The Effect Of Hydrogen-Rich Water Administration On Serum Glutathione (Gsh) Levels And Lung Histopathology In Male Wistar Rats Exposed To Cigarette Smoke

Zulaichah[1], Ermi Girsang[2], Sri Wahyuni Nasution[3]

[1,2,3,]Faculty of Medicine, Prima Indonesia University, Medan, Indonesia

**E-mail: Zulaichahamir@gmail.com*

ABSTRACT

Environmental factors such as exposure to cigarette smoke can result in decreased antioxidant concentrations, one of which is: glutathione(GSH). Water with a higher pH and hydrogen ion content than regular water is known as hydrogen-rich water (HRW). Also one of the functions of hydrogen ions is as an antioxidant. This study was conducted to see how the effect of hydrogen-rich water on serum GSH levels and histopathological changes in the lungs of male Wistar rats exposed to cigarette smoke for 28 days. This study used 20 rats in 4 groups, control group 1.2 and treatment group 1.2. In this study, hydrogen-rich water (HRW) was given and serum GSH levels were examined using the ELISA method. Then observations were made after the experimental animals were exposed to cigarette smoke, which was then examined for lung histopathology in rats. The results showed a significant difference in serum GSH levels in the four groups of rats with a p-value <0.001 supported by a large effect size (ES = 0.935) so it can be concluded that HRW intervention provides a large antioxidant effect through serum GSH levels in rats and lung histopathology results also showed no significant difference in the degree of inflammation K1, K2, KP1, and KP2 with a p-value = 0.599 and a small effect size (ES = 0.418)

Keywords: *Hydrogen-rich water, Glutathione, Cigarette Smoke, Lungs*

INTRODUCTION

Smoking causes disorders in the body, especially in the respiratory system such as Chronic Obstructive Pulmonary Disease (COPD). *Glutathione* is an antioxidant found in almost every cell in the body. Water with a higher pH and hydrogen ion content than ordinary water is known as hydrogen-rich water (HRW). Hydrogen in HRW ranges from

0.55 to 0.65 mM. HRW is used because it is inexpensive, readily available, free of dangerous compounds, and possesses anticancer and Alzheimer's disease preventive qualities. The results of GSH measurements in the positive group showed a significant decrease in GSH levels, and it can prove that exposure to cigarette smoke for 14 days can increase free radicals, thereby reducing GSH levels. The antioxidant effect of hydrogen in hydrogen-rich water preparations observed from GSH levels and lung histopathology of rats exposed to cigarette smoke has not been widely studied, so researchers are interested in researching the effect of hydrogen-rich water on serum GSH levels and histopathological changes in the lungs of male Wistar rats exposed to cigarette smoke for 28 days.

LITERATURE REVIEW

Smoke from cigarettes contains three extremely risky chemical compounds, such as nicotine, tar, and carbon monoxide. Nicotine is the largest component in cigarette smoke and is an additive, tar is a mixture of several hydrocarbon substances, and carbon monoxide is a toxic gas that has a strong affinity for hemoglobin levels in red blood cells, therefore it can form carboxyhemoglobin. In addition to these three dangerous compounds, cigarette smoke contains other compounds such as ammonia, pyridine, carbon dioxide, aldehydes, ketones, nickel, cadmium, zinc, and nitrogen oxides. In different amounts, all of these compounds can interfere with the mucous membranes in the mouth and respiratory tract (Hussain, 2016). Tobacco smoke consists of a gas phase and a particulate phase, and the latter contains nicotine and heavy metals such as cadmium (Cd) and lead (Pb). Depending on indoor ventilation, nicotine concentrations vary from 2 to 10 mg/m3 (Leung, 2013).

