Effects of low-dose filtered kretek cigarette smoke on the bronchoconstriction in *Sprague Dawley* rats

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Abstracts:

Background: Many Indonesians are smokers, both with low doses and high doses. Exposure to cigarette smoke is mainly in the respiratory tract which results in histometric changes. The aim of this study is to determine the effect of filtered kretek cigarette smoke on the changes of bronchial histometric in *Sprague Dawley* rats.

Methods: *Sprague-Dawley* rats in group 1 as a control group, while group 2 were exposed to filtered cigarette smoke at a dose of 1 cigarette/day, as well as in group 3 was exposed of 2 cigarettes/day. Treatment time for all groups is 30 days.

Results: The bronchial lumen area of rats in group 1 was higher than groups 2 and 3 (P=0.000), but group 2 was not different from group 3 (P=0.508). The bronchial lumen perimeter of rats in group 1 was higher compared to groups 2 and 3 (P=0.000), similarly group 2 also higher than group 3 (P=0.028). Bronchial mucous layer area of rats in group 1 was higher than that of group 2 (P=0.000), as was group 2 higher than group 3 (P=0.001). Bronchial smooth muscle area in rats in group 1 was lower than group 2, similarly group 2 was lower than group 3 (P=0.000). Rats in the treatment group showed that bronchial mucosal layer undergoes hyperplasia, as well as bronchial smoot muscle layer and bronchial lumen undergoes bronchoconstriction.

Conclusion/Conclusions: Exposure to filter cigarette smoke 1 cigarette/day as well as 2 cigarettes/day for 30 days caused bronchial mucosal hyperplasia and bronchoconstriction.

Key words: filtered kretek cigarette, low-dose filtered kretek cigarette smoke, bronchial histometric, bronchial hyperplasia, bronchoconstriction.

Introductions:

Even though local governments in Indonesia are required to establish smoking-free areas, the results of the 2018 National Basic Health Research show that tobacco consumption is still quite high. In more detail it is explained that smoking is associated with diabetes, hypertension and pulmonary tuberculosis.[1] In Indonesia, 4.8% of women and 62.9% of men are aged 15 years and over tobacco users.[2] Tobacco consumption causes illness, disease and death in many countries, including the US,[3] Japan,[4] and China.[5]

There are more than 4000 types of chemical substances contained in cigarettes that have been identified.[6] Cigarette smoke contains about 8% solid particles and 92% gas.[7] In addition, nicotine in cigarettes is important to pay attention to because it is addictive.[8] In addition to these additives, there is also a radioactive component, namely ^{210}Po, which is a carcinogen.[9] These substances in cigarette smoke are inhaled from the mouth to the alveoli so that they experience deposition along the respiratory tract.[10] As a result, cigarette smoke causes abnormalities, and can cause diseases of the respiratory system.[11] In addition, cigarette smoke also contains free radicals. These free radicals are one of the causes of permanent lung tissue damage called chronic obstructive pulmonary disease (COPD).[12] Chronic obstructive pulmonary disease (COPD) is a chronic lung disease leading to irreversible destruction of the terminal bronchioles.[13] It has been proven that the main cause of COPD is cigarette smoke.[14] In the application of COPD diagnosis in humans, qualitative and quantitative assessments are required.[15]

It has been stated that irreversible destruction of the bronchial caused of smoking. The results of previous studies indicate that cigarette smoke also causes a decrease in airway diameter.[16] Other results show that smoking actively affects the human bronchial epithelium.[17] In addition to cigarette smoke, 2,3-pentanedione vapor causes injury to the epithelium lining the respiratory tract of rats.[18] Cigarette smoke also causes a stress effect on the respiratory system, causing bronchopulmonary dysplasia.[19] It has also been demonstrated that degenerative bronchioles can occur in a rat lung injury.[20]

Previous studies have demonstrated the effect of cigarette smoke on airway smooth muscle cell proliferation and function.[21] The thickness of the smooth muscle layer in the bronchioles can be influenced by several factors, including exposure to the chemical.[22] Other studies have shown that smooth muscle bronchioles affect the morphology and function of the rat airways.[23] Changes in the thickness of the smooth muscle in the bronchial are important to know due to exposure to cigarette smoke. This relates to the effect of smooth muscle thickness on the area and perimeter of the bronchial.

In addition to what has been described previously, cigarette smoke induces dysregulation that affects function in respiratory epithelial cells. Toxic substances in cigarette smoke are inhaled to the epithelial cells of the respiratory tract,[24] for example ciliated cells and goblet cells. [25, 26] Previous studies demonstrated that ciliated epithelial cells are positively correlated with bronchial lumen area, and negatively correlated with airflow through the bronchi.[26] Therefore, it is important to measure the area and perimeter of the bronchial lumen. This is related to respiratory epithelial cells affecting the lumen of the bronchial.

