Author’s response to reviews

Title: Preventive Effects of the Novel Antimicrobial Peptide Nal-P-113 in a Rat Periodontitis Model by Limiting the Growth of Porphyromonas gingivalis and Modulating IL-1β and TNF-α Production

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Reviewer reports:

Gill Diamond (Reviewer 1): A very nice study. One small issue in the materials and method section: it wasn’t clear how the model was carried out with respect to time. How many days was the bacterial treatment and the drug treatment? Also, please correct the English grammar and usage.

Response: We thank the reviewer for the suggestion. We added the details on bacterial treatment and drug treatment in “materials and methods”(Page 5 Line 115-116). Meantime, we have corrected the English grammar and usage mistakes and we hope our writing is now of the standard for the Journal.

Marianna Kulka (Reviewer 2): In this work, Wang et al have evaluated the antimicrobial effect of a modified peptide Nal-P-113 that was derived from histatin 5 in a periodontitis rat model induced by Porphyromonas gingivalis.
They investigated the effect of a series of Nal-P-113 concentrations on alveolar bone loss and IL-1β and TNF-α expression. The study shows an interesting in vivo result, which significantly limited periodontitis by decreasing the amount of Porphyromonas gingivalis. However, the authors have not adequately examined the inflammatory cytokine expression to fully ascertain its effect on inflammation. Furthermore, some of the data represented is unclear, or missing from the manuscript.

1. The figure resolution in some cases is poor. Please insert higher resolution figures.
Response: As requested, we have now presented higher resolution figures in the revised version.

2. The treatment group "f", is described as "f, without P. gingivalis W83 or Nal-P-113." (P2 L 41 and P6 L125). However, the authors also mentioned this group to be "without P. gingivalis W83 and Nal-P-113" in P20 469…Please check the use of "or" and "and" in these cases.
Response: Correction made. We have changed “and” to “or” in Figure Legends, Page 23 Line 531,539.

3. Figure 1, a better explanation of panel B is required. How were the images obtained and under what circumstances? In the current figure legend it is unclear which information pertains to panel A and B.
Response: We apologize for the confusion in the description. As suggested, we have added the discription of panel B in the revised manuscript (Materials and Methods, Page 6 Line 121-125, Results, Page 9 Line 194-199, and Figure Legends, Page 23 Line 532-533).

4. Figure 2. The SEM images in this figure are impressive. However, the notations below the figures are too small to read and the scale bar is very unclear. The authors should provide labels to indicate the bacteria and the tissue morphology that they wish the read to focus on.
Response: We thank the reviewer for pointing out the problem in our manuscript. We have added different colours of arrows in the figure 2 to lable bacteria and explained the arrows in Figure Legends, Page 23 Line 539-541. However, the notations below the figures generated by SEM software automatically, we cannot change them artifically.
5. Figure 3. The authors did not provide information of how IL-1β and TNF-α expression was measured. What kind of staining method and which color represents which cytokine in the images? The graph in section C is very poor resolution and the X and Y axis labels are not legible.

Response: We thank the reviewer for this comment. As suggested, we have added the relevant explanation in the revised manuscript. (Materials and Methods, Page 7 Line 159-161, and Figure Legends, Page 23 Line 544-548). Meanwhile, we have improved the resolution of Figure 3C.

6. The authors mentioned that they observed "significantly higher numbers of neutrophils and other inflammatory cells", yet there is no discussion or explanation of how this was determined. The authors should at least mention how they characterized these different cell populations and how they define "significantly higher" in this context. Also, what kind of inflammatory cells were they expecting to see and why?

Response: We thank the reviewer for pointing out this in our research. As suggested, we have corrected the vague descriptions in the revised manuscript (Results, Page 10 Line 220-225). We randomly selected 6 views at 400x magnification, inflammation degrees were divided into mild, moderate and severe scores according to the percentage of inflammatory cells (mainly including neutrophils, lymphocytes and monocytes). The inflammatory cells less than 50% was mild score, 50%-75% was moderate score, >75% was severe score. Because neutrophils, lymphocytes and monocytes reflect the degrees of local inflammation induced by bacteria.

