The Anti-inflammatory Effects of Hydrogen-Rich Water Acts in a Volume-Dependent Manner on Rats’ Lungs Exposed to Cigarette Smoke

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Abstract
Cigarette smoke can activate various ROS-sensitive signaling pathways in the lungs, triggering airway inflammation and leading to mucus hypersecretion. Meanwhile, hydrogen has shown anti-inflammatory activity in various injury models. This study aims to determine the anti-inflammatory effect of hydrogen-rich water on lungs exposed to cigarette smoke in rats. Male wistar rats were divided into four groups randomly (n=5), namely CI group (given aquades + exposed to free air), CII group (given aquades + exposed to cigarette smoke), HI group (given HRW 5 mL once a day + exposed to cigarette smoke), and HII group (given HRW 5 mL twice a day + exposed to cigarette smoke). HRW/aquades was administered orally 30 minutes before cigarette smoke exposure. Exposure to cigarette smoke lasts about 15-25 minutes with 5 cigarettes/day for 28 days. Subsequently, we examine the levels of lung NF-κB p65 using the ELISA method and perform pulmonary histopathological examination. The results showed that the administration of HRW reduced the levels of NF-κB p65 induced by cigarette smoke exposure, which is significant in the HII group (p<0.01). From the histopathological examination, administration of HRW significantly reduced the degree of lung inflammation caused by cigarette smoke exposure in the HI and HII group (p<0.05).

Keywords: anti-inflammatory, cigarette smoke, Hydrogen-rich water, Nuclear Factor kappa B

Introduction
Smoking can cause disease, disability, and harm almost every organ in the body (Rockville, 2020). The incidence of smoking-related diseases in Indonesia is estimated to be still high. In 2016, about 39.5 % of Indonesia's population aged 15 years and older were active smokers (Holipah et al., 2020). Data from the Global Adults Tobacco Survey (GATS) shows that overall, the prevalence of tobacco smokers has not changed significantly from 2011 to 2021, precisely 34.8% in 2011 and 33.5% in 2021 (WHO, 2022).

One of the main effects of cigarette smoke on the respiratory tract is triggering airway inflammation, leading to the recruitment and activation of inflammatory cells and mucus...
hypersecretion (Park et al., 2017; Yang et al., 2021). More than 5,000 constituents have been identified in cigarette smoke. They are significant sources of free radicals, including reactive oxygen species (ROS) and reactive nitrogen species (RNS) such as hydrogen peroxide (H₂O₂), superoxide, hydroxyl radicals, and nitric oxide (Rom et al., 2013). These reactive substances can enter the bloodstream and cause oxidative stress directly (Alharbi et al., 2021). Then, it stimulates the activation of Nuclear Factor kappa B (NF-κB), a transcription factor involved in inflammation, immune response, cell adhesion, growth signalling, cell proliferation, cell differentiation, and apoptosis (Rom et al., 2013; Alharbi et al., 2021). Nuclear Factor kappa B (NF-κB) activation triggers an increase in pro-inflammatory cytokines, including tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) (Helianti, 2020). Other inflammatory mediators and chemotactic factors activated by cigarette smoke, such as IL-8, MCP-1, LTB4, as well as secretion of proteolytic enzymes (especially MMP-9 and MMP-12) also contribute to lung damage (Silva et al., 2019).

In 2019, Li Ge et al. first provided evidence that hydrogen can selectively reduce hydroxyl radicals and peroxynitrite (Silva et al., 2019). Molecular hydrogen is currently known to have protective effects, including acting as an antioxidant, anti-inflammatory, and antiapoptotic (Barancik et al., 2020). The anti-inflammatory activity was seen in various injury models. Specifically, hydrogen inhibits oxidative stress-induced inflammatory tissue injury through the downregulation of pro-inflammatory and inflammatory factors/cytokines, one of which is NF-κB (Ge et al., 2017).

Hydrogen can be administered in various ways, including inhalation, injection of Hydrogen-rich saline (HRS), drinking Hydrogen-rich water (HRW), bathing with Hydrogen water, or using HRS as eye drops (Yang et al., 2020). Inhaled hydrogen gas is not practical in daily life for continuous consumption even though it can work faster, and administration of hydrogen by injection is inconvenient and has a potential risk of cross-infection (Yang et al., 2020; Tian et al., 2021) Thus, hydrogen water may be helpful due to its portability, ease of administration, and safety (Tian et al., 2021). Such multifaceted beneficial effects of hydrogen water led us to investigate its effectiveness on the fine particle burden in the lungs and blood, and underlying mechanisms. Studies show a link between air pollution and the incidence of respiratory, cardiovascular, neurological, and some cancers. (Choi et al., 2017)

