Peer Review File
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First Round Peer Review

Reviewer A

This article about the possible use of Oxy-Hydrogen Medical Technology to reduce/treat COPD is very interesting.

However, I would like to comment on a few key points:

Comment 1: The use of CSS administered by intraperitoneal injection is not widely accepted as a model for COPD. A few studies have indeed used it and point out that it can reproduce some of the damage seen in COPD, but not all components of the disease (including the damage to other part of the respiratory system apart of the lung parenchyma). Moreover, the method used in this article is slightly different from the one used in the reference. The lack of weight difference in the control group and might indicate a problem with this COPD.
Reply 1: Thanks for your comments. We would like to change our terms in the manuscript from COPD to COPD-like injury. This is because we found an increase in inflammatory levels and significant lung damage that consists of COPD in our model. Although we didn't do other tests such as lung function analysis to confirm the COPD, these findings still show that our model potentially develops COPD.
Changes in the text: Where the terms COPD were revised into COPD-like injury.

Comment 2: The results and discussion give a big importance for the survival rate. However, there is no description for the etiology/reason of the death of the two mice in the COPD group. Are those death related at all with COPD? If so, are those related to the lung injury of the intraperitoneal injection? What if the death rate for COPD mice model in the literature? The small number of events make it hard to really attribute those death to COPD and make an affirmation that the Oxy-Hydrogen Medical Technology can really prevented. I think you should focus in the other parameters described in the study.
Reply 2: Thanks for your comments. Unfortunately, we did not conduct an autopsy on the two dead mice, so not sure if there is any other external reason for their death. However, they had lived in the same environment and were treated like others in their group and H_2/O_2 inhalation group except for no H_2/O_2 inhalation treatment. Therefore, we believe CCS intervention may be the main cause of death. Also, thanks for your suggestion regarding the number of samples we used; we will describe these deficiencies in our limitation.
Changes in the text: we have modified our text as advised (see Page 18, lines 11-14)

Comment 3: In figure 4b you show that there is no significant difference between the

groups. However, looking at figure 4a, its clear the difference. The way this was presented can be misleading. It would be better to show a pulmonary fluorescence images for the "mean" in each group.
Reply 3: Thanks for your comments. The images we chose for figure 4a were the most closed to the mean pulmonary fluorescence in each group. According to our manuscript, the mean levels of NE680 Fast fluorescence intensity in control, COPD, and COPD+ H2/O2 groups were 163 (108 P/S/mm2), 182.1 (108 P/S/mm2), and 168 (108 P/S/mm2), respectively. The fluorescence levels of images we chose for figure 4a were 163.2 (108 P/S/mm2) in control, 177.7 (108 P/S/mm2) in COPD+ H2/O2 groups and 170.6 (108 P/S/mm2) in COPD+ H2/O2 groups.
Changes in the text: No

Comment 4: There are some grammatical errors and coloquial language in the article that should be fixed before publication.
Reply 4: Thanks for your comments.
Changes in the text: We used the tracking function to mark out all changes in language.

Reviewer B

Comment 1: Overall, this study aimed to see the effect of H2/O2 on COPD induced by cigarette smoke solution. According to the study's flowchart, a preventive method (H2/O2) was administered at the start of the study, along with the administration of cigarette smoke before any COPD developed. The preventive method should also be applied to the control group and exposed it to H2/O2 to see if there is any effect on this group. Additionally, confirmatory tests to confirm the development of COPD is required, or this may only show emphysema.
Reply 1: Thanks for your comments. The control group in this study was used to ensure our CSS intraperitoneal injection does damage the healthy mice and then the application of H_2/O_2 ameliorated the injury. The purpose of administering H_2/O_2 is to prevent the damage and make the main testing level back to normal. Therefore, a blank group without any treatment would be better for us to compare the examination data. Regarding the confirmatory tests for the development of COPD, we would like to change the terms in this study from COPD to COPD-like injury, which may be closer to the results.
Changes in the text: No

Comment 2: Introduction
• Why do authors choose different methods of cigarette smoke administration? Furthermore, a previous experimental study found no difference between whole-body exposure and intraperitoneal cigarette smoke extract injection [1]. Inhalational cigarette smoke exposure may be the most relevant to real-life situations.

