

IMPACT OF FREE RADICALS ON LUNGS AND TESTIS HISTOLOGY FROM DIFFERENT TYPES OF TOBACCO IN INDONESIA

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Abstract. Tobacco is the main raw material in the manufacture of cigarettes. Various kinds of cigarette products in Indonesia have been widely known and exported to foreign countries. Geographical conditions, and planting techniques for the processing of tobacco leaves can give different flavors to each cigarette product. However, this does not change the negative impact caused by smoking. The resulting free radicals would give changes to the lungs and testes histologically. This study aimed to analyze histological changes caused by free radicals from various types of tobacco in Indonesia. Wistar rats were exposed to smoke from 3 types of tobacco leaves in Indonesia, namely *Rejeb*, *Jinten*, and *Melati Tumpeng*, then the histological changes in lung and testicular organs were examined. Exposure to tobacco leaves was carried out for 30 days. The parameters in this study were the mean numbers of alveolar macrophages in the lungs and sperm in the testes in 10 visual fields. The results showed that there was a difference between the control group and the treatment group ($p=0.004$), where there was an increase in malondialdehyde levels and a decrease in sperm count in experimental animals exposed to tobacco leaves. Various types of tobacco leaves in Indonesia, which are used as raw materials for cigarettes, can pose a health impact by increasing free radicals and decreasing sperm count.

Keywords: alveolar macrophages, cigarettes, histology, tobacco, sperm

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INTRODUCTION

Smoking is a long-standing health problem which does not come to solution until now. Various preventive measures in reducing the number of smokers through education and even policies show unsatisfying results (Lorensia *et al*, 2021). The number of female smokers at reproductive ages also increases as the number of male smokers does (Aitken *et al*, 2014; Jafari *et al*, 2021). Diseases caused by smoking are non-infectious ones, but they have caused seven million deaths annually. By 2030, it is anticipated that a higher number of smokers may result in eight million deaths (WHO, 2017). Almost a half of million active smokers in the United States of America died, and the other half who died are referred to as passive smokers (Courtney, 2015). Indonesia is known to have a high number of smokers. The number of active smokers aged above 15 years reached 39.5% of the total population in 2016, higher than the average number of smokers worldwide (Holipah *et al*, 2020). Besides, tobacco cigarette consumption by male smokers reached 20-76.5% in period of 2000 to 2015 (Holipah *et al*, 2020). Some health problems caused by smoking include hypertension, chronic obstructive pulmonary disease (COPD), and stroke which account for 21.05% of chronic diseases in Indonesia (Kristina *et al*, 2018).

Indonesia is the largest tobacco producer in the world, and tobacco is the main material in cigarette production. As a big commodity, the tobacco industry provides job opportunities for low socio-economic groups to be tobacco farmers and cigarette laborers (Astuti *et al*, 2020). As a result, Indonesia is ranked the fourth largest number of active smokers, a third of the total population, who consume tobacco. Some regions are transformed into tobacco production centers and distributors by cigarette manufacturers (Biswas *et al*, 2017; Hurt *et al*, 2012). The East Java Province is the largest tobacco producer which has a lot of clove cigarette manufacturers (Sahadewo *et al*, 2020). The most planted tobaccos used for clove cigarettes in East Java include *Rejeb*, *Jinten*, and *Melati Tumpang*. All of these tobaccos are species of *Nicotiana tabacum* from different regions. *Rejeb* is mostly planted in Magetan

District, *Jinten* in Jombang District, and *Melati Tumpang* in Madura Island (Hamida *et al*, 2020; Utari and Slamet, 2019). Tobacco plantation relies on the fertility and nutrients of soil which contributes to different substances in the tobacco leaves. Besides, the tobaccos differ in the plucking, drying, and production processes which are traditionally done in each region. Such a method is believed to reduce the negative health impacts of smoking (Zeng *et al*, 2014; Lisuma *et al*, 2021).