Oxidative stress refers to a state in which excessive reactive oxygen species (ROS) exceed the biological antioxidant capacity, leading to disruption of ROS homeostasis and cell damage. (Sies, 2015). Cells need to maintain moderate levels of ROS to perform normal physiological functions (Schieber, M. & Chandel, 2014). Excessive ROS levels are responsible for oxidative damage to DNA and lipids, which can lead

to cell death. Similarly, oxidative stress can trigger inflammatory responses. (Hussain, 2016)which can further increase oxidative stress

Almost all of the body's cells contain glutathione, an antioxidant. In addition to acting as a hydrogen donor in the detoxification of hydrogen peroxide, this molecule aids in the detoxification of pharmaceuticals and xenobiotics (Pizzorno, 2014). As a protein naturally produced by the body, GSH has three important roles as protection in the body, it is called an antioxidant, immune system booster, and detoxification (Aurelia, 2019).

Hydrogen-rich water (HRW) is a type of water that has a higher pH and hydrogen ion content than ordinary water. One of the functions of hydrogen ions is as an antioxidant(Shirahata, 2012). HRW contains hydrogen of 0.55-0.65 mM(Nakao, 2010).

The most common rat strains used for research are the Wistar and Sprague Dawley. The Sprague Dawley white rat (Rattus norvegicus) was bred from the Wistar white rat, with the characteristics of the Wistar strain being a long body shape, smaller head, thick and short ears with fine hair, red eyes, and a tail no longer than its body. The body weight of male rats at 12 weeks of age reaches up to 240 grams, while female rats reach up to 200 grams. These rats survive for around 4-5 years (Sri Luliana, 2017).

METHODS

This study was conducted as an experimental laboratory study with a Post-Test Control Group Design. This study will observe the antioxidant effects of hydrogen-rich water by assessing GSH levels and lung histopathology of Wistar rats exposed to cigarette smoke, where the results of this study will be compared with the control group.

Wistar rats will be divided into four groups, as follows: K1: Control (air + distilled water), namely Wistar rats exposed to ordinary air and given distilled water (control) with a volume of 5 mL/head, once a day gradually for 28 days. Rats are given standard feed and drinking water ad libitum. K2: Control (cigarette smoke + distilled water), namely rats exposed to cigarette smoke as much as 5 cigarettes/day for 28 days. Rats are given distilled water (control) with a volume of 5 mL/head once a day and standard feed and drinking water ad libitum. Group KP1: Treatment (cigarette

smoke + HRW once a day), where rats were given exposure to cigarette smoke 5 cigarettes/day and supplemented with hydrogen-rich water with a volume of 5 mL/head, once a day for 28 days. Mice were given standard feed and drinking water ad libitum outside the HRW administration time. KP2: Treatment (cigarette smoke + HRW twice daily), where rats were exposed to cigarette smoke at 5 cigarettes/day and supplemented with hydrogen-rich water at a volume of 5 ml/head twice daily for 28 days. Mice were given standard feed and drinking water ad libitum outside the HRW administration time.

This research was conducted from September 2022 to October 2022 at the Integrated Laboratory of the Faculty of Veterinary Medicine, Hasanuddin University, Makassar. PGSH level examination using the ELISA method at the HUMRC Laboratory research unit / Hasanuddin University Teaching Hospital, Makassar, and histopathological examination of lung tissue was carried out at the Anatomical Pathology Laboratory, Hasanuddin University Teaching Hospital, Makassar.

Ethical Clearance was submitted to the Health Research Ethics Commission, Prima Indonesia University. The hydrogen concentration used in this study was 1-1.5 ppm (1000-1500 ng/mL). Group K2, KP1, and KP2 will be exposed to cigarette smoke as much as 5 cigarettes/day with an exposure duration of 30 minutes for 28 days. The cigarettes used are kretek types containing 2.3 mg nicotine and 39 mg tar. Exposure to cigarette smoke given to experimental animals in this study was carried out using a room (Smoking Box). Examination of GSH levels was measured by the ELISA method using the GSH (UNEB0099) ELISA kit with the following procedures: adding samples as much as possible avoiding touching the inner walls of the foamy chamber, detection reagent A with Incubation for 1 hour at 37°C, washing by filling each well with wash buffer (about 400μL) and leaving it for 1-2 minutes. Detection Reagent B was incubated for 45 minutes at 37°C and washed, adding 90μL of the substrate solution, then measuring the OD of the microplate reader set to 450 nm. After all is done, it is then stored until it expires. Termination of experimental animals was carried out using intramuscular ketamine injection at a dose of 10-30 mg/kg BW. Histological preparations were stained with hematoxylin and eosin and examined under a microscope at 100x and 400x magnification. The degree of lung inflammation was evaluated using a subjective scale ranging from 0 to 4 by observing the infiltration of inflammatory cells and obstruction of the bronchial