• A hypothesis can be included in this section.
Reply 2: Thanks for your comments. As mentioned in our discussion section, we found that it was observed that some mice might hide in the corner of the chamber to escape from the toxic smoke when we applied the CSS exposure method. Also, it is difficult to measure the amount of toxic gas inhaled by each mouse. Both above reasons may cause uncertain doses of cigarette smoke induction. Therefore, we chose CSS IP to ensure every mouse got the same concentration of CSS.
Thanks for your comments. We will add a hypothesis in this section.
Changes in the text: we added reference to the discussion section (see Page 17, line 18). We also added a hypothesis at the end of the introduction section (see Page 6, lines 1-3)

Comment 3: Methods and materials
• Are there any functional or imaging tests available to confirm the development of COPD? The histological section revealed increased MLI, indicating the occurrence of emphysema, but additional tests may be required to diagnose COPD.
Reply 3: Thanks for your comments. Like our reply 1, the terms COPD in this study will be changed to COPD-like injury. Although some tests were missed in this study, such as lung function analysis, we found inflammatory levels and significant lung damage that consist with COPD still shows that our method could possibly develop COPD.
Changes in the text: No

Comment 4: Result
• How do the authors calculate the inflammation score in Table 1?
Reply 4: Thanks for your comments. According to section 2.6, Lung sections were stained with H&E and examined microscopically by the veterinary pathologist to score lesions. The calculated method was followed by Shackelford et al. (2002). The lesion was scored as follows: 0, normal; 1, minimal, <1%; 2, slight, 1-25%; 3, moderate, 26-50%; 4, moderately severe, 51-75%; 5, severe/high, 76-100%.
Changes in the text: No

Comment 5: Discussion
• In this section, the limitations and conclusion can be included.
Reply 5: Thanks for your comments. limitations and the conclusion have been included in the discussion section.
Changes in the text: we have modified our text as advised (see Page 18, lines 11-14 and Page 21, line 11-16)

Reference:
[1] Z.-H. He, P. Chen, Y. Chen, S.-D. He, J.-R. Ye, H.-L. Zhang, J. Cao, Comparison between cigarette smoke-induced emphysema and cigarette smoke extract-induced emphysema, Tobacco Induced Diseases 13(1) (2015).

Second Round Peer Review

This is an interesting study that investigated the impact of Hydrogen-Oxygen treatment for prevention of COPD in an animal model.

There are some important corrections needed before publication:

Comment 1: Considering that the whole article is based of the prevention of COPD, the authors should add more details and previous articles that used CSS administered by intraperitoneal injection. What are the measurements used in those study that proved the COPD model? How those measurements compared to the ones in the COPD group in your article? Was the weight gain similar? How about the Neutrophil Elastase Activity? MLI? You can compare all those variables to show that your model is valid.
Reply 2: There were two studies Liu et al. (2017) (10) and Lu et al. (2018) (11) have found positive effects of inhaling hydrogen gas in cigarette smoke-induced animal models. Also, He et al. (2015) (20) suggested that both CS exposure and CSS IP could have the same effectiveness and similar values of emphysema in the model animals, which both create COPD-like symptoms. Most of the measurement, like change of MLI of the lung & total histopathological lesion scores, in our studies showed similar trend in the control and COPD-like groups as in the previous studies. However, in our study, H2 didn't prevent the weight reduction of the COPD animal model. This might imply the toxicity of cigarettes on the weight changes independent of H2 treatment in the lung. NE 680 Fast is a novel technology used in an animal model to detect neutrophil-mediated inflammation, which is also one characteristic of COPD pathology. We ensured the inflammation changes among groups via using NE 680 Fast for confirmation of neutrophil proliferation as a COPD-like marker before sacrificing the mice. We also can see the lung section result to confirm the COPD-like change.
Change in the text: we have modified our text as advised (see Page 6, lines 1-6).

Comment 2: It is important to properly analyze the survival rate. There are statistical tools (Kaplan-Meier curves and log-rank tests) that can be used to compare the survival rate between groups. Only saying that the survival rate in one group was 80% and in the other was 100% is not enough.
Reply 2: Thanks for your comment. The data set in death is too small to run either the Kaplan-Meyer or log-rank tests. The trend, however, is intriguing. No animals died in the control or COPD + H2/O2 groups while 2 of 10 animals (20%) died in the COPD alone group. As this was a preliminary experiment, it may be in our next clinical study to perform a larger scale experiment to obtain robust survival statistics. We have revised the text to refer to this figure as preliminary data.
Change in the text: we have modified our text as advised (see Page 10, line 3 and Page 18, line 12)

Comment 3: In the results sections, many variables are described as "significantly decreased", "significantly higher". Please describe the exact value for each one of them. The same applies for the p value, especially the ones that are < 0.05.
Reply 3: Thanks for your comment. We have modified our text as advised.
Change in the text: Page 13, lines 7, 8, 11 and Page 15, line 8.

Comment 4: In figure 6, there is an arrow but no description in the legend.
Reply 4: Thanks for your comment. We have modified our text as advised.
Change in the text: Page 14, lines 3, 4 and Page 16, line 3.

Comment 5: Double check the terms used in the text (e.g., you use Oxy-Hydrogen and Hydrogen-Oxygen).
Reply 5: Thanks for your comment. We have double checked the terms and made sure that we used Hydrogen-Oxygen or H2/O2 throughout the manuscript regarding this new therapy/treatment. Only the generator used in this study has been called ”Oxy-Hydrogen generator”, which will not be changed as it’s a specific name for the device.
Change in the text: No