The health benefits of hydrogen are known based on the descriptions above. However, the anti-inflammatory effects of hydrogen in hydrogen-rich water preparations on lungs exposed to cigarette smoke have not been widely studied. In addition, studies comparing the anti-inflammatory effect of hydrogen-rich water based on the total volume consumed daily on rats' lungs exposed to cigarette smoke have not been conducted to the best of our knowledge. Therefore, this study aimed to evaluate the effects of consuming 5 mL of HRW once a day and 5 mL of HRW twice a day on lung NF-κB p65 levels and lung histopathological changes in rats exposed to cigarette smoke. This study is expected to become a reference for people who are interested in biomedical, environmental health, pulmonology specialist and public health practitioners who are concerned with cigarette smoke-related lung inflammatory diseases in providing therapy and education about the benefits of HRW to optimize patient recovery.

**Methods**

**Animal and Experimental Models**

The design of this research is Post-Test Control Group Design. A total of 20 male Wistar rats (Rattus norvegicus) aged ± 3 months weighing 150-250 grams were maintained in the Integrated Laboratory of the Faculty of Veterinary Medicine, Hasanuddin University. First, the rats were acclimatized in the cage for 7 days. Rats were given a standard diet and drinking water ad libitum. The cage environment is also arranged so that it has sufficient air circulation, the room temperature ranges from 28-32°C, and the lighting in the room is set for 12 hours of light and 12 hours
After acclimatization, rats were randomly divided into four groups (n=5 rats), namely Control I (CI) group given 5 mL of aquadest + exposed to free air, Control II (CII) group given 5 mL of aquadest + exposed to cigarette smoke, Hydrogen I (HI) group were given 5 mL of HRW once a day + exposed to cigarette smoke, and Hydrogen II (HII) group were given 5 mL of HRW twice a day + exposed to cigarette smoke. Experimental animal treatment lasted for 28 days.

**HRW Preparation**

HRW was made using Hydro-gen Fontaine PEM & Inhaler (Livewell Global, South Korea) as instructed by the manufacturer. The hydrogen concentration used in this study was 1-1.5 ppm and was measured with a hydrogen meter. HRW was prepared shortly before treatment, and after the hydrogen concentration was measured, HRW was immediately administered to rats to prevent the decrease of the hydrogen concentration any further.

**HRW/Aquadest Administration**

HRW/aquadest was administered orally through a cannula 30 minutes before exposure to cigarette smoke. HRW administration in the HII group was carried out twice a day, with a 30-minute interval between administrations.

**Cigarette Smoke Exposure**

Exposure to cigarette smoke in this study used a chamber (smoking box) made of glass sized 70 cm x 35 cm x 30 cm. The rats were put into the chamber (n=5) then the cigarettes were burned. Cigarette smoke is distributed directly into the chamber box and assisted by a modified smoke pump. Cigarette smoke exposure to rats lasts about 15-25 minutes, with 5 cigarettes/day for 28 days. The cigarettes used were kretek (containing 2.3 mg nicotine and 39 mg tar). Exposure to cigarette smoke in the HII group was conducted 30 minutes after the second administration of HRW.

**Harvested of Lungs**

On the 29th day, all rats were anaesthetized using ketamine, then terminated by cervical dislocation, and a necropsy was performed to harvest the lungs. The right lung was taken to examine the levels of NF-κB p65 as an inflammation marker using ELISA method. In contrast, the left lung was taken for pulmonary histopathological examination.

**Examination of NF-κB p65 Levels**

Lung tissue was washed with ice-cold PBS to remove excess blood. Then approximately 100 mg of lung tissue was added into a 1.5 mL microcentrifuge tube. Add 0.9 mL of PBS solution, and then the tissue was minced until it was homogenous. The homogenate was centrifuged for 1 minute at 13,000 rpm to obtain the supernatant. The supernatant from each sample was then tested with the Rat NF-κB p65 ELISA Kit (BT Lab, China) according to the manufacturer’s instructions. The optical density value was read using a microplate reader with a wavelength of 450 nm.

**Lung Histopathological Examination**

Lung tissue was made into histological preparations with hematoxylin & eosin staining. Then the results were examined by an anatomical pathologist under a microscope. The assessment of lung inflammation degree was based on research by Ning et al. (Ning et al., 2013). It was evaluated using a subjective scale from 0 to 4 by observing inflammatory cell infiltration and obstruction of the bronchiolar lumen by mucus and cell debris. The interpretation of the score of lung inflammation degree was 0=normal; 1=mild inflammation; 2=moderate inflammation; 3=severe inflammation; 4 = very severe inflammation.

