Author’s response to reviews

Title: Regulating Autonomic Nervous System Homeostasis improves Pulmonary Function in rabbits with Acute Lung Injury

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Regulating Autonomic Nervous System Homeostasis improves Pulmonary Function in rabbits with Acute Lung Injury

Dear editor,

Thank you very much for your letter and comments concerning our manuscript entitled “Regulating Autonomic Nervous System Homeostasis improves Pulmonary Function in rabbits with Acute Lung Injury” (Manuscript ID: PULM-D-16-00357). Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our researches.

We have studied the comments carefully and have made corrections which we hope meet with approval. Revised portions are highlighted by track changes mode in MS word in the paper for easy editing purpose. The point-by-point responses to the reviewers’ comments are listed below.

We are looking forward to getting your approval for its publication. If you have any additional questions, please contact us without hesitation.

Sincerely yours,

Wenzhi Li and Yulong Bo

Response to comments
Editor Comments:

1. Please make sure the abstract follows the format outlined in our submission guidelines.

Response: Thank you very much for your kind suggestion. According to your request, we have modified the “objective” into “Background” in the section of abstract.

2. In accordance with BioMed Central editorial policies (http://www.biomedcentral.com/submissions/editorial-policies#standards+of+reporting), could you please ensure your manuscript reporting adheres to ARRIVE guidelines (https://www.nc3rs.org.uk/arrive-guidelines) for reporting in-vivo animal research. This is so your methodology can be fully evaluated and utilized.

Can you please include a completed ARRIVE checklist as an additional file when submitting your revised manuscript.

Response: N/A

3. Please add a “Conclusions” section after the “Discussion” section. This should state clearly the main conclusions of the research article and give a clear explanation of their importance and relevance.

Response: Thank you very much for your advice. We have added a “Conclusion” section after the “Discussion” section as “In summary, we have gained following based on our results: firstly, both SNS and PNS activity were markedly elevated in rabbits with HCl-induced ALI and the disturbance of ANS homeostasis was attributed to a predominance of SNS activity; Secondly, all three treatment methods including VNS, THA and SGB could effectively regulate the disequilibrium of ANS, and improve pulmonary function; thirdly, SGB treatment was the most effective approach in regulating ANS balance. However, further preclinical and clinical studies are still required to further explore the efficacy and safety of these approaches in patients with inflammatory disorders, such as aspiration pneumonia”.

4. Please consider the list of authors as it currently stands with reference to our guidelines regarding qualification for authorship (http://www.biomedcentral.com/submissions/editorial-policies#authorship).

Currently, the contributions of author YLB does not automatically qualify them for authorship. Please provide clarification on their contributions, or remove their name from the list of authors and place them in the “Acknowledgements” section instead.

Response: Thank you very much. YULONG BO is one of the authors, and is also the Corresponding author together with WENZHI LI

Reviewer reports:

Reviewer 1:
1. Generally the figure legends of the complete manuscript are extremely simplified. The figures should stand alone and therefore include the description of the experiment, method, the groups, the n number and statistical analysis.

Response: Thank you very much for your comments. Because there were multiple indicators inspected in this study, it would be very confused if we ordered these figures alone. Therefore, we had organized these figures in the present form. In addition, the detailed figure legends have been added in the figure legend section, including the description of experiment, method, and the groups with participant numbers, as well as the statistical analysis.

2. To strength the observed evidences, in figure 1, 2 and 3 error bars are used but statistical analysis should be included additionally, and comparisons between the groups described and highlighted.

Response: Thank you very much for your comments. Because there were five groups included in this study, thus, the comparative results between groups were not showed in the figures. We have added the detailed statistical analysis results of figure 1, 2 and 3 in the manuscript. Because the length of revision is too huge, the detailed modification is not presented here.

3. In the results section the indexed figures are not presented consistently, (so for Fig5 indexed parameters a), b) and c) are not independently described) as well as in the figure legends.

Response: Thank you very much for your comment. We have independently describe all the figures and figure legends included in this manuscript. Because the length of revision is too large, the detailed modification is not presented here.

4. The indexing of figure 6 is barely visible, and there should be burned in on different colour of just sit in black outside of the picture. Additionally, if the condenser of your microscope is adjusted slightly lower and with increased light power, your system will produce lung pictures with even light in the centre and in the edges of your CCD camera.

Response: Thank you very much for your suggestion. We have remapped the pictures included in Figure 6, and the new figures is affiliated.

Reviewer 2:

Major comments:

1. Data on the changes of the mean arterial blood pressure are missing. Could you please add these data into the manuscript if they are available?