lumen by mucus and cell debris. The interpretation of the lung inflammation score was as follows, namely 0: normal, 1: mild inflammation, 2: moderate inflammation, 3: severe inflammation, and 4: very severe inflammation.

Data analysis using univariate analysis includes descriptive GSH levels, homogeneity test, normality test with Shapiro Wilk with data results that are normally distributed if ($p>0.05$), testing the degree of inflammation was analyzed using the Kruskall Wallis test followed by the Mann-Whitney test and performing a one-way ANOVA test to see the effect size.

RESULTS

This study involved four groups of mice with the following codes: 1) Group 1 (K1) were mice given distilled water (control) and not exposed to cigarette smoke; 2) Group 2 (K2) were mice given distilled water and exposed to cigarette smoke for 28 days; 3) Treatment group 1 (KP1) were mice given HRW 5 mL/day and exposed to cigarette smoke; 4) Treatment group 2 (KP2) were mice given HRW 5 mL twice a day and exposed to cigarette smoke. Each group consisted of 5 mice so the total sample was 20 mice.

Reporting Research Results

Antioxidant effect of hydrogen-rich water through examination of serum glutathione (GSH) levels in mice exposed to cigarette smoke for 28 days

Table 1. Description of serum GSH levels in mice (n=20)

Serum GSH levels (mmol/L)	Group			
	K1	K2	KP1	KP2
Mean	206.2	212.6	4130.4	2191.0
Standard deviation (SD)	69.0	126.1	683.1	658.4
Minimum	125.7	83.7	1584.1	1361.8
Maximum	315.6	407.7	1313.0	3173.9
Normality	0.552	0.673	0.493	0.772
Homogeneity	0.011			

Shapiro Wilk test; Buji Levene's test (based on mean)

Table 1 shows the average serum GSH levels in mice in each group. K1, the group of rats not exposed to cigarette smoke had an average GSH level of 206.2 mmol/L with the lowest level of 125.7 mmol/L and the highest level of 315.6 mmol/L. In mice exposed to cigarette smoke for 28 days and given distilled water (K2), the average GSH level was 212.6 mmol/L with the lowest level of 83.7 mmol/L and the highest of 407.7 mmol/L. In mice exposed to cigarette smoke for 28 days and given HRW 5 mL/day (KP1), the average GSH level was 4130.4 mmol/L with the lowest level of 1313.0 mmol/L and the highest of 1584.1 mmol/L. In mice exposed to cigarette smoke for 28 days and given 5 mL HRW twice a day (KP2), the average GSH level was 2191.0 mmol/L with the lowest level being 1361.8 mmol/L and the highest being 3173.9 mmol/L.