Besides causing dysregulation and affecting respiratory epithelial cells, toxic compounds in cigarette smoke also cause fibrosis of lung tissue. It has been demonstrated that toxic compounds increase pulmonary collagen accumulation, leading to pulmonary fibrosis.[27] Associated with pulmonary fibrosis, occurs due to the formation of scar tissue that interferes with lung function.[28] It has been demonstrated that inhalation of toxic compounds causes inflammation,[29, 30] progressive lung injury,[29] and fibrosis in rat.[18, 29, 31] As with COPD, morphometric measurements have been demonstrated in lung cystic fibrosis.[32]

Based on the description above, it can be stated that cigarette smoke affects smooth muscle cell thickness, induces dysregulation that affects function in respiratory epithelial cells resulting in dysplasia, and fibrosis in the lung tissue. In addition, the facts show that filtered and non-filtered kretek smokers are common among Indonesian people. In this study, we choose a low dose of filtered kretek cigarettes smoke for treatment because in the community many residents as smokers at low doses. The results of this study may explain the effect of low doses of filtered kretek cigarettes on bronchial. The selection of low doses of filtered kretek cigarettes has been done before, namely using a dose of 2

sticks/day against *Mus musculus* to see a picture of pulmonary histology.[33] Therefore, the aim of this study is to determine the effect of filtered kretek cigarette smoke on the changes of bronchial histometric in *Sprague Dawley* rats. Quantitative changes in the bronchial due to low-dose filtered kretek cigarette smoke are important so that a diagnosis related to lung tissue disorders can be determined more objectively.

Methods:

Animal

Eighteen *Rattus novergicus* rats *Sprague-Dawley* strain aged 2-3 months, body weight 150-250 grams, male. *Sprague-Dawley* rats were obtained from the Integrated Research and Testing Laboratory Unit 4, Universitas Gadjah Mada. Rats were housed in cages individually. Treatment room with temperature 26 ± 2°C, humidity 55 ± 5%, and artificial fluorescent lights (12:12 hours, light and dark cycle). Rats were given food and drink in libitum according to standard.

Treatment

At the beginning of the study, the rats were acclimatized for 1 week. During acclimation, the rats were given food, drink and no treatment. After acclimatization, random grouping was carried out. Rats were randomly grouped into 3 groups, each group consisting of 6 rats. Group 1 was the control group, the rats breathed normal air without being exposed to filtered kretek cigarette smoke. Group 2 is a group of rats exposed to filtered kretek cigarette smoke at a dose of 1 stick/day for 30 days. Group 3 is a group of rats exposed to filtered kretek cigarette smoke at a dose of 2 cigarettes/day for 30 days. Exposure to filtered kretek cigarette smoke was carried out in a smoking chamber measuring 45 x 35 x 20 cm (31500 cm3). The oxygen valve in the smoking chamber is opened first, then the filtered kretek cigarettes are installed at the end of the pipe connected to the pump. After the pump is turned on, the filter kretek cigarettes are burned so that the smoke enters the smoking chamber to be inhaled by the rats. Cigarette smoking was carried out on groups 2 and 3 for 10 minutes in the morning. After 30 days of treatment, the rats were anesthetized to death. Anesthesia uses ketamine 100 mg/kg body weight, and xylazine 10 mg/kg body weight intra peritoneally. Furthermore, the animals were sacrificed by means of euthanasia, then the lung organs were taken. Lung collection was carried out in 10% neutral buffered formalin (NBF) solution, to make histological preparations.

Hematoxylin eosin staining

Right lobe lung tissue was fixed in 10% NBF. Next, the tissues were transferred to dehydrated alcohol with alcohol concentrations of 70%, 80%, 90%, 96%, respectively. Dehydration time is 2 hours for each alcohol concentration. The next step is clearing using xylol, then embedding and blocking. Lung tissue in the paraffin block was cut using a microtome, with a thickness of 5 µm. Stain with hematoxylin for 5 minutes, then wash with running tap water for 5 minutes. Dip in 1% acidic alcohol (1% HCl in 70% alcohol) for a few seconds. Bluing is done by rinsing under running tap water. Dip in ammonia water until the area turns blue, followed by washing with tap water. Counter stain with 1% Eosin for 10 minutes, then wash with tap water for 5 minutes. After the counter stain is dehydrated with alcohol, then cleaned with xylol. The last step is mounting and labeling.

Trichrome Masson's staining

Pieces of lung tissue 5 µm thick from paraffin blocks were deparaffinized, washed with water, then de-paraffinized with mercury pigment with Lugol's iodine for 15 minutes, washed with water. Enter the 5% sodium thiosulphate solution for 3 minutes, wash with water for at least 10 minutes. Paint with hematoxylin 3 minutes, dip quickly into 1% acid alcohol 3 times, wash with water. Soak in fuchsin acid solution 5 minutes, wash with distilled water. Soak in phosmolybdic acid solution for 5 minutes, dry. Soak in methyl blue solution for 5 minutes, wash with distilled water. Soak in 1% acetic acid for 2 minutes, then dry with alcohol and wash with xylol. The last step is mounting, and labeling.

Scanning Electron Microscope

Samples were taken from the right lobe of lung tissue. After fixation, the sample is positioned, then coated, and analyzed with a scanning electron microscope (SEM). The SEM used for observation is the JEOL 6510 LA series made in Japan.

