Peer Review File

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Reviewer A

In this paper, Dr Zhang and colleagues investigate the effects of an extract of a traditional Chinese medication on LPS induced injury in mice. In general, I appreciate the overall goal of utilizing these traditional medications in more experimentally controlled studies. Unfortunately, the current study is somewhat limited due to methodological issues as well as a lack of a clear rationale of the specific outcomes.

Comments:

Comment 1: The animal model is introduced as an ARDS-model. I do not believe this is accurate. There are clear requirements for an animal model to be designated a model of ARDS (see American thoracic society guidelines). The current model is simply one of intratracheal instillation of LPS. Furthermore, the level of inflammation induced in this model (figure 1) is extremely low. In most ARDS-like scenario, inflammatory mediators will increase many-fold over baseline values rather than the 50% - 250% increases seen here.

Reply 1: Thanks very much for your kindly review and concern. LPS is a glycolipid present in the outer membrane of gram-negative bacteria that is composed of a polar lipid head group (lipid A) and a chain of repeating disaccharides. Previous studies have shown that intratracheal instillation of LPS to induce ARDS model is a commonly used operation. LPS has been administered to humans via the intravenous and the intratracheal routes. The intratracheal instillation of LPS at doses ranging from 1 to 4 ng/kg is followed by an early phase characterized by increases in BAL fluid PMN, albumin, and proinflammatory cytokines and a later phase 24–48 h after instillation characterized by normalization of the BAL fluid cytokine concentrations and increases in the BAL fluid PMN, monocyte, macrophage, and lymphocyte counts [1, 2]. Similarity with human ARDS is that neutrophilic inflammatory response with increase in intrapulmonary cytokines while the difference is that the changes in alveolar-capillary permeability are mild. With the development of technology, advances in imaging, genetic tools, “omics” technologies, and cellular biology have provided new insights into lung injury both in preclinical models and in humans. The purpose of 2022 update to the 2011 Workshop report is to provide an updated frame work for defining experimental ALI [3].

In our study, after LPS instillation, it was observed that the mice showed symptoms such as shortness of breath, cyanosis of lips, and increased respiratory rate, and the inflammatory indicators and pathological examination results of the mice changed significantly. However, some indicators did not meet the requirements of the update guidelines, which is one of our limitations. Thank you for your valuable comments. In the future research, we will apply a more thorough study design to conduct more convincing research.

[1] Matute-Bello G, Frevert C W, Martin T R. Animal models of acute lung injury. AJP Lung Cellular and Molecular Physiology, 2008, 295(3):L379-99.

[2] Jiang L, Yao M, Yang M, et al. Study on rats with acute respiratory distress syndrome induced by intratracheal instillation of lipopolysaccharide in different ways. Clin J Crit Care Med 2019;12:80-4.

[3] Kulkarni HS, Lee JS, Bastarache JA, et al. Update on the Features and Measurements of Experimental Acute Lung Injury in Animals: An Official American Thoracic Society Workshop Report. Am J Respir Cell Mol Biol. 2022;66(2):e1-e14.
Changes in the text: No.

Comment 2: The methods, as described are very unclear. Just to provide some examples:
Reply 2: Thanks very much for your kindly review and concern. We have modified our text as advised.

A) The sex of the mice should be disclosed, if only male or only female are utilized the rationale for that approach should be provided.
Reply A: Thanks very much for your kindly review and concern. We have added an explanation of this point.
Changes in the text: Please check them in Page 7, line139-141.

B) The 20 animals (line 137) seem different than what is described below (line 148-150).
Reply B: Thanks very much for your kindly review and concern. The processing method in line 137 is the feeding environment and method, while the following processing methods (line 148-150) are modeling and grouping methods, which we have provided a clearer explanation.
Changes in the text: Please check them in Page 7, line138.

C) Were animals randomized?
Reply C: Yes. We have provided a clearer explanation.
Changes in the text: Please check them in Page 8, line154-155.

D) Why was there no control group with treatment?
Reply D: Thanks very much for your kindly review and concern. There are three groups in our experiment: the Control group 、 the Model(LPS) group and the Treatment group (LPS+FSM).The control group does not require treatment as it is a normal mouse and does not have ARDS.
Changes in the text: No.

E) The terminology for the experimental group is a bit strange. I would prefer more descriptive names such as: Control, LPS, and LPS + FSM.
Reply E: Thanks very much for your kindly review and concern. We have provided additional explanations.
Changes in the text: Please check them in Page 8, line156-157.