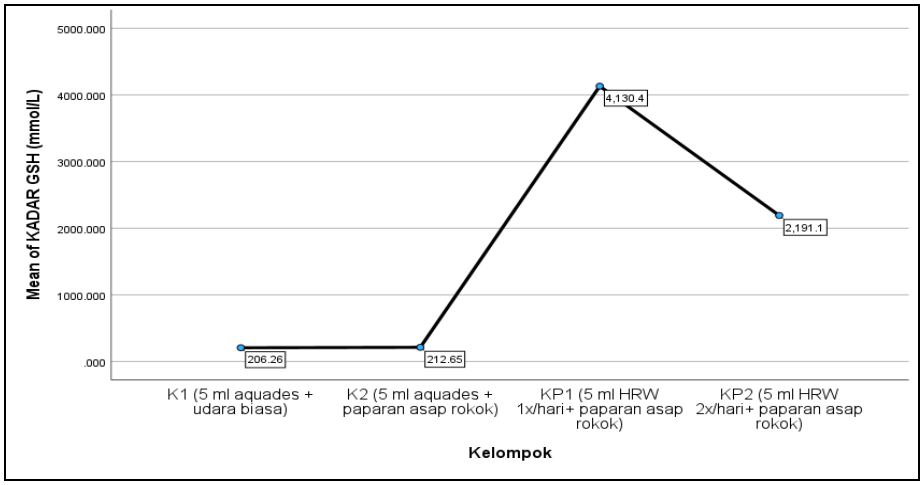

Figure 1. Graph of average GSH levels for each group of mice.

The image above shows that the highest average serum GSH levels were found in mice in treatment group 1 (given 5 mL/day), followed by mice in treatment group 2 (given HRW 5 mL twice a day), then mice in group 2 (given distilled water) and the lowest in mice in group 1 (which were not exposed to cigarette smoke).

The results of the one-way ANOVA test showed a significant difference in serum GSH levels in the four groups of mice with a p-value <0.001 supported by a large

effect size (ES = 0.935). To observe the differences between groups, it was continued the Bonferroni post hoc test because all groups had different baseline data, the test results are presented in the following figure:

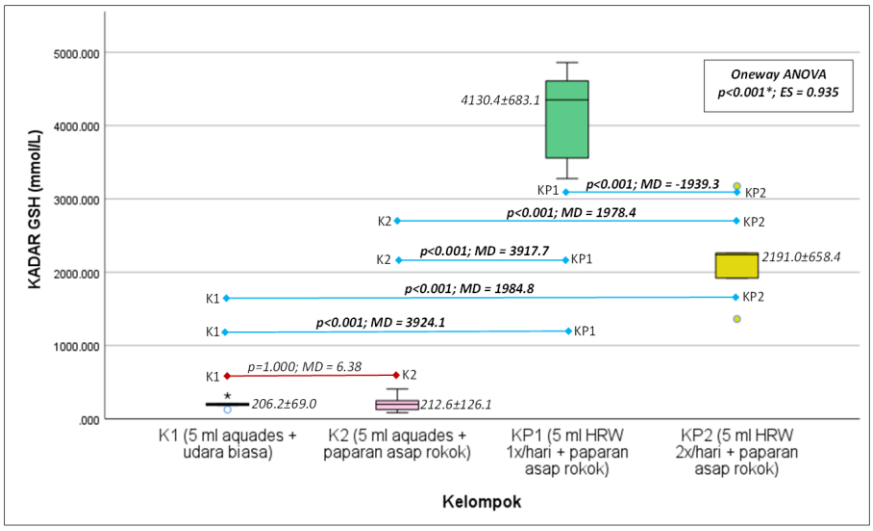

Figure 2 Oneway Anova test results

The image from serum GSH levels in the four groups of mice with a p-value <0.001 supported by large effect size (ES = 0.935). The Bonferroni post hoc test will be used to analyze the differences between groups of animals, as all groups have different baseline data. The figure shows the boxplot of serum GSH levels for each study group. Of the four groups, treatment group 1 (KP1) was seen to have higher serum GSH levels compared to other groups and there was a significant difference between serum GSH levels of KP1 and KP2, K2 and K1 ($p<0.05$).

The antioxidant effect of hydrogen-rich water through histopathological examination of mouse lung tissue by observing alveolar destruction and inflammatory cell infiltration.