Bronchial histometric measurement

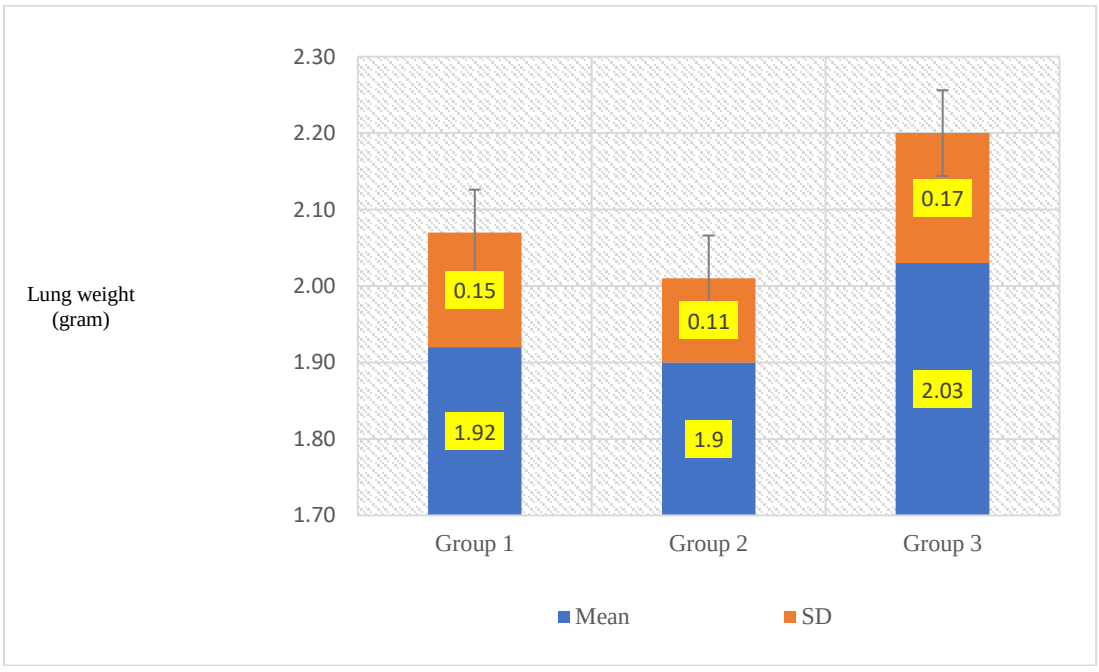
Images of preparations documented with Optilab Advance Plus and Image Raster 3 by PT MICONOS, Special Region of Yogyakarta, Indonesia. The program is available at https://miconos.ac.id/new/support/download. Lumen of bronchial histometric measurements include, length, width, area, and perimeter. In addition, area and perimeter measurements of bronchial mucous layer and bronchial smooth muscle layer were also measured. Bronchial histometric measurements of rat lung performed by three observers. The measured data is expressed as the mean ± standard deviation. Data analysis between groups used the one way-analysis of variance (one way-ANOVA) test. The difference between groups is significant if the test results show P<0.05.

Result:

Comparison of the appearance and weight of the lungs of *Sprague Dawley* rats are presented in **Figure 1.**

Group 1 Group 2 Group 3

A.

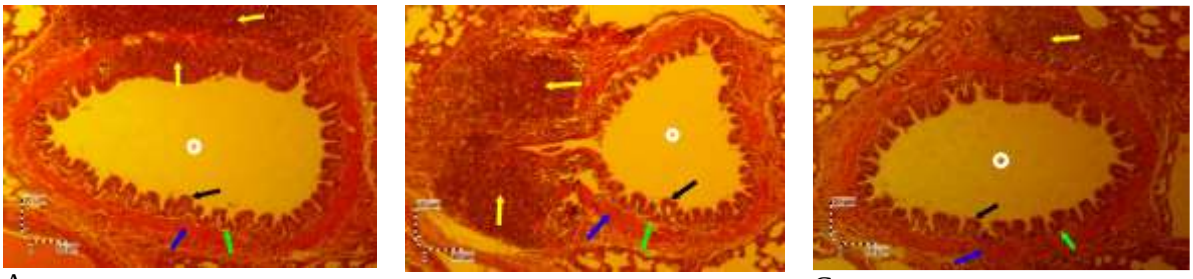


B.

Figure 1. Comparison of the appearance and weight of the lungs of *Sprague Dawley* rats. A=Macroscopic image of rat lungs. B=comparison of the lung weight of rats between groups. Group 1 (control), rats breathe using ordinary air without exposure to filtered kretek cigarette smoke. Group 2, the group of rats exposed to filtered kretek cigarette smoke 1 stick/day for 30 days of treatment. Group 3, group of rats exposed to filtered kretek cigarette smoke 2 sticks/day for 30 days of treatment.

There was no difference in the lung weight of *Sprague Dawley* rats between groups 1, 2, and 3 (P=0.281).

