Author's response to reviews

Title: Short-term glutamine feeding decreases lung inflammation and the receptor for advanced glycation end-products (RAGE) expression in direct acute lung injury in mice

Authors:

Yin C Chuang Dr (chuangkenneth@hotmail.com)
Huey M Shaw Dr (mei@mail.chna.edu.tw)
Chi C Chen MS (ccomm2@yahoo.com.tw)
He J Pan MS (camille3935@hotmail.com)
Wei C Lai MS (laiweijii@hotmail.com)
Hui L Huang Dr. (cherry85@mail.chna.edu.tw)

Version: 3 Date: 20 February 2014

Author's response to reviews: see over
Reviewer:
We thank the reviewer for the careful examination of our work. The constructive criticism provided has allowed us to significantly improve our manuscript and hope that the reviewer will find the changes undertaken satisfactory.

Review's report:
The authors are to be congratulated for their excellent work on an important topic in clinical research, which will enrich literature. There were no flaws in design nor in description of the data as far as I could see. However, there are several language and grammar errors. I would suggest reviewing of the manuscript by a native speaker.

Response:
We are sorry for our poor writing. We have corrected my manuscript by an English editing teacher. Thanks very much.

Thank you very much. ^🐟^
Reviewer: Arie J. Hoogendijk

We thank the reviewer for the careful examination of our work. The constructive criticism provided has allowed us to significantly improve our manuscript and hope that the reviewer will find the changes undertaken satisfactory.

Minor comments:
1. In the introduction it seems some references are not provides for statements made (e.g.: line 55, 56, 61, 63 and 65).
Response: We are sorry for our poor writing. It has been added in the revised version (Line 54-85). Thanks for being so careful.

2. For lung measurements, unchallenged data are provided, however not for the BALF measurements, is this due to these being undetectable?
Response: Yes, we did not analyze the unchallenged control groups in the study. The reasons are described as bellow:
(1) The aim of this study was major to investigate whether GLN feeding had a ameliorative effect by ALI challenge in mice.
(2) In our previous study (as shown in table 1, 2), we found those cytokines of unchallenged controls were very low or undetectable and induced significantly in ALI-challenged mice.
(3) The other reason is just for reducing this experimental cost and assay loading. Therefore, we decide not to analyze the two unchallenged controls, then caused this experimental design defect. We apologize for no unchallenged control for the BALF measurements in this study.
### Table 1. Cytokines contents in the BALF of lung.

<table>
<thead>
<tr>
<th></th>
<th>unchallenged control (H₂O/Saline-treated for 3 hr)</th>
<th>ALI challenge (HCL/LPS-treated for 3 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAGE (ng/mL)</td>
<td>1.12 ±0.31</td>
<td>10.1 ±4.2*</td>
</tr>
<tr>
<td>IL-1β (pg/mL)</td>
<td>ND</td>
<td>11.3 ±6.5</td>
</tr>
<tr>
<td>IL-6 (ng/mL)</td>
<td>ND</td>
<td>9.78 ± 5.32</td>
</tr>
<tr>
<td>TNF-α (ng/mL)</td>
<td>0.53 ± 0.45</td>
<td>3.86 ±1.23*</td>
</tr>
</tbody>
</table>

1. Data are presented as means ± S.D.; n=6. ND, not detected.
2. The effect of ALI challenge was evaluated by Student’s t-test (*p <0.05 vs. the unchallenged control group).

### Table 2. The relative mRNA levels of genes in the lungs of ALI-challenged and unchallenged mice.

<table>
<thead>
<tr>
<th></th>
<th>unchallenged control (H₂O/Saline-treated for 3 hr)</th>
<th>ALI challenge (HCL/LPS-treated for 3 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS</td>
<td>1.09 ± 0.47</td>
<td>1.97 ± 0.64*</td>
</tr>
<tr>
<td>COX2</td>
<td>1.06 ± 0.34</td>
<td>5.77 ± 2.20*</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.01 ± 0.31</td>
<td>191 ± 89*</td>
</tr>
<tr>
<td>IL-1β</td>
<td>1.04 ± 0.32</td>
<td>38.0 ± 10.5*</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.00 ± 0.28</td>
<td>102 ± 49*</td>
</tr>
<tr>
<td>GPx</td>
<td>1.08 ± 0.47</td>
<td>0.58 ± 0.22*</td>
</tr>
<tr>
<td>Catalase</td>
<td>1.00 ± 0.57</td>
<td>0.12 ± 0.55*</td>
</tr>
</tbody>
</table>