7. Figure 4: This figure shows a linear correlation curve of the amount of P. gingivalis' DNA copy and cytokines in periodontal tissue (IL-1β and TNF-α). A significant positive linear correlation was identified for DNA copy of P. gingivalis and cytokines levels in periodontal tissue. In the figure legend, the authors need to better explain panel A and panel B.

Response: We have added the explanations in Figure Legends (Page 24 Line 554-563)

8. The SEM protocol used in this study relied on washing with PBS and then dehydration. This could lead to salt crystals, which can be seen in e/f. One step of washing in water can avoid this.

Response: We thank the reviewer for pointing out this to us. In the future scientific research, we will carry out SEM protocol following the reviewer’s recommendations.
9. P13, L289 "intro"? Should it be "in vitro"?

Response: We apologize for the typo. Yes, it should be “in vitro”. We have corrected that in the revised version (Discussions, Page 12 Line 282).

10. The mechanism behind the therapeutic effect is unclear. What is the effect of Nal-P-113 without P. gingivalis? Does it upregulate the production of these cytokines on its own?

Response: We agree with the reviewer that the effect of Nal-P-113 without P. gingivalis is important to take into consideration in this research. We have verified that 25μg/mL or 400μg/mL Nal-P-113 did not increase IL-1β and TNF-α expression levels in immortalized human gingival epithelial cells or macrophages. But TNF-α levels was raised in human periodontal ligament stem cells when co-cultured with 25μg/mL or 400μg/mL Nal-P-113 for 2 h. The data and relevant descriptions have been added in supplementary materials and discussions (Supplementary Figure 1 and Discussions, Page 14 Line 313-317). In this study, we focused on the effect of Nal-P-113 on P. gingivalis- induced rat periodontitis models and comparing the different levels of bacteria and cytokines when applying with Nal-P-113 or without Nal-P-113. Therefore, in this research, we did not set up the control group with Nal-P-113 alone.

11. Does the peptide directly interact with P. gingivalis? Or can this peptide also interact with immune cells? Although the authors have mentioned "One of the reasons may be that antimicrobial peptides themselves can induce a slight up-regulation of TNF-α expression,[30]", so the question is, up regulated by which cell, through what possible receptor? There are many antimicrobial peptides and signaling pathways that could be involved and examining at least one of these would be very helpful to this study. One possibility are macrophages and mast cells, both of which express antimicrobial peptide receptors and are able to fight bacterial infections in tissues.

Response: We thank the reviewer for the suggestion. In the previous study, we have confirmed that Nal-P-113 interacted with P. gingivalis directly, which can kill planktonic state and biofilm state P. gingivalis (PMID: 26210284). In this study, we found that 25μg/mL and 400μg/mL Nal-P-113 may increase TNF-α expression levels in human periodontal ligament stem cells, but not in immortalized human gingival epithelial cells or macrophages (Discussions, Page 14 Line 313-317, and Supplementary Figure 1). Also, We thank the reviewer for pointing out the importance to add mechanistic insight on how Nal-P-113 peptide regulates immune response. However, the major goal of this study is to investigate the potential use of Nal-P-113 peptide to inhibit rat periodontitis models. Under these circumstances, we added some descriptions in discussions concerning signaling pathway and receptors of other antimicrobial peptides (Discussions, Page 13 Line 298-309 and Page 14 Line 313-317).
12. What happens when the authors use the unmodified version of this peptide? Does it have the same effect?

Response: Moffa EB et al have verified that the antifungal activity of histatin 5 was reduced when facing salivary amylase (PMID: 26544073). We have previously shown that unmodified P-113 had weaker bactericidal capacity than Nal-P-113 in vitro (PMID: 21768519). Also, we confirmed that P-113 degraded completely if it presented in human serum for 2 h. Therefore, in this study, we didn’t use histatin 5 or P-113.

13. What happens to the production of other cytokines and chemokines such as IFN-gamma and MCP-1? What about the production of other host-produced antimicrobial peptides?

Response: We agreed with the reviewer that other cytokines and chemokines is important to take into consideration. In the future studies, we will explore the effects of Nal-P-113 on production of IFN-gamma and MCP-1. Nevertheless, IL-1β and TNF-α are recognized as the main inflammatory factors of bone destruction. Therefore, in this study, we only detected these two cytokines.