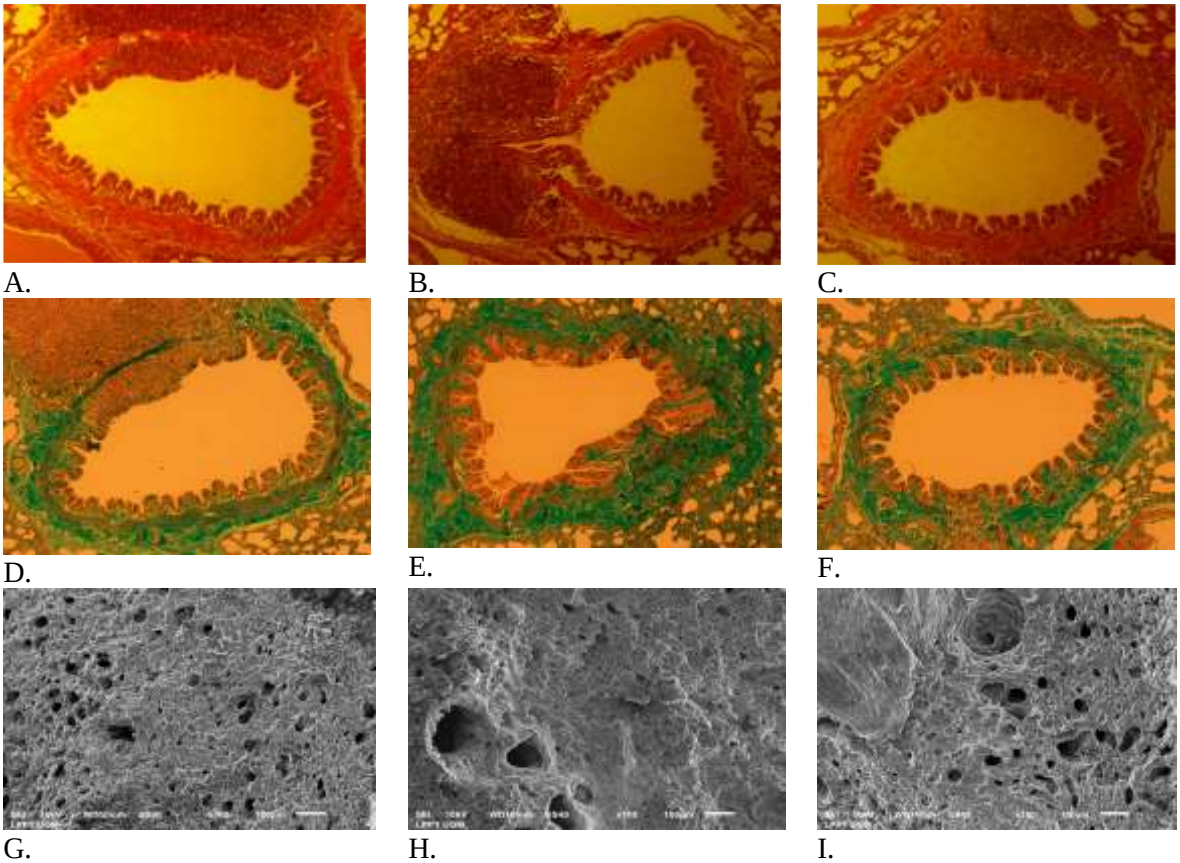
Bronchial wall appearance of a *Sprague Dawley* rat, presented in **Figure 2.**



A. B. C.

Figure 2. Bronchial wall appearance of a *Sprague Dawley* rat with H&E stained, magnification of 100 x. A=Group 1 (control), rats breathe using ordinary air without exposure to filtered kretek cigarette smoke. B=Group 2, the group of rats exposed to filtered kretek cigarette smoke 1 stick/day for 30 days of treatment. C=Group 3, group of rats exposed to filtered kretek cigarette smoke 2 sticks/day for 30 days of treatment. H&E=hematoxylin & eosin; black arrow=respiratory epithelium layer; lime arrow=lamina propria layer; blue arrow=smooth muscle layer; yellow arrow=bronchus-associated lymphoid tissue; white circle=bronchial lumen.

Observations of the lungs of *Sprague Dawley* rats using a light microscope, and SEM are presented in **Figure 3.**



A. B. C.

D. E. F.

G. H. I.

Figure 3. Lung appearance of *Sprague Dawley* rats. The lungs of *Sprague Dawley* rats stained with hematoxylin & eosin were observed using a light microscope with 100x magnification in group 1 (A), group 2 (B), and group 3 (C). The lungs of *Sprague Dawley* rats stained with Masson's trichrome were observed using a light microscope with 100x magnification in group 1 (D), group 2 (E), and group 3 (F). The lungs of *Sprague Dawley* rats were observed using scanning electron microscope with 100x magnification in group 1 (G), group 2 (H), and group 3 (I). Group 1 (control), rats breathe using ordinary air without exposure to filtered kretek cigarette smoke. Group 2, the group of rats exposed to filtered kretek cigarette smoke 1 stick/day for 30 days of treatment. Group 3, group of rats exposed to filtered kretek cigarette smoke 2 sticks/day for 30 days of treatment.

The appearance of the bronchial walls in group 1 showed a normal histological structure (**Fig. 2. A**), whereas in group 2 showed an abnormal histological structure, namely hyperplasia of the bronchial mucosa layer of *Sprague Dawley* rats (**Figure 2. B**). In group 3 of *Sprague Dawley* rats showed bronchoconstriction (**Fig. 2. C**). Qualitative appearance of bronchial walls showing differences in bronchial lumen, bronchial mucous layer, and bronchial smooth muscle cells clearly visible with H&E staining (**Fig. 3. A, B, C**). Likewise, it was also shown qualitatively that differences in bronchial collagen accumulation were clearly seen by Masson's trichrome staining which demonstrated that rats in groups 2 and 3 experienced more collagen accumulation than group 1 (**Fig. 3. D, E, dan F**). The results of observations using SEM showed that qualitatively there were differences in the lungs appearance of *Sprague Dawley* rats between groups 1, 2, and 3 (**Figure 3. G, H, dan I**).

