Original Article

The effects of hydrogen treatment in a cigarette smoke solution-induced chronic obstructive pulmonary disease-like changes in an animal model

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Background: Molecular hydrogen, with its antioxidant and anti-inflammatory properties, may be suitable for the prevention and treatment of chronic obstructive pulmonary disease (COPD). This study aims to investigate the therapeutic efficacy of hydrogen-oxygen (H_2/O_2) treatment in cigarette smoke solution (CSS)-induced COPD-like injury in a female BALB/c mouse model.

Methods: Thirty mice were randomly assigned to three groups: Control (n=8), COPD (n=10), and COPD + H_2/O_2 (n=12). CSS was administered by intraperitoneal (IP) injection twice weekly for 6 weeks during the COPD induction phase. Simultaneously, the COPD + H_2/O_2 group started received 75 minutes of inhalation therapy (42% H_2) delivered by the Oxy-Hydrogen Generator twice daily for 9 weeks. Mice body weights and survival were measured throughout the study period. Neutrophil elastase (NE) activity and lung histopathological changes were also evaluated.

Results: The results showed a higher survival rate in the COPD + H_2/O_2 group compared to the COPD group (100% *vs.* 80%) during the induction phase. Slight decreases in body weight gains were observed in the COPD and COPD + H_2/O_2 groups during the first 15 days of the induction phase, but there was no significant difference in mean body weights among the three groups throughout the study period. NE activity was numerically lower in the COPD + H_2/O_2 group compared to the COPD group. The histopathological evaluation showed significant improvements in the H_2/O_2-treated mice with respect to mean linear intercept (MLI) and lesion (inflammation and emphysema) scores. Improvements in goblet cell hypertrophy and hyperplasia of airway epithelium were not significant.

Conclusions: A 9-week H_2/O_2 inhalation therapy delivered by the Oxy-Hydrogen Generator to CSS-induced COPD-like injury in mice showed improvement in survival rate, alveolar structural changes, and histopathological lesion scores of the lung.

Keywords: Hydrogen gas; chronic obstructive pulmonary disease (COPD); inflammation; oxidative stress

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Introduction

Chronic obstructive pulmonary disease (COPD) is a common inflammatory disease with high morbidity and mortality. It is the third most common cause of death globally, as estimated by World Health Organization (WHO) in 2019 (1). It was reported that 10% of adults over 40 suffered from this disease (2). COPD caused by harmful particles and gases, especially tobacco smoke, is characterized by chronically abnormal respiratory responses, such as airway inflammation and emphysema (3). The Global Initiative for Chronic Obstructive Lung Disease (GOLD) science committee suggests that patients with COPD exacerbations should use systemic steroids, antibiotics, oxygen (O_2) supplementation, and other protective lung strategies (4). Currently, these treatments are intended to relieve the symptoms or prevent the exacerbation of the disease. None of these interventions reverses the damage to the lung. Therefore, there is still a need for novel therapeutics to prevent and treat COPD.

A recent review of the literature indicated that hydrogen (H_2) could be a valuable treatment for many diseases (5). H_2 can serve as a scavenger of free radicals such as hydroxyl radicals (OH) and thus exert anti-oxidative (6) and anti-inflammatory (7) effects. Moreover, the signaling-regulating (8) and anti-apoptotic (9) effects of H_2 play a significant role in a wider range of diseases. Hydrogen has been demonstrated to treat COPD, cerebral ischemia, metabolic syndrome, arthritis and side effects of chemotherapy. Many pre-clinical studies have found that inhalation of H_2 may have the potential to be a therapy for COPD. Liu *et al.* in 2017 (10) and Lu *et al.* in 2018 (11) have found positive effects of inhaling hydrogen gas in cigarette smoke (CS)-induced animal models. This paper examines the effects of hydrogen gas inhalation, administered by using the advanced technology of Oxy-Hydrogen Generator, on COPD-like injury in a mouse model. To ensure that the mice received the same level of induction, this study applied cigarette smoke solution (CSS) by intraperitoneal (IP) injection. Zhang *et al.* in 2013 (12) have demonstrated the IP injection of CS extract induced emphysema, and He *et al.* in 2015 (13) have suggested that both CS exposure and CSS IP injection modes could have caused similar levels of emphysema. It is hypothesized that inhaled hydrogen gas may reduce the mortality rate, weight loss and other lung damage associated with CSS-induced COPD-like injury in this mouse model. In addition, the hydrogen treatment was started on the first day of the experiment to demonstrate the protective effect of molecular hydrogen in COPD-like injury. We present the following article in accordance with the ARRIVE reporting checklist (available at https://jtd.amegroups.com/article/view/10.21037/jtd-22-324/rc).