Cigarette which contains mostly tobacco leads to devastating health impacts. Exposure to cigarette smoke is a source of free radicals which influence the antioxidant resistance system (Suryadinata and Wirjatmadi, 2021). The excessive amount of free radicals may cause oxidative damage because of lipid peroxidation. Superoxide free radicals are the main factor of body cell damage (Biswas *et al*, 2017). The increased number of free radicals in the lungs due to tobacco smoke may stimulate alveolar macrophages to transfer the free radicals to the lungs. It may cause pro-inflammatory cytokine secretion and thus lead to tissue injury and cell (Suryadinata *et al*, 2021b). The cell death in the lungs may result in the declined function of the lungs and other organs; therefore, it may cause various diseases such as cardiovascular, stroke, and metabolic diseases (Tantisuwat and Thaveeratitham, 2014). Analyzing the histological preparations and counting them will reveal the existence of alveolar macrophages. The greater number of alveolar macrophages observed indicates pulmonary tissue injury (Naem *et al*, 2022). Thus, the study aimed to identify the health impacts of exposure to tobacco smoke based on the number of alveolar macrophages and the first declined number of sperms.

MATERIALS AND METHODS

This study used an experimental method conducted in male Wistar rats (*Rattus norvegicus*) exposed to tobacco smoke for 30 days. Twenty-eight animals were equally divided into 4 groups: a negative control group

and three experiment groups exposed to *Rejeb*, *Jinten*, and *Melati Tumpeng* tobaccos, respectively. The animal care and treatment were conducted in a biomedical laboratory at the Faculty of Medicine, Universitas Surabaya based on Three Rs Principle (3R: (Replacement, Reduction, and Refinement) (Tannenbaum and Bennett, 2015).

The first group is a negative control group that only received daily exposure with fresh air. They were a comparison group to the experiment group to identify negative health impacts caused by tobacco smoke. The treatment was given three experiments with animal groups exposure to *Rejeb*, *Jinten*, and *Melati Tumpeng* smoke. These tobaccos were chosen as they were the most frequent material in cigarette production in Indonesia. Five grams of tobacco leaves (equivalent to one cigarette) were burnt and the rats in experiment groups were exposed to the cigarette smoke twice a day with 6 hours interval for a total of 30 days. Duration of exposure was 60 minutes each time. Tobacco smoke was given through a smoking chamber (100 × 70 × 30 cm) where the carbon dioxide (CO₂) level was controlled at 50 ppm.

After the 30-day intervention, the experiment animals were terminated by spinal dislocation and their lungs and testis were collected. Histological preparations on the lung and testis tissues were done in a histological laboratory, Faculty of Medicine, Universitas Surabaya through a histotechnic method and coloring with hematoxylin eosin (HE) using Lillie's protocol (Lillie, 1965). The parameters include the average number of alveolar macrophages in the lung tissues and sperms in the testis tissues as shown in 10 fields of view for each prepared tissue with 400x magnification. The data were then analyzed through analysis of variance (ANOVA) test to identify differences between groups, *ie* negative control group, experiment group exposed to *Rejeb* smoke, experiment group exposed to *Jinten* smoke, and experiment group exposed to *Melati Tumpeng* smoke, if the normality and homogeneity test results show a value of $p > 0.05$. But if the assumptions of normality and homogeneity tests do not meet the statistical test, Kruskal-Wallis was applied.

Ethical consideration

This research has received an ethical clearance certificate from the Institutional Ethical Committee Universitas Surabaya with Number: 33/KE/IV/2022.

RESULTS

Table 1 shows the average number of alveolar macrophages in the lung tissues and the number of sperms in the testis tissues in 10 fields of view. In the lung tissues, Group II (*Rejeb*) had the highest number of alveolar macrophages at 31.60 ± 5.98 , while Group I (Control), the control group, had the lowest number at 2.80 ± 0.837 . Meanwhile, the highest number of sperms in the testis tissues was found in Group I (Control) at 73.46 ± 4.29 and the lowest in Group II (*Rejeb*) at 56.64 ± 6.67 .

Table 2 describes the data of alveolar macrophages that were normally distributed sequentially with *p*-values of 0.314, 0.529, 0.120, and 0.613 for Group I (Control), Group II (*Rejeb*), Group III (*Jinten*) and Group IV (*Melati*

Table 1
Average numbers of alveolar macrophages and sperms in each group

Group	Alveolar macrophages Mean \pm SD	Sperms Mean \pm SD
I Negative control group (<i>n</i> = 7)	2.80 ± 0.837	73.46 ± 4.29
II Experiment group exposed to <i>Rejeb</i> smoke (<i>n</i> = 7)	31.60 ± 5.98	57.72 ± 6.70
III Experiment group exposed to <i>Jinten</i> smoke (<i>n</i> = 7)	23.40 ± 3.847	56.64 ± 6.67
IV Experiment group exposed to <i>Melati Tumpang</i> smoke (<i>n</i> = 7)	27.00 ± 3.80	58.76 ± 6.29