Response: Thank you very much for your comment. We have added the mean arterial blood pressure as Figure 1f, and the corresponded description has been provided as “In addition, the mean arterial pressures (MAPs) in groups were also continuously monitored throughout the experiment. According to the recordings, MAPs with HCl aspiration were stable compared with control group, but in the THA group, a significant decrease was shown at 30 min after treatment...”
while compared with control and HCl groups (P < 0.05). However, both in VNS and SGB groups, the MAPs were obviously decreased throughout the experiment, but there were no remarkable differences identified between them and control group, as well as HCl group (P > 0.05)” in the results section of manuscript. Also, the Figure legend has been provided.

2. Data and Methods (Par. 4 Measurements): It is not clear where the leukocyte count and examination of PMNs were done (in the blood? in the BAL fluid?). What was the methods of these investigations?

Response: Thank you very much for your comments. The leukocyte count and PMNs percent were examined in BAL fluid and we have added this information in the manuscript. The specific detection means has been provided as “After the BALF obtained, a centrifugation at 4°C, 2000 rpm for 10 min was performed. The sediment for this centrifugation was utilized to evaluate the leukocyte counts and percentage of PMNs by smear stained with Giemsa and Wright. All the detections were performed by two independently experienced inspectors via microscope” in the manuscript.

3. HRV methodology is not sufficiently described. Specifically, the part related to methodological processing for ECG recording is missing. Furthermore, it is not clear which method for linear HRV analysis was used - autoregressive analysis or Fast Fourier transformation?

Response: Thank you very much for your comment. We have provided the methodological processing for ECG recording and HRV analysis as “During the procedure, the electrocardiograph (ECG) was inspected and recorded by multi-functional physiological detector MP150 (BIOPAC MP Hardware, MP150, 40 Aero Camino, Goleta, USA). After recording, 5 min of ECG without interpretation was selected and the R-wave was defined artificially. Then, the heart rate variability (HRV) analysis was performed using Kubios HRV software 2.0 (Biosignal Analysis and Medical Imaging Group, Department of Applied Physics, University of Eastern Finland, Kuopio, Finland)” in the manuscript. In addition, the Fast Fourier transformation was used for linear HRV analysis in this study.

4. Major criticism is related to frequency band in HF-HRV ranged from 0.15-0.4 Hz that is appropriate for analysis of RR intervals in humans. The HF-HRV strongly depends on respiratory rate, therefore, the frequency range used in this study is not appropriate to respiratory rate in rabbits. In this context, the interpretation of HF-HRV is vague. I suggest to add publications where the heart rate variability has been evaluated in rabbits, e.g., J Physiol Pharmacol. 2013;64(6):751-759, Can J Physiol Pharmacol. 2008;86(11):804-814, Acta Vet. Brno 2006, 75: 3-12. Moreover, the authors should describe procedure for removal of potential artefacts from ECG recording.

Response: Thank you very much for your comment. Actually, Four HF-HRV ranges had been investigated, including very low frequency (VLF: 0-0.04 Hz), low frequency (LF: 0.04-0.15), high frequency (HF: 0.15-0.4 Hz) and very high frequency (VHF: 0.4-3.0 Hz). But because there were no obvious difference observed in VLF and VHF, the related data was not shown.
addition, all parameters measured in this manuscript had been standardized in clinical settings, so that can provide a visualized alteration for clinical therapy.

In order to remove the potential artefacts from ECG recording, the ECG recording was performed firstly before other detections were carried out. The related information has been provided in the manuscript as “To exclude the man-made influence, the ECG was recorded before the detections of lung compliance, discharge frequency and blood sampling”.

5. The length of RR intervals used in HRV analysis (short-term or long-term analysis) is crucial for correct interpretation of the results. This information is missed in the manuscript.

Response: Thank you very much for your kind suggestion. We have provided this information in the manuscript as “After recording, 5 min of ECG without interpretation was selected and the R-wave was defined artificially”.

6. In the part Data and methods, the authors should describe calculation of LFnu and HFnu.

Response: Thank you very much for your suggestion. In this manuscript, frequency-domain analysis was utilized. We chose 5 min of ECG signal without distribution to analyzed HRV and LFnu as well as HFnu. The R-wave was defined artificially and the tiny alteration of RR interval was assessed by Kubios Heart Rate Variability software. The corresponding description has been added as “Meanwhile, measurement of low frequency (LF) by frequency bands ranged from 0.04 Hz to –0.15 Hz and of high frequency ranged from 0.15 Hz to 0.4 Hz in this 5 min of ECG were also examined” in the manuscript.