Methods

Animals

Thirty female BALB/c mice (6 weeks old) were purchased from BioLasco Taiwan Co., Ltd. And quarantined for one week. Animals were group-housed (4–5 mice in one cage) in the Taiwan Mouse Clinic, at a constant temperature (18–20 ℃) and humidity (30–70%) with 12 h/12 h light/dark cycles. Animals had free access to rodent pellet food (PicoLab® Rodent Diet 20) and drank water ad libitum. All animal study protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Development Center for Biotechnology (IACUC-2020-R501-043), in compliance with Guideline for the Care and Use of Laboratory Animals, Council of Agriculture, Executive Yuan, Taipei.

Preparation of CSS

CSS was prepared, with modifications, from a previously described protocol (12). Cigarettes (brand: New Paradise; tar: 10 mg; nicotine: 0.8 mg) were burned, and the smoke was collected via peristaltic pump (35 mL/min). The smoke was filtered to remove particles and delivered into a vessel with phosphate-buffered saline (10 mL). The average nicotine concentration in the CSS was 114 μg/mL as measured by high-performance liquid chromatography (HPLC).

Oxy-Hydrogen Generator

The Oxy-Hydrogen Generator (HOHO Biotech Co., LTD., Taipei) produces hydrogen and oxygen gases from reverse osmosis of water. The output gas was 42% H_2 and 21% O_2 and delivered with 43% air through a Y-shaped tube at a flow of 0.9 L/min to the group of mice housed in an inhalation box. Mice body weights were measured twice weekly.

Treatment regimen of CSS in mice

Figure 1 shows the study flow. Mice were randomly divided

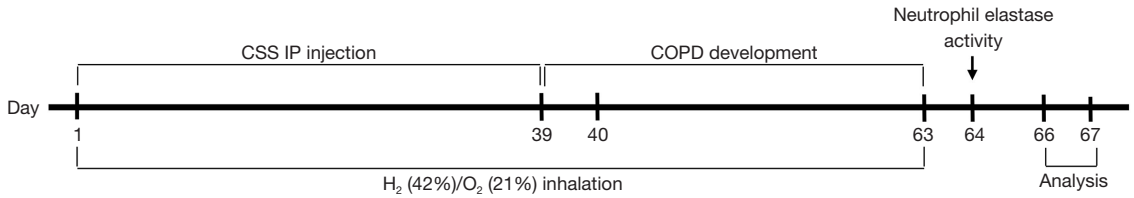

Figure 1 The protocol for the hydrogen gas inhalation experiment in the CSS-induced COPD-injury mouse model. NE activity in each group was pre-analyzed by using fluorescence X-ray before sacrifice. CSS, cigarette smoke solution; IP injection, intraperitoneal injection; COPD, chronic obstructive pulmonary disease. H_2/O_2, hydrogen-oxygen; NE, neutrophil elastase.

into three groups: Control group (n=8), COPD group (n=10) and COPD + H_2/O_2 group (n=12). The average body weight of the mice was 21.6±0.3 grams. Mice were treated with 0.48 mL of double-distilled water (Control group) or CSS (COPD induction groups) twice weekly via IP injection for 39 days (*Figure 1*). The COPD + H_2/O_2 group was also placed into an inhalation box connected to the Oxy-Hydrogen Generator. The H_2/O_2 dosing regime was 75 minutes, twice daily, with four hours in between, for 63 days.

Neutrophil elastase (NE) activity

Mouse thorax fur was removed prior to fluorescence imaging analyses. NE 680 FAST (NEV11169, PerkinElmer) was used as an imaging agent and administered (100 µL) via tail vein injection. Mice were anesthetized with an inhalation anesthetic (isoflurane) and placed under the *in vivo* X-treme imaging system to detect fluorescent signals. Image analyses were performed with molecular imaging software (Bruker). The region of interest (ROI) was drawn in the thorax, and the fluorescence intensity within the ROI was calculated.