SD: standard deviation

Table 2
Kruskal Wallis test results in the alveolar macrophage group

Group	Multiple Comparison				Normality* Homogeneity ⁺ p-value [‡]
	Group I	Group II	Group III	Group IV	
I Negative control group	-	-	-	-	0.314 0.018 0.004
II Experiment group exposed to <i>Rejeb</i> smoke	<0.001	-	-	-	0.529
III Experiment group exposed to <i>Jinten</i> smoke	<0.001	0.185	-	-	0.120
IV Experiment group exposed to <i>Melati Tumpang</i> smoke	<0.001	0.033	0.175	-	0.613

*Normality test $p > 0.05$ indicates the assumption of normally distributed data distribution is met; ⁺Homogeneity test $p < 0.05$ indicates the assumption of homogeneous data distribution; [‡] p-value of < 0.05 indicates the effect of cigarette smoke on the number of alveolar macrophages

tumpang), respectively. While the homogeneity test showed the data were not homogenous ($p= 0.004$), so that the statistical analysis used in this study used a non-parametric test. The result of Kruskal-Wallis test was done to identify differences between groups ($p=0.004$); hence, it can be concluded that there were significant differences among groups.

Table 2 also shows differences in the number of alveolar macrophages. Group I (Control) showed differences from others (*ie* Groups II (*Rejeb*), III (*Jinten*), and IV (*Melati tumpang*)), and so did Groups II (*Rejeb*) and IV (*Melati tumpang*) ($p=0.003$), while other groups, *ie* Groups II (*Rejeb*) and III (*Jinten*), Group III (*Jinten*) and IV (*Melati tumpang*) showed no significant differences in the number of alveolar macrophages.

Fig 1 shows the histopathological comparison of the numbers of alveolar macrophages in the lung tissue of control and intervention groups.

Table 3 shows the amounts of sperms in seminiferous tubules of testis

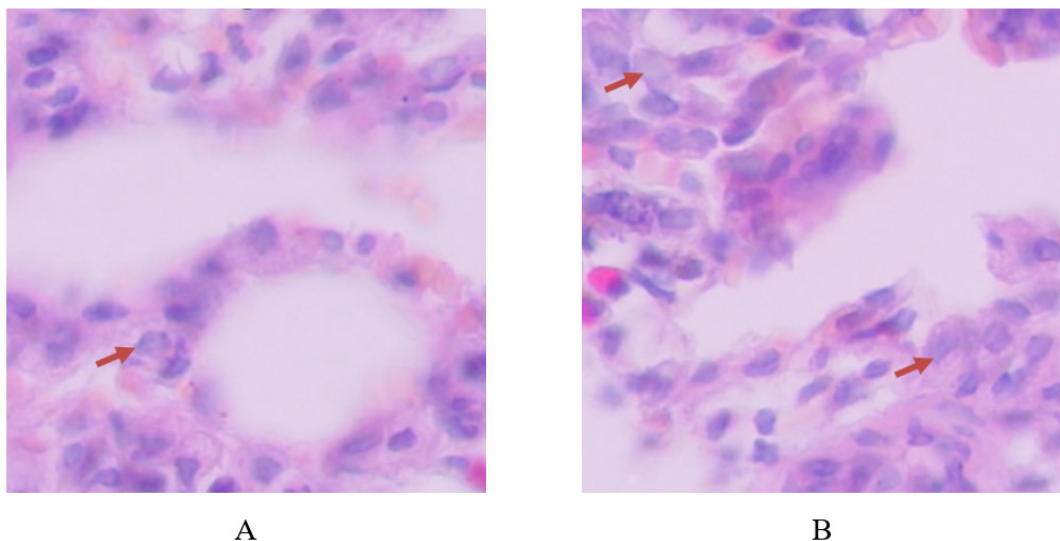


Fig 1 - Histopathology of the alveolar macrophages (Hematoxylin & Eosin – 400x; red arrows indicate alveolar macrophages)