Bronchial histometric comparison of *Sprague Dawley* rats are presented in **Figure 4.**

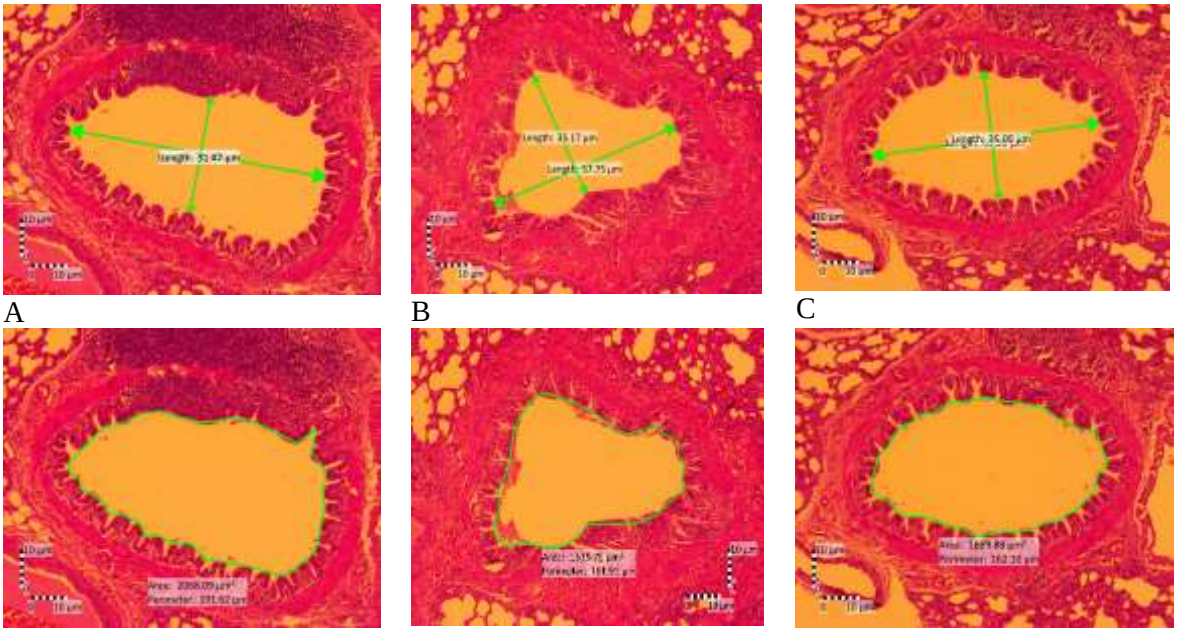


Figure 4. Bronchial histometric comparison of the *Sprague Dawley* rats. The length and width of the bronchial lumen stained with hematoxylin & eosin, 100x magnification, in group 1 (A), group 2 (B), and group 3 (C). The area, and circumference of the bronchial lumen stained with hematoxylin & eosin, magnification 100x, in group 1 (D), group 2 (E), and group 3 (F). Group 1 (control), rats breathe using ordinary air without exposure to filtered kretek cigarette smoke. Group 2, the group of rats exposed to filtered kretek cigarette smoke 1 stick/day for 30 days of treatment. Group 3, group of rats exposed to filtered kretek cigarette smoke 2 sticks/day for 30 days of treatment.

Effect of filtered kretek cigarette smoke on the bronchial lumen in *Sprague Dawley* rats is presented in **Table 1.**

Table 1. Effect of filtered kretek cigarette smoke on the bronchial lumen in *Sprague Dawley* rats

Bronchial lumen	**Group 1**	**Group 2**	**Group 3**
Length (µm)	83.94±4.94	65.61±7.72**	54.94±6.43**
Width (µm)	52.06±2.83	35.96±5.98**	35.42±7.54**
Area (µm^2)	2048.43±63.73	1514.79±57.98**	1541.07±78.18**
Perimeter (µm)	189.79±4.04	161.66±1.48**	156.19±5.21**

Abbreviations: Group 1 (control), rats breathe using ordinary air without exposure to filtered kretek cigarette smoke. Group 2, the group of rats exposed to filtered kretek cigarette smoke 1 stick/day for 30 days of treatment. Group 3, group of rats exposed to filtered kretek cigarette smoke 2 sticks/day for 30 days of treatment. **P<0.01.

Bronchial lumen length, bronchial lumen width, bronchial lumen area, and bronchial lumen perimeter of *Sprague Dawley* rats differs between groups (P=0.000) (**Table 1**). The bronchial lumen length of *Sprague Dawley* rats in group 1 was higher than group 2 and 3 (P=0.000), as was group 2

higher than group 3 (P=0.012). The bronchial lumen width of *Sprague Dawley* rats differed between groups (P=0.000). The bronchial lumen width of *Sprague Dawley* rats in group 1 was higher than that of groups 2 and 3 (P=0.000), but group 2 was no different from group 3 (P=0.874). The bronchial lumen area of *Sprague Dawley* rats differed between groups (P=0.000). The bronchial lumen area of *Sprague Dawley* rats in group 1 was higher than groups 2 and 3 (P=0.000), but group 2 was not different from group 3 (P=0.508). The bronchial lumen perimeter of *Sprague Dawley* rats differed between groups (P=0.000). The bronchial lumen perimeter of *Sprague Dawley* rats in group 1 was higher compared to groups 2 and 3 (P=0.000), similarly group 2 also higher than group 3 (P=0.028). Based on **Fig. 4**, and **Table 1,** shows that the bronchial lumen of rats undergoes bronchoconstriction.