Table 2. Frequency distribution of inflammation degree in mice (n=20)

Degree of inflammation	**Group**				**Total**
	K1	K2	KP1	KP2	
Normal	0 (0%)	0 (0%)	0 (0%)	2 (40%)	2 (10%)
Mild inflammation	2 (40%)	2 (40%)	3 (60%)	1 (20%)	8 (40%)

Moderate inflammation	1 (20%)	2 (40%)	1 (20%)	1 (20%)	5 (25%)
Severe inflammation	1 (20%)	1 (20%)	1 (20%)	1 (20%)	4 (20%)
Very severe inflammation	1 (20%)	0 (0%)	0 (0%)	0 (0%)	1 (5%)
Homogeneity	0.682				

Levene's test (based on median)

Table 2 shows the distribution of the degree of inflammation in mice in each group. Of 20 mice, the most experienced mild inflammation (8 mice), and 3 of them came from treatment group 1 (which was given 5 mL/day), while for groups K1 and K2, there were 2 mice each that experienced mild inflammation. In addition, 5 mice experienced moderate inflammation and were dominant in group 2 (given distilled water and exposed to cigarette smoke). There was 1 mouse in each group that showed severe inflammation and the very severe inflammation was only experienced by mice from group 1 (not exposed to cigarette smoke). There were only 2 mice that did not experience inflammation and all of them were in treatment group 2 (exposed to cigarette smoke for 28 days and given 5 mL twice daily). The data variance for inflammation scores also looks homogeneous (relatively the same in all groups). The distribution of inflammation levels is visually depicted in the following diagram:

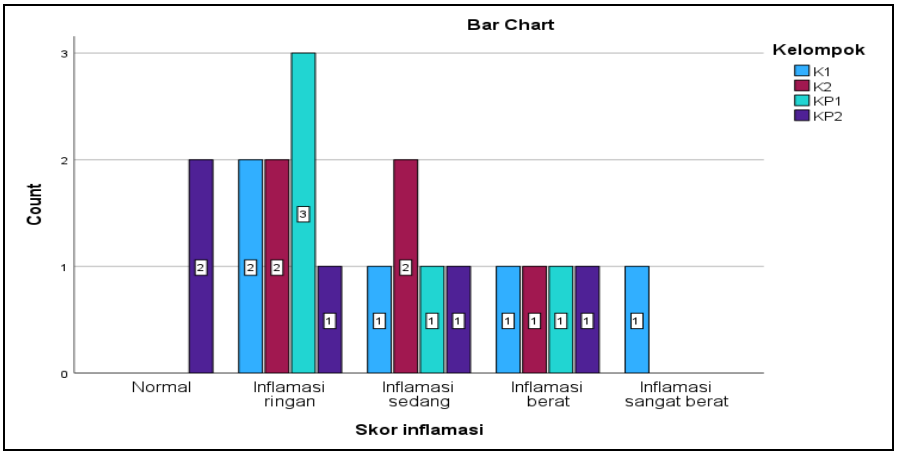

Figure 3. Visual distribution of inflammation degrees

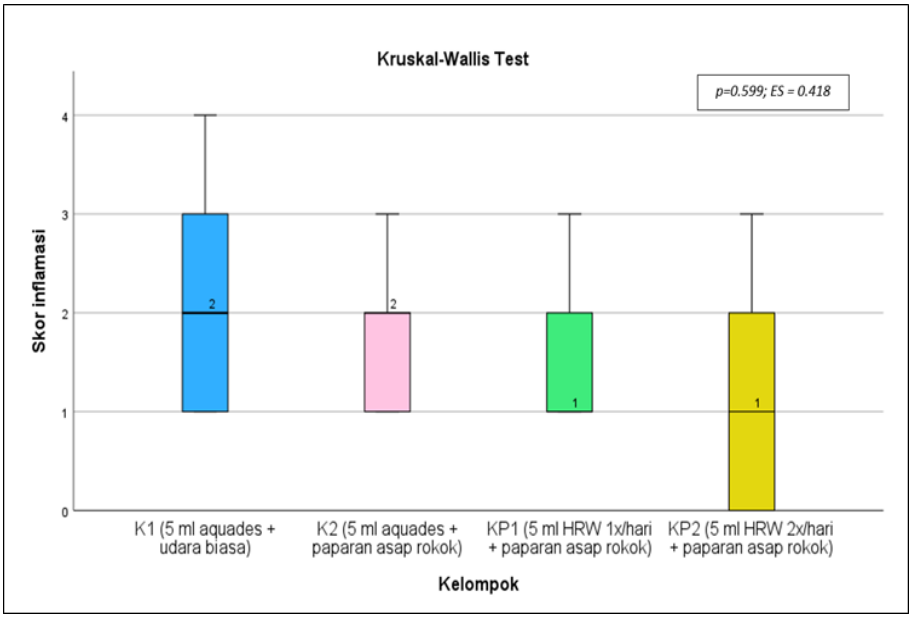

Figure 4. Comparison diagram of the degree of inflammation in each research group from the results of the Kruskal-Wallis test with a p value>0.05 (not significant)

The image above is a comparison diagram of the degree of inflammation in each research group from the results of the Kruskal-Wallis test with a value of p>0.05 (not significant). Data is presented in the form of an average score. Displays the boxplot of the degree of inflammation in each research group. The average score is the same in K1 and K2, namely an inflammation score of 2 (moderate degree of inflammation) while the average score of KP1 and KP2 is 1 (mild degree of inflammation). This is what causes the insignificant difference in the degree of inflammation of K1, K2, KP1, and KP2 with a p-value = 0.599 and a small effect size (ES = 0.418) so that it is interpreted that the intervention given provides a relatively small antioxidant effect through histological examination of lung tissue (degree of inflammation). The Mann-Whitney test was not carried out because there was no statistically significant difference in the degree of inflammation of mice in all groups

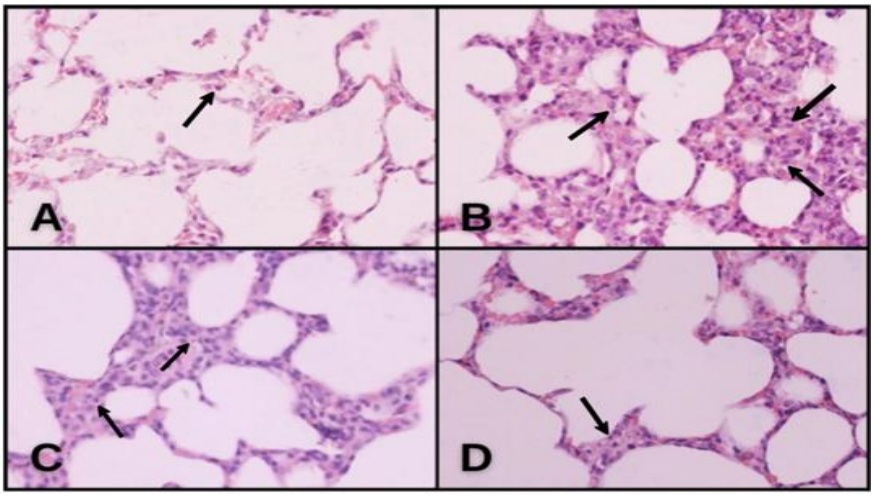

Figure 5 Results of histopathological examination of the lungs in mice

The results of histopathological examination in the KI group (5 mL/day of distilled water and exposure to normal air for 28 days) showed infiltration of inflammatory cells and obstruction of the bronchiolar lumen by mucus and cell debris which were still mild, thus obtaining an inflammation score of 1 (mild inflammation). Figure 12 (b) shows the results of histopathological examination in the KII group (5 mL/day of distilled water and exposure to cigarette smoke of 5 cigarettes/day for 28 days), with an inflammation score of 3 (severe inflammation) which was seen from the infiltration of many inflammatory cells and obstruction of the bronchiolar lumen by mucus and cell debris which was quite severe. Figure 12 (c) shows the results of histopathological examination in the KPI group (5 mL/day of HRW and exposure to cigarette smoke of 5 cigarettes/day for 28 days), obtaining an inflammation score of 2 (moderate inflammation) which showed infiltration of inflammatory cells and obstruction of the bronchiolar lumen by mucus and cell debris of a moderate degree. Figure 12 (d) shows the results of histopathological examination in the KPII group (HRW 5 mL twice/day and exposure to cigarette smoke of 5 cigarettes/day for 28 days), obtaining an inflammation score of 1 (mild inflammation) which shows inflammatory cell infiltration and milder bronchiolar lumen obstruction compared to other groups exposed to cigarette smoke.