**Data analysis**

All data obtained were processed using the SPSS version 24.0 application (IBM Corporation, Armonk, NY, USA). Normality test for the data using the Shapiro-Wilk test. Comparative test for NF-κB p65 levels using One-way ANOVA (Analysis of Variance) test with a significance level of 5% (p<0.05 ), followed by Post Hoc test using LSD test to see the significance of each group. Comparative test for inflammation scores from histopathological examination using the Kruskal Wallis test, followed by the Mann-Whitney U test to see the significance of each group.
Results

The average of NF-κB p65 levels and inflammation scores of each group can be seen in Table 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>NF-κB p65 levels</th>
<th>Inflammation Score</th>
</tr>
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<tbody>
<tr>
<td>CI</td>
<td>1.20 ± 0.23</td>
<td>0.80 ± 0.45</td>
</tr>
<tr>
<td>CII</td>
<td>2.44 ± 0.95</td>
<td>2.60 ± 0.89</td>
</tr>
<tr>
<td>HI</td>
<td>1.74 ± 0.26</td>
<td>1.40 ± 0.55</td>
</tr>
<tr>
<td>HII</td>
<td>1.28 ± 0.36</td>
<td>0.80 ± 0.84</td>
</tr>
</tbody>
</table>

**Lung NF-κB p65 Levels**

![Image of bar chart showing NF-κB p65 levels in different groups.]

*Figure 1. Effect Of HRW on Lung NF-κB p65 Levels in Rats Exposed to Cigarette Smoke*

Data expressed as mean ± SD. Significance was tested by Oneway ANOVA followed by Post Hoc Test (LSD). *(p<0.01) compared with the CI group. *(p<0.01) compared with the CII group.

This study examined each group's lung NF-κB p65 levels as the inflammatory marker. The average levels of NF-κB p65 in the CII, HI, and HII groups exposed to cigarette smoke were higher than the average levels of NF-κB p65 in the CI group that were not exposed to cigarette smoke (Table 1). The highest average levels of NF-κB p65 was shown in the CII group (Aquadest + Cigarette smoke) at 2.44 ± 0.95 ng/mL, and statistically significant compared to the CI group (p<0.01) (Figure 1). There were decreases in the average levels of NF-κB p65 in the treatment group given HRW compared to the CII group, the HI group at 1.74 ± 0.26 ng/mL, followed by the HII group at 1.38 ± 0.43 ng/mL, which was statistically significant (p<0.01) compared with the HII group (Figure 1).
Figure 2. Lung Tissue Histopathology

Figure 3. Effect of HRW on lung histopathology in rats exposed to cigarette smoke. (A) Inflammation score of rats' lungs exposed to cigarette smoke. (B) Representative of H&E staining of lung section. Data expressed as mean ± SD. Significance was tested with Kruskal-Wallis, followed by the Mann-Whitney U test. * (p<0.01) compared with CI group. ## (p<0.05) compared with the CII group.

Table 1 shows that the highest average inflammation score was obtained from the CII group at 2.6 ± 0.89, who received aquadest and exposure to cigarette smoke for 28 days. This value is statistically significant compared to the average inflammation score in the CI group who were exposed to free air (p<0.01) (Figure 2). Figure 2 also shows that the average inflammation score of the HI group given 5 mL of HRW once a day decreased to 1.4 ± 0.55 and significantly differed from the CII group (p<0.05). Similarly, the HII group given 5 mL of HRW twice a day had an even lower score at 0.8 ± 0.84 and was significantly different from the CII group (p<0.05).

Figure 3 represents H&E staining from
histopathological examination on each group. In the CI group, inflammatory cell infiltration and obstruction of the bronchiolar lumen by mucus and cellular debris were still mild, thus getting an inflammation score of 1 (mild inflammation). In the CII group, the lung section shown obtained an inflammation score of 3 (severe inflammation), observed from the prominent infiltration of inflammatory cells and obstruction of the bronchiolar lumen by mucus and quite heavy cellular debris. In the HI group, moderate infiltration of inflammatory cells and obstruction of the bronchiolar lumen by mucus and cellular debris were seen, resulting inflammation score of 2 (moderate inflammation). As for the HII group, it was seen that inflammatory cell infiltration and bronchiolar lumen obstruction were lighter than other groups exposed to cigarette smoke, so this sample obtained an inflammation score of 1 (mild inflammation).

**Discussion**

This study showed that cigarette smoke could increase NF-κB p65 levels. The highest levels of NF-κB p65 were shown in the CII group (Aquadest + Cigarette smoke), and administration of hydrogen-rich water could reduce the increase of NF-κB p65 levels induced by cigarette smoke exposure in a volume-dependent manner. The greater volume of HRW consumed daily, the more significant the decrease in lung NF-κB p65 levels in rats exposed to cigarette smoke.