1. Each value was normalized to that for GAPDH, and then the relative mRNA abundance was expressed as a fraction of that in the control group and assigned a value of 1.
2. Data are presented as means ± S.D.; n=6.
3. The effect of ALI challenge was evaluated by Student’s t-test (*p <0.05 vs. the unchallenged control group).
3. Figure 2 depicts IL1-beta levels as mg/mg, this seems to be very high, it this correct?
Response: We apologize for this unit error. I agree with review's opinion that ng/mL is more appropriate in the expression of those results. Our results have been re-analysis and reconfirmed recently. It has been revised as suggested (Fig 2). Thanks for your good comment.

![Figure 2](image)

**Figure 2** RAGE concentrations in the BALF of lung in ALI-challenged mice. (A) RAGE;
4. Line 239 states that because of the severity of the model GLN could not increase overall survival. Currently this is written as an overstatement, as no data is provided to as to the effects of GLN in a less severe survival experiment.

Response: We agree with your opinion. The survival result is shown in Figure S1. It has been corrected in the revised version (Line 239-243 of the revised version).

Figure S1 Survival rates for 32 h of animals after ALI challenge.
5. IL-1alpha is mentioned in line 266 but not shown.
Response: We are sorry for this typing error. IL-1 beta has been corrected in line 273.
"Second, we found that IL-1β and IL-6 levels in the lung were significantly lower in the GLN group with corresponding decreases in their mRNA than was found in those of the control group. "

Thank you very much. ^___^
Reviewer: Samuel Valenca

We thank the reviewer for the careful examination of our work. The constructive criticism provided has allowed us to significantly improve our manuscript and hope that the reviewer will find the changes undertaken satisfactory.

Major comments:


Response:
Thank you for providing this relevant paper. We have known the BALB/c mice with more susceptibility by CLP challenge, and C57BL/6 mice had a higher increase in inflammatory cytokines by LPS challenge. We had also seen that BALB/c mice were used as experimental animals from many previous reports. The other reason is that BALB/c mice are relatively tame animals compared with C57BL/6 mice, so we are easy to establish the HCl/LPS-induced ALI model.

2. There is no support in the paper that RAGE is regulated by glutamine in challenge-induced acute lung injury.

Response:
We are postulated that GLN was to reduce inflammatory response by inhibiting RAGE expression and pro-inflammatory cytokines production. There are relationship between glutamine feeding and the decrease of RAGE, but the possible action mechanism of glutamine is unclear until recently. Therefore, there are no direct evidences that elucidate glutamine regulates the RAGE in this paper. Effects of glutamine remains to be explored in future studies.

3. There are too much texts reporting literature with no references in introduction section.

Response:
We are sorry for our poor writing. It has been added in the revised version (Line 54-85). Thanks for being so careful.
4. The authors need to write approval protocol number from ethics committee.
   Response:
   It has been added in Line 101.
   "The protocols for animal care and handling were approved by the Institutional Animal Care and Use Committee of the Chia-Nan University of Pharmacy and Science (CN-IACUC-101028)"

5. The authors killed mice with carbon dioxide asphyxiation. This is an erroneous method for those are studying mouse lung. I realize that a limitation of the study, and the authors should address this point in the discussion section.
   Response:
   We apologize for this writing error. It is an inconceivable negligence. Thanks for this correcting. We have the other study (DSS-induced colitis) that use CO\textsubscript{2} asphyxiation. In ALI model, we are indeed using sodium pentobarbital asphyxiation. It has been corrected in Line 120.
   It has been corrected as bellow:
   "The mice were sacrificed by sodium pentobarbital asphyxiation 3 hrs after ALI challenge."

6. The English language and grammar should be improved.
   Response:
   We are sorry for our poor writing. We have corrected my manuscript by an english editing teacher. Thanks very much.

7. I observed all mice died after challenge (Figure S1). What the importance of glutamine in this case? I realize that your challenge is very hard. I suggest the authors to repeat experimental procedures with reduced dose of LPS/Acid. By the way, what kind of challenge was that, from Figure S1?
   Response:
   The method of ALI challenge in Figure S1 was the same as this paper. We suggested that all mice died may be a result of more severe injury in this model of ALI. So we couldn't see the ameliorated effect of glutamine on survival finally. We will accept your idea and try to reduce the dose of LPS/Acid in next study. Thanks for your good comments.
8. Serum IL-6 and TNF-α concentration, BALF RAGE, IL-1β, IL-6, and TNF-α content in ALI-challenged mice should be shown in unchallenged mice also. mRNA abundances of the lung in ALI-challenged mice should be shown in unchallenged mice also.