DISCUSSION

This study conducted the administration of hydrogen-rich water (HRW) to treatment mice to determine whether HRW administration could increase antioxidant levels

such as GSH and repair lung organ damage caused by exposure to cigarette smoke given to mice. Hydrogen-rich water (HRW) is alkaline water processed through electrolysis. Hydrogen-rich water contains more hydrogen than regular water. Hydrogen is an abundant antioxidant.

Mice that were not exposed to cigarette smoke (K1) and mice that were exposed and then given distilled water (K2) had low GSH levels, meaning that there were few antioxidants produced naturally by the body, resulting in low protection of cells from damage, whereas mice that were exposed to cigarette smoke and then given. HRW 5 mL/day (KP1) and the mice that received HRW 5 mL twice a day (KP2) have high GSH levels, meaning that it contains a lot of antioxidants naturally produced by the body, thereby increasing cell protection against damage.

Of the four groups, it can be seen that treatment group 1 (KP1) has higher serum GSH levels compared to other groups. There is a significant difference between serum GSH levels of KP1 with KP2, K2, and K1 ($p<0.05$), supported by a fairly large mean difference, especially between KP1 and K1 (MD=3924.1 mmol/L) which means that the group of mice that have been exposed to cigarette smoke and received an intervention of 5 ml HRW/day (KP1) have higher serum GSH levels with a difference of 3924.1 mmol/L when compared to the group that was not exposed to cigarette smoke (K1). This is inversely proportional to the average difference between KP1 and KP2 (MD = -1939.3 mmol / L) meaning that the group of mice that had been exposed to cigarette smoke and received 5 ml HRW intervention twice a day (KP2) had lower serum GSH levels with a difference of -1939.3 mmol / L when compared to the group of mice that received 5 ml HRW / day (KP1) so that it can be said that the 5 ml HRW / day intervention provides a better hydrogen-rich water antioxidant effect compared to other interventions (K1, K2 and KP2) when viewed from serum GSH levels.

In the treatment group 2 (KP2), relatively high serum GSH levels were seen when compared to K1 and K2, this was proven by a significant difference in serum GSH levels of KP2 with K1 and K2 ($p<0.05$), supported by a fairly large mean difference between KP2 and K1 (MD=1984.8 mmol/L) which means that the group of mice that had been exposed to cigarette smoke but received 5 ml HRW intervention twice a day (KP2) had higher serum GSH levels with a difference of 1948.8 mmol/L when compared to the group that was not exposed to cigarette smoke (K1). The same thing

also happened when KP2 was compared to K2, which had a difference in serum GSH levels of 1978.4 mmol/L. When we compared group 1 (K1) and group 2 (K2), an insignificant difference was seen ($p>0.05$) due to the relative mean values in K1 and K2, although there was a slight difference in the mean.

This study showed a significant difference in serum GSH levels in the four groups of mice with a p-value <0.001 supported by a large effect size (ES = 0.935) so we can conclude that HRW intervention provides a large antioxidant effect through serum GSH levels in mice.

CONCLUSION

1. Giving hydrogen-rich water to mice exposed to cigarette smoke can provide antioxidant effects as evidenced by analysis of the increase in serum GSH levels. There was a significant difference in serum GSH levels in the four groups of mice with a p-value <0.001 supported by a large effect size (ES = 0.935).
2. There was no significant difference in the degree of inflammation K1, K2, KP1, and KP2 with a p-value = 0.599.

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