Lung histological evaluations

Four mice in the control group, 4 in the COPD, and 6 in the COPD + H_2/O_2 group with higher fluorescence intensity levels from the X-treme imaging system were sacrificed. The lungs were preserved in 10% neutral buffered formalin (NBF), trimmed, embedded in paraffin, sectioned, stained with hematoxylin and eosin (H&E) and Periodic Acid-Schiff (PAS) stains, and examined microscopically with an optical microscope (Leica DM2700M, USA) by a veterinary pathologist. (BioLASCO Taiwan Co., Ltd., Pathology & Toxicology Laboratory).

Lung sections were stained with H&E to score lesions and with PAS to determine goblet cell density. According to Shackelford *et al.* in 2002 (14), 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%. Mean linear intercept (MLI), assessed by ImageJ software, was calculated by the total length of alveoli divided by the number of alveoli per field under light microscopy. This value gives a measure of the enlargement and destruction of alveoli.

Statistical analysis

All data were presented as mean ± standard deviation (SD). Statistical comparison among groups was carried out by two-way analysis of variance (ANOVA) (SPSS, Ver. 22.0) and Bonferroni's test. The histopathologic scores were analyzed by Wilcoxon ranked sum test. Statistical significance was set at $P<0.05$.

Results

The effect of H_2/O_2 inhalation on survival rates and body weight

The experiment lasted 67 days: 39 days for CSS induction, 24 days for the development of COPD-like pathological changes, and a final 4 days for analyses (*Figure 1*). During the CSS-induction period, the COPD group had two deaths that occurred on the 8th and 15th days, respectively, with an 80% survival rate (*Figure 2*). The COPD + H_2/O_2 group had a 100% survival rate. This preliminary result suggests that H_2/O_2 inhalation may reduce the risk of death from COPD-like injury in this model.

Hydrogen inhalation did not prevent the decrease in body weight caused by CSS (*Figure 3*). There was a slight decrease in body weight gain in COPD and COPD + H_2/O_2 groups during the CSS injection period.

The effect of H_2/O_2 inhalation on NE activity

The level of NE680 Fast fluorescence reflects NE enzyme activity, which plays a major role in pulmonary inflammation in COPD. NE activity in the lungs of the CSS-induced COPD-like injury in mice was detected after the end of the treatment (day 64). *Figure 4A* shows representative images of NE activity. *Figure 4B* is a dot plot showing the fluorescence intensity of animals in each group. The mean levels of NE680 Fast fluorescence intensity in control, COPD, and COPD + H_2/O_2 groups were 163, 182.1, and 168 ($\times 10^8$ P/S/mm^2), respectively (*Figure 4B*). There was no statistically significant difference in the mean levels of fluorescence intensity among the three groups.

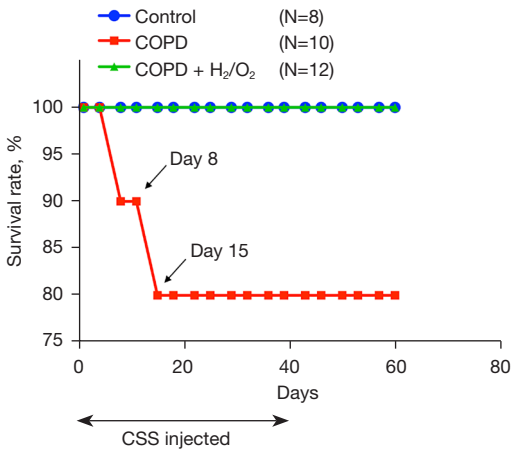

Figure 2 Effect of H_2/O_2 treatment on survival rates. COPD, chronic obstructive pulmonary disease; H_2/O_2, hydrogen-oxygen; CSS, cigarette smoke solution.

H_2/O_2 inhalation reduces CSS-induced inflammation and emphysema

Figure 5A shows histopathologic sections in the three groups. The lung section in the COPD group had alveolar wall damage, consistent with inflammation and emphysema. The MLI of the lung in the COPD + H_2/O_2 group (36.254±7.487) was significantly decreased compared to the COPD group (52.068±16.473) ($P<0.05$, *Figure 5B*).

The total histopathological lesion scores (inflammation and emphysema) were significantly higher in the COPD group compared to the control group ($P<0.05$), and a significantly lower lesion score (3.17±0.98) was noted in the COPD + H_2/O_2 group compared to the COPD group (5.25±0.96) ($P<0.05$) (*Table 1*).

H_2/O_2 inhalation reduces CSS-induced goblet cell hypertrophy and hyperplasia of airway epithelium

The PAS-stained lung sections showed histopathological changes in the COPD-like injury mice, with goblet cell hypertrophy and hyperplasia of airway epithelium (*Figure 6A*). The lesion score was significantly greater in the COPD group (1.75) than in the control group (0.25) ($P<0.01$).