Response:
Yes, we did not analyze the unchallenged control groups in the study. The reasons are described as below:
(1) The aim of this study was major to investigate whether GLN feeding had a ameliorative effect by ALI challenge in mice.
(2) In our previous study (as shown in table 1, 2), we found those cytokines of unchallenged controls were very low or undetectable and induced significantly in ALI-challenged mice.
(3) The other reason is just for reducing this experimental cost and assay loading. Therefore, we decide not to analyze the two unchallenged controls, then caused this experimental design defect. We apologize for no unchallenged control for the BALF measurements in this study.

<table>
<thead>
<tr>
<th>Table 1. Cytokines contents in the BALF of lung.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>RAGE (ng/mL)</td>
</tr>
<tr>
<td>IL-1β (pg/mL)</td>
</tr>
<tr>
<td>IL-6 (ng/mL)</td>
</tr>
<tr>
<td>TNF-α (ng/mL)</td>
</tr>
</tbody>
</table>

4. Data are presented as means ± S.D.; n=6. ND, not detected.
5. The effect of ALI challenge was evaluated by Student’s t-test (*p <0.05 vs. the unchallenged control group).
Table 2  The relative mRNA levels of genes in the lungs of ALI-challenged and unchallenged mice.

<table>
<thead>
<tr>
<th>Gene</th>
<th>unchallenged control (H₂O/Saline-treated for 3 hr)</th>
<th>ALI challenge (HCL/LPS-treated for 3 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.09 ± 0.47</td>
<td>1.97 ± 0.64*</td>
</tr>
<tr>
<td>iNOS</td>
<td>1.06 ± 0.34</td>
<td>5.77 ± 2.20*</td>
</tr>
<tr>
<td>COX2</td>
<td>1.01 ± 0.31</td>
<td>191 ± 89*</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.04 ± 0.32</td>
<td>38.0 ± 10.5*</td>
</tr>
<tr>
<td>IL-1β</td>
<td>1.00 ± 0.28</td>
<td>102 ± 49*</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1.08 ± 0.47</td>
<td>0.58 ± 0.22*</td>
</tr>
<tr>
<td>GPx</td>
<td>1.00 ± 0.57</td>
<td>0.12 ± 0.55*</td>
</tr>
</tbody>
</table>

3. Each value was normalized to that for GAPDH, and then the relative mRNA abundance was expressed as a fraction of that in the control group and assigned a value of 1.

4. Data are presented as means ± S.D.; n=6.

6. The effect of ALI challenge was evaluated by Student’s t-test (*p <0.05 vs. the unchallenged control group).

9. The authors claim to be this first study about glutamine and ALI. See the follow studies:

Response:
Thank you for providing those relevant papers.
We consider that there is no the same dosage in previous reports as our ALI model, so describing the first finding. We have seen those papers and agree with your opinion. It ("the first") has been deleted as suggested in our revised manuscript.
10. There is no direct evidence of RAGE over acute lung injury model as proposed by authors.

Response:
It has been revised as suggested (Line 87-91 of the revised version)

"We hypothesized that GLN may have anti-inflammatory effects on a mouse model of direct acid and LPS-induced ALI. To test this idea, the mRNA and protein expressions of pro-inflammatory cytokines and RAGE were measured. We try to determine whether short-term GLN supplementation has a protective effect in the early stages of injury."

11. No make sense kill the mice after 3 h of injury since survival is so far.

Response:
In a previous pretest, mice were killed at 3, 6 and 24 h after ALI. Our results indicated that the RAGE, TNF-α and IL-6 were good markers of the early stage (3 and 6 h of injury) in ALI-challenged mice. We suggested that glutamine could reduce the expression of those cytokines at an early stage, consequently improving the outcome of ALI. So the time point of 3h after ALI has special significances.

Thank you very much. "^_^
Reviewer: Ruud Veldhuizen

We thank the reviewer for the careful examination of our work. The constructive criticism provided has allowed us to significantly improve our manuscript and hope that the reviewer will find the changes undertaken satisfactory.