Comparison of the thickness of bronchial mucous layer, and bronchial smooth muscle layer of *Sprague Dawley* rats between groups is presented in **Figure 5.**

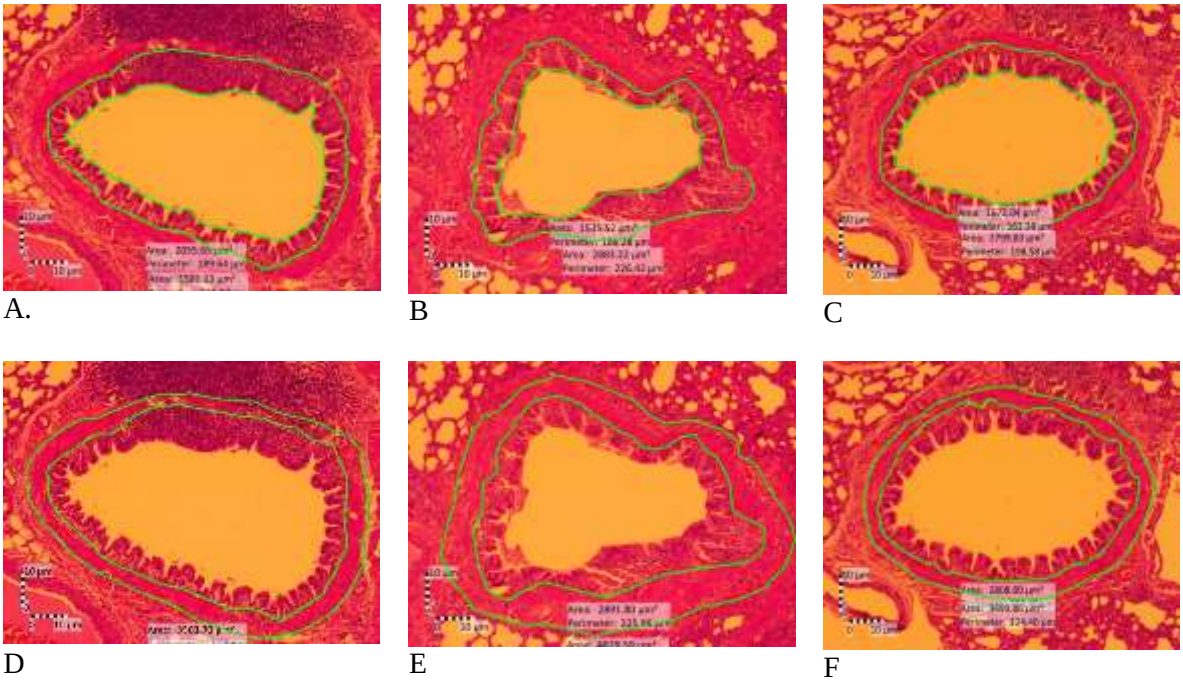


Figure 5. Comparison of the thickness of bronchial mucous layer, and bronchial smooth muscle layer of *Sprague Dawley* rats. The area and perimeter of the bronchial mucous layer with H&E staining, magnification of 100 x in group 1 (A), group 2 (B), and group 3 (C). The area and perimeter of the bronchial smooth muscle with H&E staining, magnification of 100 x in group 1 (D), group 2 (E), and group 3 (F). Group 1 (control), rats breathe using ordinary air without exposure to filtered kretek cigarette smoke. Group 2, the group of rats exposed to filtered kretek cigarette smoke 1 stick/day for 30 days of treatment. Group 3, group of rats exposed to filtered kretek cigarette smoke 2 sticks/day for 30 days of treatment. H&E=hematoxylin & eosin.

Based on the **Fig.** 5. bronchial mucous layer in in group 1 is normal, while in group 2 and 3 seen hyperplasia. Bronchial mucous layer area, Δ mucous layer perimeter, smooth muscle area, and Δ smooth muscle perimeter of *Sprague Dawley* rats differs between groups (P=0.000) (**Table 2**). The area of the bronchial mucous layer of *Sprague Dawley* rats in group 1 was higher than that of group 2 (P=0.000), as was group 2 higher than group 3 (P=0.001). The Δ mucous layer perimeter of bronchial *Sprague Dawley* rats in groups 1 and 3 was lower than that of group 2 (P=0.000), but group 1 was no different from group 3 (P=0.763). Bronchial smooth muscle area in *Sprague Dawley* rats in group 1 was lower than group 2, similarly group 2 was lower than group 3 (P=0.000). The Δ smooth muscle perimeter of bronchial *Sprague Dawley* rats in groups 1 was lower than that group 3 (P=0.018), similarly group 1 was lower than that group 3 (P=0.001), but group 1 was no different from group 3 (P=0.170).