Cigarette smoke can increase intracellular ROS in lung cells and activate various ROS-sensitive signaling pathways, such as nuclear factor kappa B, thus inducing lung inflammation (Liu et al., 2017). NF-κB p65, one of the five NF-κB family of transcription factors, has been known to play a crucial role in airway inflammation and airway remodelling, including acting as an antioxidant and excess mucus production by the airways through inhibition of pathways involved in oxidative stress (Ning et al., 2013). Those may explain why the inflammatory score from lung histopathology examination in rats exposed to cigarette smoke given HRW in our study was antioxidant properties, molecular hydrogen is currently known to have protective effects, including acting as an anti-inflammatory (Ohsawa et al., 2007; Barancik et al., 2020). Hydrogen can penetrate the biomembrane and effectively reach the nucleus, then selectively neutralize OH and ONOO- from exogenous factors such as cigarette smoke. Therefore, hydrogen inhibits oxidative stress-induced inflammatory tissue injury through the downregulation of pro-inflammatory factors, one of which is NF-κB (Ge et al., 2017), as shown in this study.

Previous studies have also found that hydrogen can decrease or inhibit NF-κB activation in models of pulmonary inflammation. Chen et al. observed increased expression of NF-κB p65 in the lungs after smoke inhalation injury, whereas administration of Hydrogen Saline significantly slowed that condition (Chen et al., 2015). Hydrogen-rich Saline administered intraperitoneally at higher doses (10 mL/kg) decreased phosphorylated NF-κB p65 activation in asthmatic rat models compared with lower doses (5 mL/kg), suggesting that the effect of hydrogen-rich saline on airway inflammation and lung remodelling is dose-dependent (Xiao et al., 2013).

From histopathological examination in this study, cigarette smoke could cause lung inflammation, which was ameliorated significantly by HRW administration. Similar to NF-κB p65 levels, HRW decreased lung inflammation scores in a volume-dependent manner. In addition to inhibiting the synthesis and release of various pro-inflammatory factors, proteins, and chemokines, hydrogen also promotes the phagocytosis of macrophages at the lesion site as well as inhibits the recruitment of neutrophils and M1 macrophages to inflamed lesions (Fu & Zhang, 2022). The study by Ning et al. found that hydrogen protects the epithelium and excess mucus production by the airways through its antioxidant abilities and possibly through inhibition of pathways involved in oxidative stress (Ning et al., 2013).
lower than in rats exposed to cigarette smoke without HRW. 

Prior research by Ning et al., as mentioned above, reported that after four consecutive weeks of repeated exposure to cigarette smoke, the observed pulmonary inflammation scores through inflammatory cell infiltration and obstruction of the bronchiolar lumen by mucus and cellular debris in rats exposed to cigarette smoke were significantly higher than the control group, and these changes are effectively alleviated by intraperitoneal injection of HRS (Ning et al., 2013). A 2017 study showed that the administration of hydrogen-rich water reduced lung damage in cigarette smoke-induced SMP30-KO male mice and reduced the destructive index, oxidative DNA damage, and premature senescence in the lungs (Suzuki et al., 2017). The male mouse with paraquat-induced acute lung injury model showed that the histological features of inflammatory cell infiltration and fibrosis were relatively lower in the HW-treated group than in the non-HW-treated group (Sato et al., 2015).

In line with previous studies about advantageous effects of hydrogen, especially hydrogen-rich water, our study strengthens that HRW has anti-inflammatory effects and may be beneficial as adjuvant therapy in cigarette smoke-related lung inflammatory diseases. To the best of our knowledge, this is the first study that comparing the anti-inflammatory effect of hydrogen-rich water based on the total volume consumed daily on rats' lungs exposed to cigarette smoke. However, we realized that our study had some limitations such as the small sample size and the relatively short intervention period. Therefore, it is necessary to conduct further research on the anti-inflammatory effects of hydrogen-rich water with a larger number of sample size, longer duration of intervention, and more varied volume of administration and concentration of HRW in order to obtain better results.

Conclusion

Giving hydrogen-rich water can provide anti-inflammatory effects in the lungs of rats exposed to cigarette smoke, judging by their ability HRW in reducing lung NF-κB levels and reducing degree. Lung inflammation from histopathological examination. So that the effect of HRW can be a drug in patients with lung disease caused by cigarette smoke.

The anti-inflammatory effect of hydrogen-rich water is related to the total volume of HRW consumed per day. The greater the volume of HRW consumed per day, the greater the anti-inflammatory effect on the lungs of rats exposed to cigarette smoke. From this conclusion the government can seek treatment with HRW measures in every government hospital.

References


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