Major comments:

1. The manuscript misses essential non-injured control groups in the data presented in figures 1 - 3. The authors have included some unchallenged controls in the data tables 3-5, similar controls should be provided for the remainder of the data to provide a perspective how the changes observed are relative to unchallenged animals. One could also argue that the controls should undergo all of the procedures except for acid/lps administration, rather than being simply unchallenged animals.

Response:

Yes, we did not analyze the unchallenged control groups in the study. The reasons are described as bellow:

(1) The aim of this study was major to investigate whether GLN feeding had an ameliorative effect by ALI challenge in mice.

(2) In our previous study (as shown in table 1, 2), we found those cytokines of unchallenged controls were very low or undetectable and induced significantly in ALI-challenged mice.

(3) The other reason is just for reducing this experimental cost and assay loading. Therefore, we decide not to analyze the two unchallenged controls, then caused this experimental design defect. We apologize for no unchallenged control for the BALF measurements in this study.

Table 1. Cytokines contents in the BALF of lung.

<table>
<thead>
<tr>
<th></th>
<th>unchallenged control</th>
<th>ALI challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(H2O/Saline-treated for 3 hr)</td>
<td>(HCL/LPS-treated for 3 hr)</td>
</tr>
<tr>
<td>RAGE (ng/mL)</td>
<td>1.12 ±0.31</td>
<td>10.1 ±4.2²</td>
</tr>
<tr>
<td>IL-1β (pg/mL)</td>
<td>ND</td>
<td>11.3 ±6.5</td>
</tr>
<tr>
<td>IL-6 (ng/mL)</td>
<td>ND</td>
<td>9.78 ± 5.32</td>
</tr>
<tr>
<td>TNF-α (ng/ mL)</td>
<td>0.53 ± 0.45</td>
<td>3.86 ±1.23³</td>
</tr>
</tbody>
</table>

7. Data are presented as means ± S.D.; n=6. ND, not detected.
The effect of ALI challenge was evaluated by Student’s \( t \)-test (*\( p < 0.05 \) vs. the unchallenged control group).

**Table 2**  The relative mRNA levels of genes in the lungs of ALI-challenged and unchallenged mice.

<table>
<thead>
<tr>
<th></th>
<th>unchallenged control (( H_2O/\text{Saline-treated for 3 hr} ))</th>
<th>ALI challenge (( \text{HCL/LPS-treated for 3 hr} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS</td>
<td>1.09 ± 0.47</td>
<td>1.97 ± 0.64*</td>
</tr>
<tr>
<td>COX2</td>
<td>1.06 ± 0.34</td>
<td>5.77 ± 2.20*</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.01 ± 0.31</td>
<td>191 ± 89*</td>
</tr>
<tr>
<td>IL-1( \beta )</td>
<td>1.04 ± 0.32</td>
<td>38.0 ± 10.5*</td>
</tr>
<tr>
<td>TNF-( \alpha )</td>
<td>1.00 ± 0.28</td>
<td>102 ± 49*</td>
</tr>
<tr>
<td>GPx</td>
<td>1.08 ± 0.47</td>
<td>0.58 ± 0.22*</td>
</tr>
<tr>
<td>Catalase</td>
<td>1.00 ± 0.57</td>
<td>0.12 ± 0.55*</td>
</tr>
</tbody>
</table>

Each value was normalized to that for GAPDH, and then the relative mRNA abundance was expressed as a fraction of that in the control group and assigned a value of 1.

Data are presented as means ± S.D.; \( n=6 \).

The effect of ALI challenge was evaluated by Student’s \( t \)-test (*\( p < 0.05 \) vs. the unchallenged control group).

The method of killing the animals by \( \text{CO}_2 \) asphyxiation is not appropriate for lung injury studies. This method of euthanasia can cause lung edema and hemorrhage and thus impact the outcomes of the study.

**Response:**

We apologize for this writing error. It is an inconceivable negligence. Thanks for this important comment. We have the other study (about DSS-induced colitis) that use \( \text{CO}_2 \) asphyxiation. In ALI model, we are indeed using sodium pentobarbital asphyxiation. So, it has been corrected in Line 120.