Effect of filtered kretek cigarette smoke on the bronchial mucous layer and bronchial smooth muscle layer in *Sprague Dawley* rats is presented in **Table 2.**

Tabel 2. Effect of filtered kretek cigarette smoke on the bronchial mucous layer and bronchial smooth muscle layer in *Sprague Dawley* rats

Bronchial thickness	**Group 1**	**Group 2**	**Group 3**
mucous layer area (µm^2)	1527.04±77.30	1303.08±68.02**	1164.28±30.20**
Δ **mucous layer perimeter (µm)**	45.12±1.74	72.78±4.87**	44.22±3.18
smooth muscle area (µm^2)	1092.52±64.16	1473.33±50.07**	850.63±78.88**
Δ **smooth muscle perimeter (µm)**	26.30±3.69	21.32±5.72*	35.49±7.82**

Abbreviations: Group 1 (control), rats breathe using ordinary air without exposure to filtered kretek cigarette smoke. Group 2, the group of rats exposed to filtered kretek cigarette smoke 1 stick/day for 30 days of treatment. Group 3, group of rats exposed to filtered kretek cigarette smoke 2 sticks/day for 30 days of treatment. Mucous layer area (µm^2)=outer mucous layer area (µm^2)-inner mucous layer area (µm^2). Δ mucous layer perimeter (µm)=outer mucous layer perimeter (µm)-inner mucous layer perimeter (µm). *P<0.05; **P<0.01.

Discusion

This study obtained an overview of changes in lung organ weight and structural abnormalities in the bronchial of *Sprague Dawley* rats with treatment of filtered kretek cigarette smoke, low doses of 1 stick/day as well as 2 sticks/day for 30 days. Exposure to filtered kretek cigarette smoke 1stick/day as well as 2 sticks/day for 30 days in this study did not affect the lung weight of rats. As a comparison, the results of previous studies showed that the average weight of rat children ranged from 0.34 – 0.40 grams with a lung weight/body weight ratio ranging from 1.51 – 1.82%.[34] In addition, it was also demonstrated that Wistar rats with a body weight of 64 ± 6.5 gr had a lung wet weight of 0.69 ± 0.09 gr.[35] The short exposure time to filter clove cigarette smoke in this study, both 1 cigarette/day as well as 2 cigarettes/day for 30 days, had no effect on changes in lung weight. Although it has been stated that nicotine in cigarette smoke affects the central nervous system and various molecular mechanisms of peptide compounds that regulate food intake so that it affects body weight.[36]

Filtered kretek cigarette smoke 1 stick/day and 2 sticks/day for 30 days of treatment affected on the rat bronchial walls (**Fig. 2; Fig. 3. A, B, C**). This influence is clearly seen in the size of the bronchial mucous layer, smooth muscle layer, and bronchial lumen. A deviation from the metabolic steady state leads to a condition of oxidative stress. The source of oxidative species can be endogenous or exogenous. A major exogenous source of these species is tobacco smoking. Oxidative damage can be induced in cells by chemical species contained in smoke through the generation of pro-inflammatory compounds and the modulation of intracellular pro-inflammatory pathways, resulting in a pathological condition.[37] More clearly it has been demonstrated that cigarette smoke induces lung inflammation by various mechanisms.[38, 39]

As a result of exposure to filtered kretek cigarette smoke specifically to the bronchial mucous layer. A previous study demonstrated that exposure to cigarette smoke 2 sticks/day for 14 days caused changes in epithelial structure in the airways of rats.[33] Our results are consistent with the results of studies showing that cigarette smoke increases lung epithelial cell activation and hyperplasia.[40] Epithelial structure in the bronchial part of the rat affects bronchial histometric. We suggest that the occurrence of bronchoconstriction is primarily driven by smooth muscle cells. Bronchoconstriction is associated with bronchial hyperactivity, which in turn causes bronchial hyperplasia. As a result of exposure to filtered kretek cigarette smoke specifically to the bronchial mucous layer, namely the occurrence of hyperplasia. This statement is in line with studies showing that growth of the smooth muscle layer of the airways is mediated by hypertrophy, and subsequent hyperplasia.[41] The results of other studies have also shown that cigarette smoke is associated with inflammation and hyperplasia of bronchial mucosal cells.[42]

Although our study demonstrated exposure to low doses of filtered kretek cigarette smoke, namely 1 stick/day as well as 2 sticks/day for 30 days on the bronchial wall of rats, the results showed a noticeable accumulation of collagen on the bronchial wall. Collagen accumulation in the bronchial walls is clearly visible in the smooth muscle layer (**Fig. 3. D, E, dan F**). Related to the data in this study, it has been demonstrated that exposure to cigarette smoke twice daily, with one day off per week, for 90 days affects the proliferation and synthetic function of airway smooth muscle cells.[21] This fact shows an abnormality of collagen deposition on the bronchial wall due to exposure to low-dose filtered kretek cigarette smoke. The appearance of bronchial collagen in rats in this study was evident through Masson's trichrome staining which was the choice for visualizing bronchial collagen accumulation, as was done for visualizing collagen accumulation in mice lungs.[43] The results of this study show the dangers of low-dose filtered kretek cigarette smoke because it can result in collagen deposition of the bronchial walls. Although in this study used low doses of filtered kretek cigarettes, but the toxic substances contained in them were shown to cause the accumulation of bronchial collagen. The results of this study are in line with studies showing the accumulation of bronchial collagen in rats due to other toxic substances, namely sulfur mustard,[29] and silica nanoparticles.[31] Based on the results of this study, research is needed to prove molecularly that low-dose filtered kretek cigarette smoke, namely 1 cigarette/day or 2 cigarettes/day for 30 days has affected the genes that control collagen accumulation resulting in bronchial fibrosis in rats. This is consistent with the results of research showing that exposure to cigarette smoke controls the gene expression of the heat shock protein gene,[44] collagen type I,[31] collagen type III,[31, 45] DNA methylation and histone modifications.[46]