F) It is not clear when treatment starts, relative to the injection of LPS.
Reply F: Thanks very much for your kindly review and concern. After the modeling is completed, we will undergo treatment.

G) I am not sure what is meant by “A protocol was prepared before the study without registration (line 157).

Reply G: Thanks very much for your kindly review and concern. We add an explanation.
Changes in the text: Please check them in Page 8, line162-165.

H) Specific of the fixation should be provided, (was this done by inflation or perfusion or ??).
Reply H: Thanks very much for your kindly review and concern. Conduct IHC experiments on mice from the three groups mentioned above.
Changes in the text: Please check them in Page9, line179.

I) Details of processing the lung for western blotting should be provided, was this done on samples from separate animals, or on pieces of lung from the same animals as the histology.
Reply I: Details of processing the lung for western blotting has been provided below: Total proteins of the lung tissues were extracted using radioimmunoprecipitation assay (RIPA) solution and the protein concentration was quantified by bicinchoninic acid (BCA) reagents. After separation of the proteins bands by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the target protein bands were blotted on a polyvinylidene fluoride (PVDF) membrane, and subsequently incubated with primary antibodies of AQP-5, SP-C, and Notch1, respectively. Then, the PVDF membrane was incubated with HRP-conjugated secondary antibodies, and followed visualized by chemiluminescence with the enhanced chemiluminescence (ECL) chemicals. Besides, this done on samples was from separate animals.
Changes in the text: No.

J) The source of the antibodies should be provided. Importantly, housekeeping protein should be mentioned in the methods as well. For SP-C specifically, was this an antibody against the preprotein, or to the mature peptide?
Reply J: The source of the antibodies and housekeeping protein has been provided in the corresponding sections. For SP-C, this was an antibody against the mature peptide.
Changes in the text: Please check them in Page 9, Line 183-185. Thank you again for your constructive suggestions!

Comment 3: Data presentation can be improved.
a) For bar graphs a scatter plot is more informative.
Reply a: Thanks very much for your kindly review and concern. We quite agreed with your suggestion. However, in this study, bar graphs can also reflect data information, so bar graphs are chosen. In future studies, we will be more careful about how we present the data. Thank you again for your constructive suggestions!
Changes in the text: No.

b) Histology pictures require a scale bar.
Reply b: Scale bars of histology pictures have been supplemented in the revised figures.
Changes in the text: Please check them in the revised figures.

c) Molecular weights of the western blots would be helpful. In terms of quantification, Western blots do not provide information on “relative expression”, only on “relative abundance”.

Reply c: Thanks very much for your kindly review and concern. Molecular weights of the western blots have been supplemented in the revised figure 3. In addition, "relative expression" is replaced by "relative abundance".
Changes in the text: Please check them in the revised figure 3.

Comment 4: The purpose of the HPLC of the FSM is unclear. Although, in general, investigation into the active ingredients of the mixture may be useful, this particular analysis just represent an incremental step towards this goal.
Reply 4: Thanks very much for your kindly review and concern. HPLC has been widely used in the field of traditional Chinese medicine research, capable of accurate and efficient separation and quantitative analysis of chemical components in traditional Chinese medicine, with authority. Several chemical substances identified through HPLC analysis may become future research directions.
Changes in the text: No.

Comment 5: there is no hypothesis. There is a rationale for the general goal, but there is no strong justification for the target molecules (SP-C, aquaporin, Notch 1). Each one has a potential role in ARDS, but so have many other molecules. The current molecules seem to be selected arbitrarily and as such do not really answer the objective the authors are trying to address "to explore the potential pharmacological mechanisms of FSM for treating ARDS".
Reply 5: Fusu mixture (FSM) is an effective TCM formula for treating ARDS in the clinic that is derived from the ancient prescriptions of Qian-yang-dan recorded in a TCM monograph of Yilizhenchuan written in the Qing dynasty. However, the related mechanism of FSM is complex, and there are few relevant studies and relatively superficial. Thus the detailed pharmacological mechanisms and active substances of FSM are still difficult to make it clear in the initial study. The pulmonary alveolar surface is lined primarily by two epithelial cell types, the alveolar type I cell and the alveolar type II cell. The type I cell express type I–specific cell markers such as AQP-5, and the SP-C is a kind of pulmonary surfactant, which can be used to observe the function of type II cells. Importantly, Notch signaling is involved in lung development and dysregulation of Notch is known to be involved in lung disease [1, 2].