7. Data and methods (Par. 5 Histological assessment): For how long and at what temperature was the lung tissue dried for estimation of wet-dry lung weight ratio? What pathological changes in the lung tissue observed by light microscope and electron microscope do you mean? More details should be provided on the methods.

Response: Thank you very much for your comments. We have dried the lung tissue at 80 °C for 48 h to estimate the wet-dry lung weight ratio. The related description has been added as “After dried at 80 °C for 48 h, tissues were measured for dry weight. The wet weight/dry weight (W/D) ratio was used to evaluate pulmonary edema”. For the analyses of light microscope and electron microscope, there were no specific indicators observed at the beginning of experiment design. We just want to identify if there were some differences between different treated groups, so that can provide us some new insights into the mechanism of ALI.

8. In the part Discussion, the interpretation of LF/HF as an index of sympathovagal balance is controversial. Importantly, short-term HRV is predominantly mediated by parasympathetic activity, therefore, the description of changes in sympathetic nervous system from short-term HRV analysis is questionable.

Response: Thank you very much for your comment. So far, we have known that LF is an important indicator for the activity of sympathetic nervous system and HF is a crucial indicator of the para-sympathetic nervous system activity. The ratio of LF/HF could reflect the balance of
nervous activity. In this study, although we have examined the HRV analysis only with 5 min of ECG signaling at different time point, the total HRV analysis have been insisted for 6h. We think that the trend of HRV during the whole procedure will also provide a promising result for the activity of both sympathetic and para-sympathetic nervous systems.

9. How can you explain the second peak of HRV response at 6 h after the treatment?

Response: Thank you very much for your comment. Among the five groups, only HCl group obviously showed a second peak of HRV at 6h. This may indicate that the three treatments can dynamically regulate the imbalance of autonomic nerve activity for more than 6h. The occurrence of second peak of HRV response in HCl group was mainly reduced by the further severe acute lung injury, inflammatory response and extreme stress. During the preliminary experiment, animals with poor state would present agonal stages at 5-6h after ALI model constructed. At this time, the sympathetic nervous system will show an extremely excited state. This alteration in preliminary experiment may be a potential evidence for the second peak of HRV response.

10. The part of Discussion (Par. 6), where changes in the parameters of HRV are given into the relationship with inflammatory markers should be little enlarged. I suggest to add information from the review in Respir Physiol Neurobiol. 2013;187(1):78-81.

Response: Thank you very much for your comments. We have carefully read the review you recommend and added some related information in the manuscript as “Sloan et al have documented that IL-6 levels were inversely related with vagus nerve activity indexed by HF-HRV [22]. Meanwhile, the Weber et al have reported that low HRV can delay the recovery of TNF-α after stressor ending compared with ones with high HRV [23]. In addition, Tonhajzerova et al demonstrated that IL-6 is an independent risk factor for cardiovascular mortality [24]”. Thanks again for your kind suggestion.

11. Generally, English should be improved throughout the manuscript including Abstract.

Response: Thank you very much for your kind advice. We have carefully revised the manuscript. Meanwhile, we also have sought the help from our colleagues who is proficient in English to improve our proof.

Minor comments:

1. Please use „autonomic nervous system" instead of „automatic nervous system".

Response: Thank you very much for your kind advice. We have replaced the “automatic nervous system” by “autonomic nervous system” in the manuscript.

2. In Data and Methods (Par. 1 Animals), I suggest to explain again the abbreviations for expression of the groups 3 (VHS), 4 (THA) and 5 (SGB) for better understanding.
Response: Thank you very much for your comment. According to your request, we have added the specific information for VNS, THA and SGB in the manuscript as “electronic vagus nerve stimulation (VNS) group, VNS and intratracheal injection of HCl; (4) tetrahydroaminoacridine (THA) group, THA (Shanghai Hanxiang, Shanghai, China) and intratracheal injection with HCl; and (5) stellate ganglion block (SGB) group, SGB and intratracheal injection with HCl”.

3. In Data and methods (Par. 2 Surgical Procedure), I suggest to explain why the lactated Ringer’s solution was infused into the trachea of rabbits at that dose (10 ml/kg/h).

Response: Thank you very much for your comment. This does was selected according to the preliminary experiment and the studies published previously. After searching for several published articles, several recommended doses with small discrepancy were screened. During the preliminary experiment, we have considered several potential influence factors, including animal abrosia, physiological needing, fluid volatizing during mechanical ventilation and surgery, bleeding, and blood sampling during surgery, and the dose of 10 ml/kg/h was finally chosen.

4. Data and methods (Par. 3 ALI model and treatment): It is not clear from the text how bupivacaine was administered - continuously or as a bolus injection?