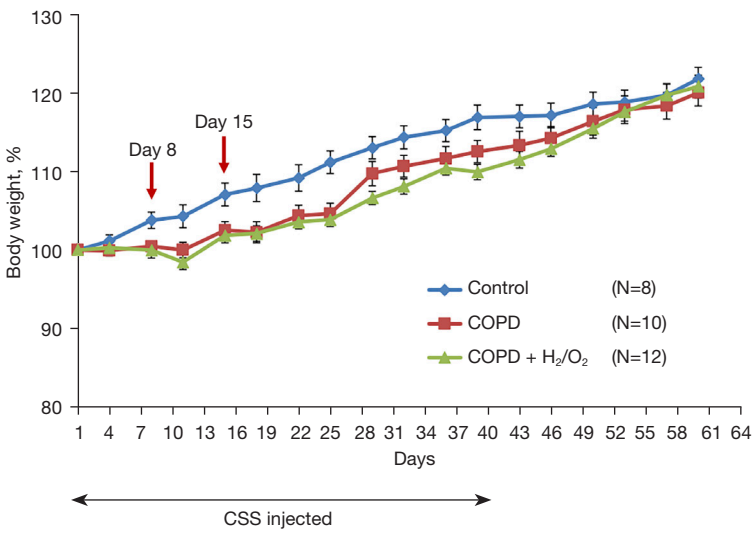

Figure 3 Changes in body weight, expressed as the percentage of initial body weight, during the study period. COPD, chronic obstructive pulmonary disease; H_2/O_2, hydrogen-oxygen; CSS, cigarette smoke solution.

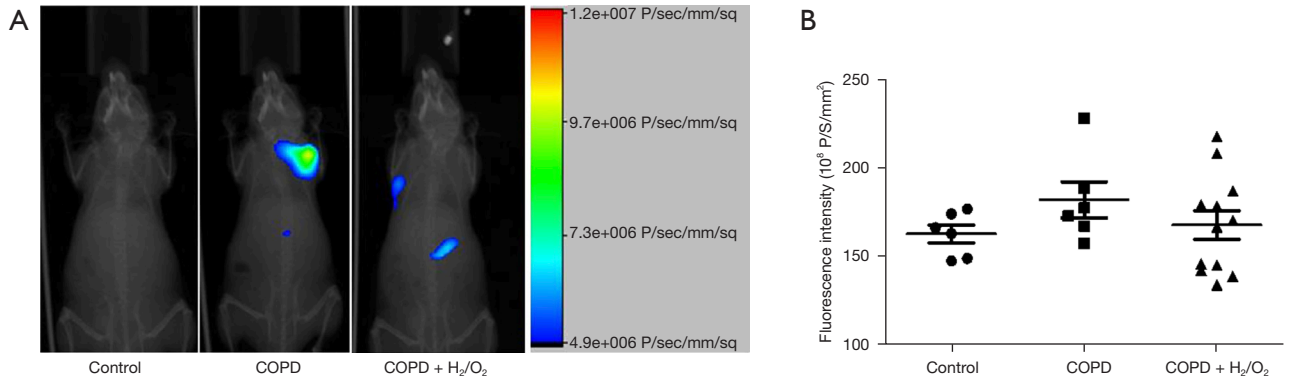

Figure 4 The effects of COPD-like injury and H_2/O_2 treatment on NE activity. The area of pulmonary fluorescence images of NE activity was detected by *in vivo* X-treme imaging system. (A) Representative fluorescence images of NE activity. (B) A dot plot is showing the fluorescence intensity of animals in each group (n=6 for control and COPD group, and n=12 for COPD + H_2/O_2 group). COPD, chronic obstructive pulmonary disease; H_2/O_2, hydrogen-oxygen; NE, neutrophil elastase.