This study aimed to investigate the potential pharmacological mechanism of FSM in the treatment of ARDS by exploring the use of FSM in the process of ARDS. The relationship between FSM and proliferation of alveolar epithelial cells was explored by the changes of SP-C, AQP-5, and Notch1.

[1] Siebel C, Lendahl U. Notch Signaling in Development, Tissue Homeostasis, and Disease. Physiol Rev. 2017;97(4):1235-1294.
[2] Liu X, Zhu X, Zhu G, Wang C, Gao R, Ma J. Effects of Different Ligands in the Notch Signaling Pathway on the Proliferation and Transdifferentiation of Primary Type II Alveolar Epithelial Cells. Front Pediatr. 2020;8:452. Published 2020 Aug 6.
Changes in the text: No.

Comment 6: There are a large number of grammatical errors, to list a few:
Line 33 and 80: Common
Line 47: delete "Besides" or use "In addition,"

Reply 6: Thanks very much for your kindly review and concern. We have modified our text as advised.

Changes in the text: We have corrected the above syntax error according to the comments of the reviewers. Please check them in Page3, line34; Page3, line48; Page5, line81; Page5, line93; Page7, line61; Page8, line167.

Comment 7: The authors use the term "obviously" a lot. I am not sure if this reflects statistics, but if so they should use "significantly".
Line 81: delete "clinical"
Line 92 change second "of" to "for". Also delete "desperate"
Line 138: "are" should be "were"
Line 141 Delete –
Line 156: delete the last "the"
Line 159: "centrifugation"
Etc.

Reply 7: Thanks very much for your kindly review and concern. We have modified our text as advised.

Changes in the text: We have corrected the above syntax error according to the comments of the reviewers. Please check them in Line 82; Line 93, Line 138, Line 141, Line 156, Line 159.

Reviewer B

In this article, Zhang et al. suggested that Fusu mixture (FSM) alleviates inflammatory reactions and promotes the proliferation of alveolar epithelial cells in LPS-induced ARDS mice via regulation of SP-C, AQP-5, and Notch1 in lung tissues. Overall, FSM selected in the experiments seemed to have a good therapeutic effect in ARDS and the manuscript was relatively well written. However, the following comments need to be addressed before this manuscript is accepted for publication.

Major：

Comment 1: It is not clear where the answers to the research question are. The authors could not tell whether FSM promotes the proliferation of alveolar epithelial cells by animal experiments alone.

The authors should continue to complete the experiment by alveolar epithelial cells to intuitively detect whether FSM regulates SP-C, AQP- The authors should 5, and Notch1 in LPS stimulated cells, and the relationship between FSM and the proliferation of alveolar epithelial cells.

Reply 1: Thanks very much for your kindly review and concern! We quite agreed with your suggestion. The related mechanism of FSM is complex, and there are few relevant studies and relatively superficial. Thus the detailed pharmacological mechanisms and active substances of FSM are still difficult to make it clear in the initial study. In future studies, we will continue to complete the experiment by alveolar epithelial cells, the relationship between FSM and the proliferation of alveolar epithelial cells will be further explored.

Changes in the text: Please check them in the Page 14, Line 312-315.

Comment 2: In Figure 2, Figure 4, Figure 5, Figure 6, the author only showed qualitative pictures without quantitative data analysis.
The authors should conduct quantitative statistical analysis of the related content.
Reply 2: Thanks very much for your kindly review and concern! We quite agreed with your suggestion. However, limited by funding and laboratory conditions, we are currently unable to conduct quantitative data analysis, which is also one of our limitations. In the future research, we will fully mobilize all resources to analyze the data at multiple levels, so as to make the conclusions more accurate and reliable.
Changes in the text: No.

Comment 3: In Figure 4, Figure 5, Figure 6, IHC pictures demonstrated by author could not show the relationship between proteins (SP-C, AQP-5, and Notch 1) and alveolar epithelial cells. Related mark should be added.
Reply 3: Thanks very much for your kindly review and concern! Given laboratory conditions and funding constraints, we were unable to label the relationship between proteins and alveolar epithelial cells by fluorescent staining. But under the current conditions, IHC images can still show that after FSM treatment, the SP-C, AQP-5 and Notch 1 were significantly increased, which was reflected by the deepening of tissue staining in the treatment group.
Changes in the text: No.