Response: Thank you very much for your comment. The bupivacaine was firstly injected at a bolus injection of 5 ml, and then continuously administrated at 0.5 ml/h. The description of this procedure has been modified as “In the SGB group, 0.25% bupivacaine (Sigma, Taufkirchen, Germany) was continuously administered (0.5 ml/h) after a bolus injection of 5 ml through the catheter during the experiment” in the manuscript.

5. Data and Methods (Par. 4 Measurements): Please use „blood gases” instead of „blood gas”.

Response: Thank you very much for your kind suggestion. We have revised the “blood gas” to “blood gases” in the Data and Methods section.

6. Discussion, Par. 3, Sentence 3. I suggest to change it to : „However, a local anesthetic (bupivacaine) was given ... „

Response: Thank you very much for your kind advice. We have change the “However, a local anesthetic was given” into “However, a local anesthetic (bupivacaine) was given...” in the section of Discussion.

7. Discussion, Par. 4, Sentence 3: „Lyudmila et al. suggested... (15)” Are you sure that this is a correct reference? Lyudmila et al. is not present in the list of references.

Response: Thank you very much for your comment. We have revised “Lyudmila et al” to “Ma P et al” in the section of Discussion.

Reviewer 3:

Major:
1. Methods p4/5, "Surgical procedure": Here the description is rather short and therefore also incomplete. Please add the following descriptions and parameters:

   a. Where has venous access been established? What catheter? Were the animals awake during this procedure or anesthetized, e.g. by an inhalational agent?

   b. What was the FiO2? This information is crucial in any study regarding lung injury.

   c. Did the animals receive any form of pain treatment?

Response: Thank you very much for your comments.

a: The venous access had been established via ear vein. The catheter was inserted into trachea. During the surgery, the rabbits were first anesthetized by intravenous injection of pentobarbital sodium (30 mg/kg, Sigma, St. Louis, USA) and maintained with continuously pumping of 0.1 mg/kg/h pipecuronium (Arduan, Gedeon-Richter, Hungary) and 5 mg/kg/h pentobarbital during surgery. This information has been provided in the manuscript.

b: The FiO2 was set as 1.0 in the mechanically ventilation. This information has been provided in the manuscript.

c: Because the depth of anesthesia in this study can hold up more than 6h, and the rabbits were sacrificed after 6h of surgery. Thus, there was no other pain treatment utilized.

2. Methods p5, L33: The authors state that bupivacaine "was continuously administered […] as a bolus injection". This is confusing. Has there been a bolus administration or a continuous administration or a repeated bolus? Please clarify.

Response: Thank you very much for your comment. The bupivacaine was firstly injected at a bolus injection of 5 ml, and then continuously administrated at 0.5 ml/h. The description of this procedure has been modified as “In the SGB group, 0.25% bupivacaine (Sigma, Taufkirchen, Germany) was continuously administered (0.5 ml/h) after a bolus injection of 5 ml through the catheter during the experiment” in the manuscript.

3. Methods p5, L41: How was the discharge frequency in the specific nerve trunks measured?

Response: Thank you very much for your comment. In this study, the electrocardiograph (ECG) was inspected and recorded by multi-functional physiological detector MP150 (BIOPAC MP Hardware, MP150, 40 Aero Camino, Goleta, USA). After recording, 5 min of ECG without interpretation was selected and the R-wave was defined artificially. Then, the heart rate variability (HRV) analysis was performed using Kubios HRV software 2.0 (Biosignal Analysis and Medical Imaging Group, Department of Applied Physics, University of Eastern Finland, Kuopio, Finland). We have provided this information in the manuscript.

4. Methods p6, L4: How were the leukocytes and PMNs stained and analyzed? Please add this information.
Response: Thank you very much for your comment. The leukocyte count and PMNs percent were examined in BAL fluid and we have added this information in the manuscript. The specific detection means has been provided as “After the BALF obtained, a centrifugation at 4°C, 2000 rpm for 10 min was performed. The sediment for this centrifugation was utilized to evaluate the leukocyte counts and percentage of PMNs by smear stained with Giemsa and Wright. All the detections were performed by two independently experienced inspectors via microscope” in the manuscript.

5. Methods p6, "Histological assessment": Besides several language issues, this paragraph contains several points that will need clarification:

a. L29: The lung W/D ratio is a measure to estimate the amount of pulmonary edema, not necessarily "lung injury" as stated by the authors.

b. L32: after "4 µm" the word "slices" or something similar should be added.

c. There is no detailed description about the assessment of either the H&E sections or the EM sections. How were these slices evaluated? A scoring system? If there was no scoring system applied, this might be something the authors could consider adding, as it might help with quantification and subsequent analysis.