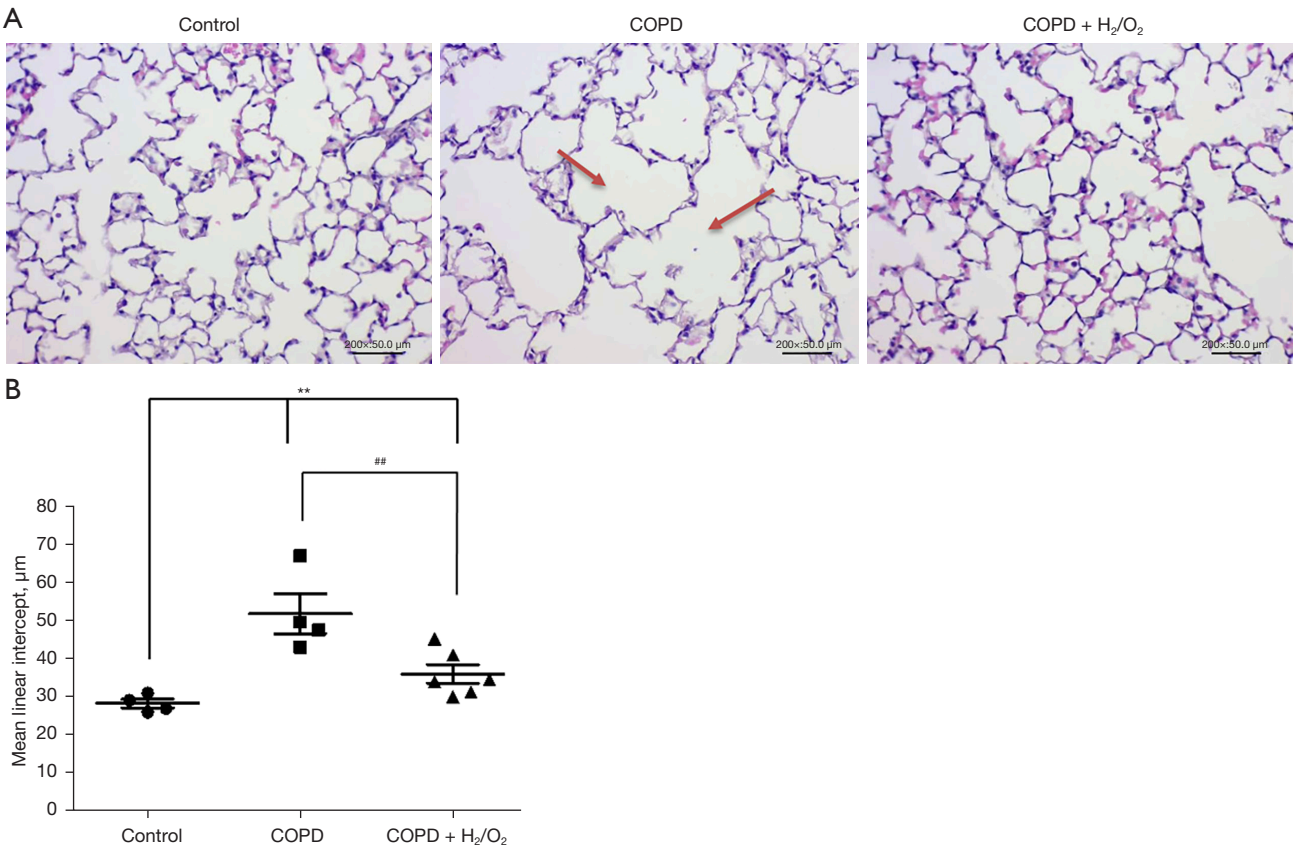

Figure 5 The effect of H_2/O_2 treatment on the histopathologic lung changes in CSS-induced COPD-like injury in mice. (A) Representative microphotographs showing histochemical staining of mouse lung tissue sections (H&E, ×200). The arrows indicate the alveolar wall destruction and airspace enlargement. (B) A dot plot is showing the MLI of animals in each group. **, P<0.05 as compared to the control; ##, P<0.05 as compared to the COPD group. n=4 for control and COPD group, n=6 for COPD + H_2/O_2 group. COPD, chronic obstructive pulmonary disease; H_2/O_2, hydrogen-oxygen; CSS, cigarette smoke solution; H&E, hematoxylin and eosin stain; MLI, mean linear intercept.

Table 1 The effect of H_2/O_2 treatment on the histopathologic scores of CSS-induced COPD-like injury in mice

Measurements	Groups		
	Control (mean ± SD)	COPD (mean ± SD)	COPD + H_2/O_2 (mean ± SD)
Inflammation	1.75±0.50	2.50±0.58	1.67±0.52$^{\#}$
Emphysema	0.75±0.96	2.75±0.50*	1.50±0.55$^{\#\#}$
Total histological score	2.50±1.29	5.25±0.96*	3.17±0.98$^{\#}$

The mean lesion scores of the H&E-stained lung tissues for each group were presented. *, P<0.05 (significant difference between control and other groups); $^{\#}$, P<0.05; $^{\#\#}$, P<0.01 (significant difference between the COPD and the COPD + H_2/O_2 groups). n=4 for control and COPD group, and n=6 for COPD + H_2/O_2 group. H_2/O_2, hydrogen-oxygen; CSS, cigarette smoke solution; COPD, chronic obstructive pulmonary disease; SD, standard deviation; H&E, hematoxylin and eosin stain.