The results of our study further reinforce that low-dose filtered kretek cigarette smoke of 1 stick/day as well as 2 stick/day for 30 days against rat lungs causes bronchoconstriction. These results are similar to the treatment of high doses of toxic agents (ammonia) that cause bronchoconstriction in rats.[47] In addition, bronchoconstriction in rat lungs has been demonstrated that short-term smoking increases the risk of insulin resistance,[48] the subsequent increase in insulin acutely causes smooth muscle contraction in rats.[49] The results of our study are different from studies on the effects of high intensity interval training on adult rats. Adult rats exposed to high intensity interval training increased the thickness of the bronchial epithelial and bronchial muscles.[35]

The limitations of this study include no measurement of the levels of substances in filter kretek cigarette smoke. However, that bronchoconstriction in this study showed a clear reduction in area and perimeter of the bronchial lumen, bronchial mucosal layer area and smooth muscle area. This fact can be used as a warning to novice smokers and smokers with low doses. Research is needed with human subjects on low-dose kretek smokers and novice kretek smokers. We know that novice kretek smokers usually take a low-dose as the start of their addiction to kretek cigarettes. Subsequent research is also focused on histometric abnormalities in the airways other than bronchial, for example trachea, bronchioles, respiratory bronchioles, and pulmonary alveoli.

Conclusion

Based on the research data, it can be concluded that exposure to low-dose filtered kretek cigarette smoke of 1 stick/day as well as 2 sticks/day for 30 days to *Sprague Dawley* rats does not affect changes in lung weight. Hyperplasia of the bronchial mucous layer, as well as bronchoconstriction in *Sprague Dawley* rats which is characterized by reduction in area and perimeter of the bronchial lumen, bronchial mucosal layer area and smooth muscle area appeared in the treatment group.

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Conflict of Interests

The authors declare that they have no competing interests.

Funding source

This publication is based on work supported by Universitas Trisakti (no. 0458/PUF/FK/2022-2023.).

Ethical statement

Ethical clearance for this research was obtained from the Ethical Clearance Commission for preclinical research of the Integrated Research and Testing Laboratory, Universitas Gadjah Mada with number: 00012/04/LPPT/VI/2022.

Acknowledgments

We would like to thank the Leaders and Staff of the Integrated Research and Testing Laboratory, Universitas Gadjah Mada for the opportunity given to us so that this research could be carried out.

Authors contributions

DV, EP, and HA=Schemed and designed experiment. DV, EP, HJE, RAD, and AVO=data collecting, analysis, and interpretation of the results. HJE, RAD, JJVT, and LG=images review and processing. DV, EP, HA, RAD, and AVO=writing of the manuscript. All author's=reviewing and approved the final manuscript.

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UNIVERSITAS GADJAH MADA
LABORATORIUM PENELITIAN DAN PENGUJIAN TERPADU

KETERANGAN KELAIKAN ETIK
(Ethical Clearance)

No. Sertifikat: 00012/04/LPPT/VI/2022

Komisi Ethical Clearance untuk penelitian praklinik Laboratorium Penelitian dan Pengujian Terpadu, Universitas Gadjah Mada Yogyakarta, setelah mempelajari dengan seksama rancangan penelitian yang diusulkan, dengan ini menyatakan penelitian:

Judul Penelitian	: **Pengaruh Asap Rokok, Spray Insektisida terhadap Biometrik Cerebrum, Hippocampus, Cerebellum, dan Paru Tikus *Sprague-Dawley***
Peneliti Utama	: **dr. David Tjahyadi, M Kes.**
Asal Instansi	: **Fakultas Kedokteran, Universitas Trisakti**
Lokasi Penelitian	: **Laboratorium Penelitian dan Pengujian Terpadu (LPPT) Unit 4, Universitas Gadjah Mada**

Telah dinyatakan memenuhi persyaratan etik untuk penelitian pada hewan coba. Komisi Ethical Clearance mempunyai hak untuk melakukan pemantauan selama penelitian berlangsung. Apabila terjadi perubahan dalam hal jenis dan jumlah hewan coba serta metode perlakuan terhadap hewan coba, peneliti wajib mengajukan permohonan amandemen kepada Komisi Ethical Clearance.
Surat Keterangan ini berlaku 1 (satu) tahun sejak ditandatangani.

Yogyakarta, 10 Juni 2022
Komisi Ethical Clearance
Ketua,

Prof. Dr. Siti Isrina Oktavia Salasia, DVM.

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