Response: Thank you very much for your comments. We have replaced the “lung injury” with “pulmonary edema”. We consider that pulmonary edema is a result of inflammatory response which may induced by HCl, thus, this index has been utilized. Meanwhile, in the “Histological assessment” section, “slices” has been inserted behind 4 µm. For the H&E staining and EM observation, we consider that both of them only available for morphological observation, thus, the qualification is not appropriate here. Therefore, the qualification of both H&E staining and EM observation result are nor performed.

6. Methods p6, "Statistical analysis":

a. Reporting of a mean +/- SEM is inappropriate. Please always report mean +/- SD.

b. There is no information whether the values were assessed for normal distribution. Did the authors do such an analysis prior to statistical testing? If yes, what test was used?

c. If there should have been any non-normally distributed data, further assessment by ANOVA might be inappropriate. In that case non-parametric testing should be used. Please clarify and adjust your statistical assessment if necessary.

Response: Thank you very much for your comments.

a: SEM and SD can be mutual converted. In this study, because there were 5 groups included in this study, it will be very disordered in the figures if we showed data with mean ± SD. Therefore, the mean with SEM was chosen.
In the current study, all detected values in five groups showed with normal distribution. Thus, the one-way analysis of variance followed by a post hoc Student-Newman-Keuls test.

7. Results p7: The description of the results is not detailed enough. To me it is not clear, which group has been tested against which (at least regarding Figures 1-3). Additionally, the description of the statistical methods suggests that the groups have been tested against each other via ANOVA at a certain time point. However, no information regarding this issue is provided in the results section and figures 1-3 also do not have any marks, which might help the reader understand the conducted statistical analysis. Also, the authors should consider adding more numbers and values to the text, to enhance the reader’s ability to follow the analysis.

Response: Thank you very much for your comments. The detailed statistical analytical results for comparisons have been provided in the manuscript. But because there were multiple comparisons conducted and some of them were not significant. Therefore, only the significant ones have been presented. Meanwhile, due to the large length of modification, the specific revised contents are not affiliated here.

8. Results, general: Please consider reporting the exact p-values for ALL reported results, also the non-significant ones. The description of p-values just as > or < 0.05 is inappropriate.

Response: Thank you very much for your comment. We have provide the t and P value for all significant comparisons included in this study.

Minor:

1. Abstract p2, L15: The sentence "Then animals randomly received different treatments […]" should also mention the control groups (saline and HCL only) in order to improve understanding of the experimental setup even in the abstract.

Response: Thank you very much for your kind suggestion. We have modified the sentence as “Animals in control groups were received saline or HCl only, and the others received both HCl and followed treatment: treatments: vagus nerve stimulation (VNS), intravenous injection of tetrahydroaminoacridine (THA), or stellate ganglion block (SGB)” in the section of abstract.

2. Abstract p2, L29: "activity was more active", should be changed to "activity was higher".

Response: Thank you very much for your kind advice. We have revised “activity was more active” into “activity was higher” in the abstract section.

3. Abstract p2, L38: "alleviated" should be "alleviate" instead.

Response: Thank you very much for your kind suggestion. We have corrected “alleviated” as “alleviate” in the abstract section.

4. Background p3, L15: "play" should be "plays" or "may play"
Response: Thank you very much for your kind advice. We have revised “play” into “plays” in the background section.

5. Background, p3, paragraph starting in L41: Is acid aspiration really a common clinical feature? The paragraph might be misleading, as I assume the authors just wanted to use HCl instillation as a model (as explained well in the following paragraph on page 4). I would consider omitting this paragraph.

Response: Thank you very much for your comments. Considering your opinion, we have omitted the sentence of “In clinical practice, acid aspiration can damage lung airway and induced a chemical burn, leading to pulmonary edema and hemorrhagic pneumonia, and even resulting in acute lung injury (ALI) and acute respiratory distress syndrome”.

6. Methods p5, "ALI model": To me it is not clear, when exactly the treatment in the different groups was started after HCl instillation (immediately?). Please clarify.

Response: Thank you very much for your kind suggestion. The treatments after ALI model instillation were brought out immediately. The description of this information has been added in the manuscript as “All of these three treatments were performed immediately after ALI model constructed”.

7. Methods, general: For the most part, the description of the methods is lacking detailed information regarding manufacturers or specific description of the equipment and agents used. This should be added.

Response: Thank you very much for your suggestion. We have added the detailed information of equipment and agents used in the text.

8. Methods, p6, L24: "killed" should be changed to "sacrificed".

Response: Thank you very much for your kind advice. We have changed the word of “killed” to “sacrificed”.