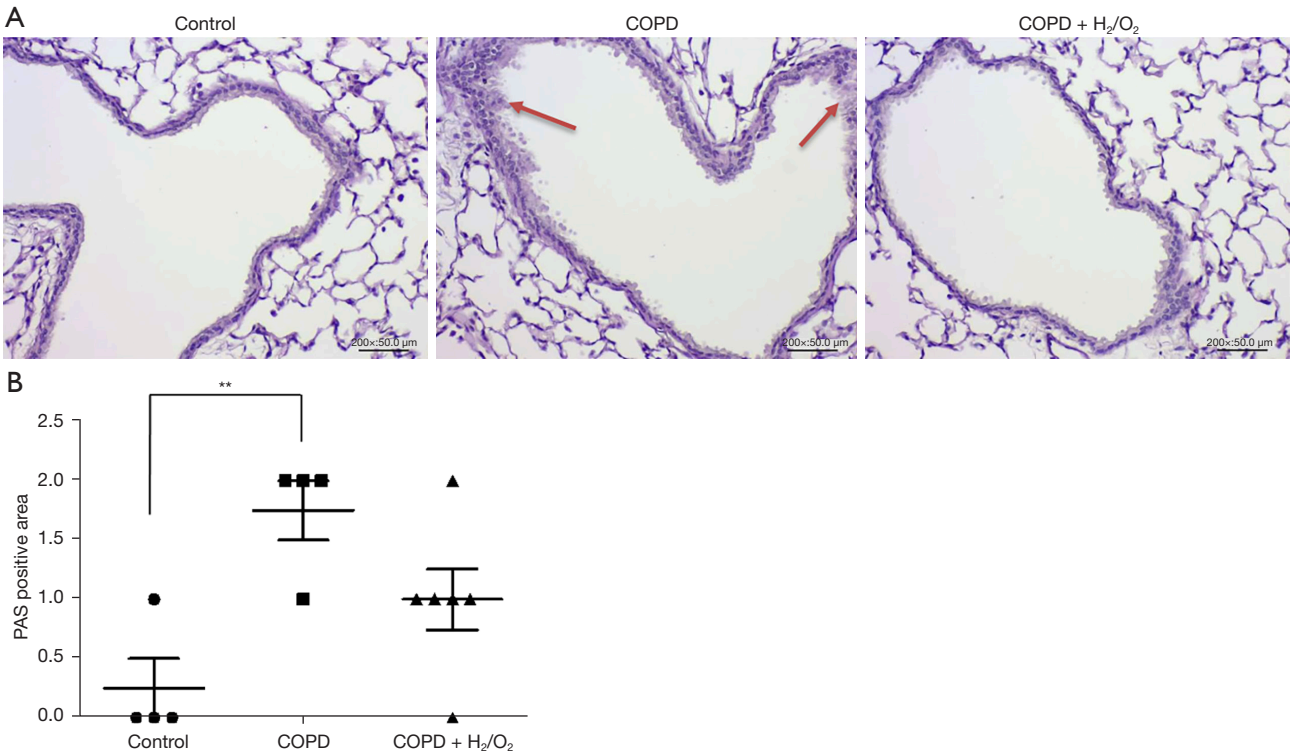

Figure 6 The effect of H_2/O_2 treatment on the histopathological changes in CSS-induced COPD-like injury in mice. (A) Representative microphotographs showing histochemical staining of mouse lung tissue sections (PAS, ×200). The arrows indicate goblet cell hyperplasia. (B) A dot plot is showing the PAS-positive area score of animals in each group. **, P<0.01. n=4 for control and COPD group, n=6 for COPD + H_2/O_2 group. COPD, chronic obstructive pulmonary disease; H_2/O_2, hydrogen-oxygen; PAS, Periodic Acid-Schiff stain; CSS, cigarette smoke solution.

There was no statistically significant difference between the COPD and the COPD + H_2/O_2 groups (*Figure 6B*).