"The mice were sacrificed by sodium pentobarbital asphyxiation 3 hrs after ALI challenge."
3. The animal model is complicated. It consists of both LPS and acid instillation, both of which may have independent as well as synergistic effects on the lung. Utilizing such complicated model, it would need to be well characterized to be able to interpret the data provided. Unfortunately, the information provided is very limited. More information on some of the following: lung histology, lung compliance, inflammatory cell infiltrates, blood gas values and other physiological measurements would be needed to better establish the relevance of this model in the context of ARDS. See the paper by Matute-Bello et al, (Matute-Bello et al on behalf of the Acute Lung Injury in Animals Study Group. An Official American Thoracic Society Workshop Report: Features and Measurements of Experimental Acute Lung Injury in Animals. Am J Respir Cell Mol Biol Vol 44. pp 725–738, 2011), for more information. It should also be noted that a strong rationale for utilizing this specific model is not provided.

Response:
We apologize that couldn't provide a strong rationale in our study. We try this ALI model for imitation clinical cases that suffered from direct gastric fluid reflux and bacterial pneumonia, and focus on the expression of inflammatory cytokines and survival. That's a serve animal model of ALI by direct acid and LPS challenge. We agree with your suggestion and will check those physiological and pathological parameters before next study. Thanks for your comment.

4. I also have to question the animal ethics in the survival studies. It is simply described as a “survival study”. The details are important here; how frequent were animals monitored, were they provided with analgesic, were there criteria for euthanasia?

Response:
In the survival study, we observed the mice once every hour and didn't provide any analgesic after ALI challenge. We think that any treatment is an extra stress for the mice. All of the mice after injury were housed individually in an quiet and comfortable environment.
Our procedure have been approved by the Institutional Animal Care and Use Committee of the Chia-Nan University of Pharmacy and Science (CN-IACUC-101028).
5. The manuscript, especially the introduction, is poorly referenced. Numerous sentences, including ones that contain statements like: ”in numerous studies”, “it has been shown”, “previous studies”, are not referenced. Examples: Line 53-55, 59-61, 61-63, 63-65, 68-69, 76-78, etc. An example in the methods is line 137-138.

Response:
We are sorry for our poor writing. It has been added in the revised version (Line 54-85, 143). Thanks for being so careful.

Minor comments:
1. The authors use the term Acute Lung Injury when referring to patients but should probably switch to the new definition of Acute Respiratory Distress Syndrome in which the term ALI is discouraged.

Response:
We agree with your suggestion. It has been revised as suggested (Line 54 of the revised version).

2. The manuscript will need some thorough proofreading

Response:
We are sorry for our poor writing. We have carefully proofread twice and hope to minimize the errors.

3. The administration of acid: was this in water or saline or another buffer?

Response:
HCl was prepared in distilled water, and saline used for LPS solution preparation. It has been added in line 116.

"HCl (pH 1.0, in sterile distilled water; 2 mL/kg BW) was administered intratracheally to each mouse."
4. The results on weight gain of the lung or not useful, the method to investigate this is wet/dry weight ratio.

Response:
We didn't assay wet/dry lung weight ratio in this study, because the lung samples are not enough. The results on weight gain of the lung are used to prove lung enlargement by ALI challenge, but not indicate edema or not.

5. N-values are confusing!! On the one hand the author state that 8 mice per group were used as unchallenged controls (line 104), yet tables 3-5 state n=10-12. Similarly, the aforementioned survival study was performed on 8 mice in each group (line 106). Yet the discussion states survival rates of 11 and 33%, both mathematically impossible with an n=8.

Response:
Thanks for your very careful review of our paper. You are right. The survival study was performed on 8 mice in two groups. The results in our paper used 10-12 mice in each group. I am sorry to let you so confuse. It has been revised. (Line 108-111).

"Finally, 10 mice in control and GLN group were sacrificed as the unchallenged control, then the remainder were given the ALI challenge. Among those mice after ALI challenge, eight mice in both groups were used for the test of survival rates. 12 mice in both groups were killed at 3 h of ALI challenge."
6. Figure 2: why is RAGE expressed in mg/ml, but all others in mg/mg. Incidentally, in the expression of this data, mg/ml is probably more appropriate considering that the protein levels in the lavage would change due to edema. At any rate, recovered lavage fluid and protein levels should probably be provided.

Response:
We apologize for this writing error. I agree with your opinion that mg/mL is more appropriate in the expression of those results. Our results have been re-analyzed and reconfirmed recently. It has been revised as suggested (Fig 2). Thanks for your good comment.

7. Figure 3, L missing in the label of Graph A
Response:
We are sorry for this typing error. It has been added in Figure 3.

Thank you very much. ^-^