also modified (page 6, line 111-115, underlined in RED).

**To Reviewer #1**

**Basic reporting:**

*1. The article is clear and english is correct.*

🡪 Thanks to your comments.

*2. Results of figure 2 should be inserted in figure 1 as panels C and D.*

🡪 As your suggestion, we now inserted Figure 2 in Figure 1 as panels C and E. We accordingly modified RESULTS (page 10, lines 204, 210, 214, 219, and page 11, line 221, underlined in RED) and Legend for Figure 1 (page 25, line 532 – page 26, line 548, underlined in RED).

*3. Similarly, data of figure 4 should be inserted in figure 3 as panels C to F.*

🡪 The data of Figure 4 also inserted in Figure 3 to generate Figure 2 as panels C, D, F, and G. Accordingly, RESULTS (page 11, lines 227, 228, 232, 233, and 243; page 12, lines 245 and 247, underlined in RED) and Legend for Figure 2 (page 27, line 551 – page 28, line 569, underlined in RED) are modified.

*4. Data presented in the supplemental figures should be inserted in the main figures.*

🡪 As suggested, we now inserted the supplemental Figure #1 and #2 in Figure 2 and 3 as panels 2E and 3B, respectively. RESULTS (page 11, line 243; page 12, lines 258, 262, 264, 266, and 269, underlined in RED) and Legends for Figure 2 and 3 (page 27, line 551 – page 28, line 569; page 29, lines 572, 574-575, 577, and 578, underlined in RED) are also modified.

*5. Some references should be added in particular on the effect of resveratrol on HMGB1 release (e.g. Xu et al. Shock 2014; Dong et al. Free Radic Biol. Med 2015).*

🡪 We now cited New references in DISCUSSION (page 15, line 322-323, underlined in RED) as suggested. The new references are now added in REFERENCE (page 19, line 397-400; page 23, line 503 – page 24, line 506, underlined in RED).

**Experimental design:**

*1. The objective of the study is to assess if formononetin modulates HMGB1 secretion in response to LPS. Overall, the methods are well described, the experiments are well designed with appropriate controls and technical replicates.*

🡪 Thanks to your comments.

*2. The authors should provide the quantification of the western-blots in figure 2A-B.*

🡪 As your suggestion, we repeated the similar experiments to quantitate the results of Western blots. The data are now included in Figure 1C and 1E, and also commented in RESULTS (page 10, lines 210 and 219, underlined in RED).

*3. In figure 2B, it seems that the total amount of HMGB1 is increased with LPS or formononetin treatment. The authors should analyze HMGB1 mRNA level upon formononetin treatment.*

🡪 As suggested, we performed additional experiments to analyze the HMGB1 mRNA levels upon formononetin treatment in RAW264.7 cells exposed to LPS. The levels of HMGB1 mRNA were not affected by formononetin in the presence or absence of LPS. These results are now included in Figure 1F, and commented in RESULTS (page 10, line 219 – page 11, line 222, underlined in RED). Accordingly, we modified the MATERIALS & METHODS (page 9, lines 174 and 181-182, underlined in RED) and added the new REGEND for Figure 1F (page 25, line 544 – page 26, line 548, underlined in RED).

*4. RAW264.7 cell line is not the most suitable model to study HMGB1 release and the effect of formononetin on HMGB1 secretion should be confirmed in human primary macrophages.*

🡪 As suggested, we carried out similar experiments to confirm the effect of formononetin on the HMGB1 secretion using primary human peripheral blood macrophages purchased from STEMCELL Technologies (Vancouver, BC, Canada) and cultured in RPMI 1640 according to manufacturer’s instructions. Consistent with results obtained from murine macrophage RAW264.7 cells, formononetin also inhibited the LPS-primed HMGB1 release in human primary macrophages. The results are now included in Figure 1D and commented in RESULTS (page 10, line 211-214, underlined in RED). Accordingly, Figure 1 is rearranged to integrate these findings and new LEGEND for Figure 1D is added (page 25, line 536-541, underlined in RED). MATERIALS & METHODS is also modified (page 6, line 111-115, underlined in RED).