Discussion

COPD is recognized as a preventable, treatable but irreversible respiratory disease mainly triggered by cigarette smoking (15). The yearly increasing mortality rate makes COPD treatment a significant worldwide issue (16). A typical characteristic of COPD is constant airflow limitation developed from obstructive bronchiolitis and emphysema (17). Patients may suffer from dyspnea and

chronic cough, which lead to comorbidities and exacerbated conditions (18). To our knowledge, there is still no therapeutic agent that can completely reverse COPD. Therefore, the searching for a novel preventive treatment to reduce the risk of COPD remains an important and urgent medical need.

Hydrogen has been found to have anti-oxidative and anti-inflammatory effects. Moreover, the safety of H_2 usage in diving has been established since 1994 (19), suggesting that H_2 is likely to be safer and thus become a more widely used therapeutics. Many of the properties of hydrogen support the benefits of medical treatment. For example, H_2 can penetrate bio-membranes and reach cytosol and organelles because of its small molecular weight. Furthermore, the compatibility of H_2 with other tissues is also higher than other oxidant scavengers (20). The potential effectiveness of H_2 inhalation in preventing COPD-like injuries, as demonstrated in this study, adds to the importance of pursuing its therapeutic benefits.

Our results are similar to two studies which analyzed the benefits of inhaling hydrogen on cigarette smoking-induced COPD disease in animal models (10,11). However, two main differences are worth noting. Firstly, the two studies used CS inhaled by animals to induce COPD, while CSS was applied via injection in our study. It has been observed that sometimes mice hide in the corner of the chamber to escape from the toxic smoke when CS is used as an inducer. Moreover, the individual differences in breath frequency and the amount of inhaled gas in each breath might increase the variability of dose administration during CS induction. However, He *et al.* in 2015 (13) suggested that both CS exposure and CSS IP injection could have the same effectiveness and similar values of emphysema. We selected the IP injection application of CSS in order to reduce the variability of the amount of CS delivered during the study. A second difference between our approach and that used in the above-mentioned two studies is the hydrogen generating technology. The hydrogen gas used in this study was provided via the Oxy-Hydrogen Generator containing a proton exchange membrane, the latest technology in generating hydrogen gas. By avoiding direct contact with metals, this equipment can produce pure hydrogen without toxic by-products. Furthermore, hydrogen could be generated at a lower temperature, allowing a safer environment when applied to the experiment.

To evaluate the preventive effect of H_2 on the risk of mortality caused by COPD-like injury, the survival rate of mice was calculated in this study. The 80% survival rate in the COPD group demonstrated the toxicity and lethal effects of CSS on the mice. H_2/O_2 inhalation might attenuate this effect and result in a 100% survival rate. However, in future studies, autopsies, to characterize the cause of death, need to be included to document the contribution of H_2/O_2 therapy in increasing the survival rate. Moreover, the small number of events also makes it challenging to obtain a statistically robust conclusion for the efficacy of the Oxy-Hydrogen Generator on survival benefit.

One recent study demonstrated the weight loss in animal models from cigarette smoking treatment and ameliorating effects of the H_2 inhalation (10). However, in this study, only a slight decrease in weight gain was found in groups when treated with CSS IP injection, and no protective effects of H_2 inhalation were observed. This result is consistent with the finding made by Ardite *et al.* (21), which strongly suggested that the weight gain reduction of the COPD animal model may be due to the toxicity of cigarettes and appears to be independent of the changes and treatment in the lung.

Pulmonary function analysis is a standard tool for evaluating lung-related diseases, including COPD. Taking forced vital capacity (FVC) value for example, COPD patients usually have a lower FVC value than healthy people. Thus, pulmonary function analysis is also applied in animal models to develop novel medicines and devices for lung treatment. However, the different ways of measuring lung functions in human and animal models are of concern. The values measured from humans focus on active respiration values, while only passive respiration values can be detected from animals. This defect can be seen in Lu *et al.* in 2018 (11), where an increase in FVC value was found in the CS-induced mice. This result may be because a forced expiration maneuver was used, causing CS-induced mice with airspace enlargement to have an increase in FVC value. Based on the above consideration, we did not measure pulmonary function in this study.

NE 680 Fast is a novel technology used in an animal model to detect neutrophil-mediated inflammation. It was first used in a mice lung injury experiment in 2011 to image and quantify NE activity (22). COPD is well known to have lung inflammation associated with NE; therefore, it is reasonable to use NE 680 Fast as a pre-analysis before the histopathology study. It is generally believed that healthy animals also have chronic inflammation because of oxidative effects inside the body. In the study, mice in the control group had mean fluorescence intensity of 163 ($\times 10^8$ P/S/mm^2). Although the result in this study

shows that there is no significant difference between the three groups on mean values of the fluorescence intensity, more mice with lower fluorescence intensity than the mean value of control were found in the COPD + H_2/O_2 group, suggesting possible inflammatory protection of H_2 inhalation from CSS-induced COPD-like injury. Interestingly, some mice in the COPD + H_2/O_2 group even had fluorescence intensity lower than mice in the control group. This result proposes a possibility that H_2/O_2 inhalation may not only reduce the damage from CSS-induced COPD-like injury but also reduce inflammation in a healthy mice body.

Histopathologic changes can be used to determine the seriousness of COPD development. Inflammatory and emphysema, two major lesion scores (23) in COPD development, were analyzed in this study. A well-acceptable mechanism of emphysema is the destruction of lung elastin induced by the imbalance of protease and antiprotease activity (24), leading to permanent hyperextension and loss of elastic recoil of alveolar walls (2,25). Hence, in the histopathological analysis, alveolar wall destruction and airspace enlargement can be seen in emphysema. In this study, the above emphysema features were shown in histopathologic pictures captured under a light microscope in the COPD group. Less alveolar wall damage was found in the COPD + H_2/O_2 group, indicating a preventive effect of H_2 in the development of emphysema-like changes. This result is consistent with the lesion scores reported by veterinary pathologists with significantly higher emphysema scores in the COPD group, whereas lower scores in the COPD + H_2/O_2 group. In addition, the measurement of MLI based on evaluating quantitatively histological changes (26) can objectively reflect the enlargement of alveolar spaces (25,27) associated with the development of emphysema in the lung section. A higher value of MLI indicates more severe emphysema, which can be seen in the COPD group in this study. In contrast, the COPD + H_2/O_2 group had a significant lower MLI, confirming the effectiveness of H_2 in reducing emphysema-like changes.

One symptom of COPD in patients is airflow obstruction caused by mucus hypersecretion (28). On a molecular scale, goblet cell hyperplasia leads to the hypersecretion of the mucus (29). Thus, observing hyperplasia of goblet cells in the lung section is a common way to understand the development of COPD. In this study, a significant increase of goblet cells in the COPD group was found in both visual observation and PAS score. However, the COPD + H_2/O_2 group showed an insignificant decrease in the PAS score compared to that of the COPD group. In a study by Ning *et al.*, hydrogen-rich saline injection significantly attenuated CS inhalation-induced mucus hypersecretion (30). It could be that direct CS exposure to goblet cells triggers more mucus production. And further studies are needed to confirm the optimal route and dosage of molecular hydrogen for mucus hypersecretion.

Conclusions

In conclusion, H_2/O_2 inhalation can potentially reduce the risk of developing CSS-induced COPD-like injury. This potential is particularly strong for the development of emphysema-like changes but less clear for the reduction of mucus secretion. These positive results could be attributable to the anti-inflammatory activity of hydrogen. Given the safety of hydrogen therapy, future clinical trials should be considered to confirm the safety and efficacy of H_2/O_2 in, for example, COPD patients.

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Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://jtd.amegroups.com/article/view/10.21037/jtd-22-324/rc

Data Sharing Statement: Available at https://jtd.amegroups.com/article/view/10.21037/jtd-22-324/dss

Peer Review File: Available at https://jtd.amegroups.com/article/view/10.21037/jtd-22-324/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jtd.amegroups.com/article/view/10.21037/jtd-22-324/coif). HJY, WHT, HMS, KYW, and FLD reports funding from HOHO Biotech Co., Ltd. HJY is the medical advisor of HOHO Biotech. KYW is the founder of HOHO Biotech. WHT is the employee of HOHO Biotech. HMS and FLD are board members of HOHO Biotech. HMS is the person in charge for IPC Intellectual Property Connections, INC. She declares that IPC Intellectual Property Connections, INC. has no relationship with HOHO and does not have any interest in the publication of the present article. The other

authors have no conflicts of interest to disclose.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All animal study protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Development Center for Biotechnology (IACUC-2020-R501-043), in compliance with Guideline for the Care and Use of Laboratory Animals, Council of Agriculture, Executive Yuan, Taipei.

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