**Validity of the findings:**

*1. The novelty of the study is rather limited as a large number of studies already demonstrated that many herbal compounds inhibit HMGB1 release by preventing its acetylation. It might be interesting to focus on the advantage of using formononetin compared to other similar compounds. Hence, it might be useful to compare the effect of formononetin on HMGB1 release with other similar compounds.*

🡪 As suggested, we compared the effect of formononetin on HMGB1 release with other herbal compounds exhibiting anti-inflammatory activity such as curcumin and genistein. Although direct comparison is difficult because of different chemical properties of each compound itself, formononetin showed similar or superior effect in the inhibition of HMGB1 release induced by LPS. These results are now attached to this letter as Reference Figure #1.

*2. In figure 5A, treatment with formononetin alone significantly increased the level of SIRT1 and in figure 1B it seems that formononetin alone decreases the level of HMGB1 in the cytoplasm. It might be interesting to perform a pre-treatment with formononetin and then wash the cells and activate them with LPS in order to evaluate if the effect of formononetin treatment persists in time.*

🡪 As your suggestion, we performed additional experiments to test whether the effect of formononetin persists in time during the blockade of HMGB1 release induced by LPS. The inhibitory effect of formononetin was evident from 6 h pretreatment and persisted for 24 h pretreatment, in which the HMGB1 release is almost completely abolished. These results are in a time frame of our experiments, in which the HMGB1 release was detected after 24 h treatment with formononetin in the presence or absence of LPS. These results are now attached to this letter as Reference Figure #2.

*3. From authors conclusions, formononetin and resveratrol appear to modulate HMGB1 release via SIRT1 expression. Hence, why did they observe a synergic effect in figure 5D? If these two compounds modulate HMGB1 release via the same mechanism, no synergic effect should be observed. The authors should also analyze the level of SIRT1 in this experiment.*

🡪 As suggested, we performed similar experiments to analyze the level of SIRT1 in cells treated with formononetin and/or resveratrol in the presence of LPS. Although the synergistic effect of formononetin and resveratrol in the LPS-induced HMGB1 release also observed, the levels of SIRT1 were not markedly different in cells treated with both reagents compared with formononetin alone in the presence of LPS. However, when the formononetin-treated group is compared with resveratrol-treated group, the LPS-induced suppression of SIRT1 was markedly reversed by formononetin, but not resveratrol. These results indicate that resveratrol activates the SIRT1 recovered by formononetin to elicit synergistic effect compared with resveratrol alone, in which SIRT1 level is markedly suppressed by LPS. These observations are now included in Figure 3E (lower two panels) and modified the LEGEND for Figure 3 (page 29, line 577-578, underlined in RED).

**To Reviewer #2**

**Basic reporting:**

*1. The paper deals with the role of formononetin as anti-inflammatory molecule. Basically, formononetin acts by inhibiting HMGB1 release via Sirt1 up-regulation. English form is correct and appropriate. References are accurate.*

🡪 Thanks to your comments.

*2. Line 165: Please fix “. At which time”*

🡪 As your suggestion, we now corrected Line 165 as “At which time” (page 8, line 169, underlined in RED).

**Experimental design:**

*1. The study is well designed. The results are well presented and understandable. Methods are well described with the exception of statistical analysis, which should be explained in a wider way.*

🡪 As indicated, we now described more widely the statistical analysis (page 9, line 194-196, underlined in RED).

**Validity of the findings:**

*1. Authors provide a functional explanation for HMGB1 release inhibition by formononetin. All experiments are well showed, results are consistent and statistically sounding. Conclusions are well written and results support the conclusions.*

🡪 Thanks to your comments.

**Reference Figure #1**

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**Reference Figure #1. Effect of herbal compounds in the HMGB1 secretion induced by LPS.** RAW264.7 cells grown to sub-confluency were maintained in serum-free medium for 16 h and then stimulated with LPS in the presence or absence of formononetin, curcumin, and genistein for 24 h. Equal volumes of conditioned media were analyzed by immunoblotting. Ponceau S staining was used as the loading controls.

**Reference Figure #2**

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**Reference Figure #2. Effect of formononetin pretreatments in the HMGB1 secretion induced by LPS.** RAW264.7 cells grown to sub-confluency were maintained in serum-free medium for 16 h and then pre-treated with formononetin for indicated times. Following washing with fresh medium, the cells were stimulated with LPS for 24 h. Equal volumes of conditioned media were analyzed by immunoblotting. Ponceau S staining was used as the loading controls.