A: Control group; B: Experimental group

Table 3
ANOVA test results in the sperm group

Group	Multiple Comparison				Normality* Homogeneity [†]	p-value [‡]
	Group I	Group II	Group III	Group IV		
I Negative control group	-	-	-	-	0.798	0.642
II Experiment group exposed to <i>Rejeb</i> smoke	0.001	-	-	-	0.375	
III Experiment group exposed to <i>Jinten</i> smoke	<0.001	0.792	-	-	0.719	
IV Experiment group exposed to <i>Melati Tumpang</i> smoke	0.001	0.790	0.589	-	0.440	

*Normality test $p > 0.05$ indicates the assumption of normally distributed data distribution is met; [†] Homogeneity test $p < 0.05$ indicates the assumption of homogeneous data distribution; [‡] p-value of < 0.05 indicates the effect of cigarette smoke on the number of alveolar macrophages

were normally distributed sequentially with p -values of 0.798, 0.375, 0.719, and 0.440 for Group I (Control), Group II (*Rejeb*), Group III (*Jinten*) and Group IV (*Melati tumpeng*), respectively, while the homogeneity test showed the research data were homogenous ($p= 0.642$). Therefore, the ANOVA test was conducted to identify differences between groups ($p=0.001$); it can be concluded that there are significant differences in numbers of sperms among Groups I (Control), II (*Rejeb*), III (*Jinten*), and IV (*Melati tumpeng*). Table 3 explained differences in the number of sperms among groups. Control Group (Group I) showed differences from *Rejeb*, *Jinten*, *Melati tumpeng* Groups with p -values of 0.001, <0.001 and 0.001, respectively, while other groups (*Rejeb*, *Jinten*, *Melati tumpeng*) did not show any significant differences with another groups.

Fig 2 shows the histopathological comparison of the numbers of sperms in the control and intervention groups.

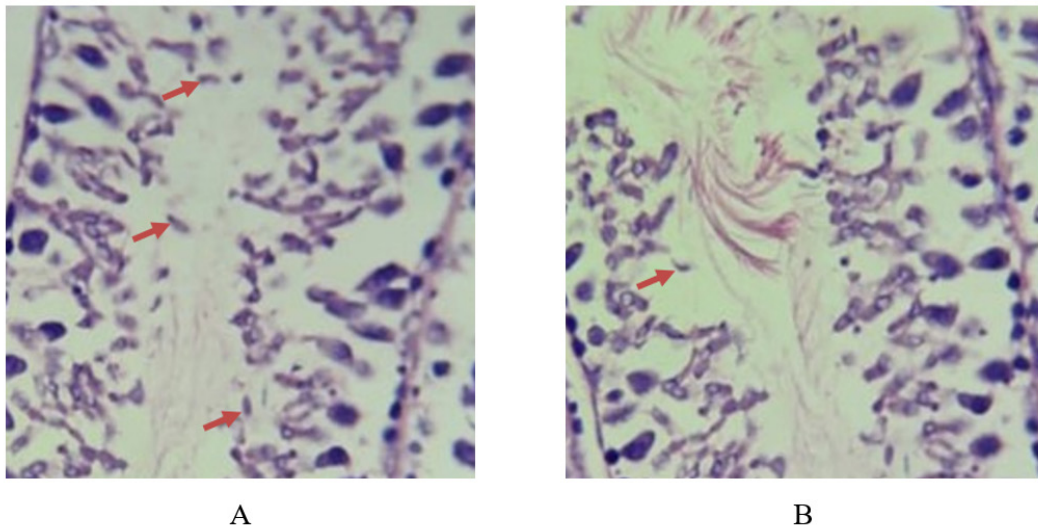


Fig 2 - Histopathology of the seminiferous tubule (Hematoxylin & Eosin – 400x; red arrows indicate spermatozoa)

A: Control group; B: Experimental group

DISCUSSION

Exposure to tobacco smoke has negative health impacts. This is indicated by the results of studies showing an increase in the number of alveolar macrophages in the lungs and a decrease in the number of sperms in the testes. The increase in alveolar macrophages has positive implications for alveolar damage in the lungs. This damage can affect the process of respiration (exchange of O₂ and CO₂). This situation, of course, will reduce the level of quality of life. Same with alveolar macrophages, sperm count can also be used as an indicator for decreased quality of life. Decreased sperm count is closely related to the quality of fertility (Suryadinata and Wirjatmadi, 2021). The increased number of alveolar macrophages was caused by free radicals entering the respiratory tract as exposed to tobacco smoke (Suryadinata *et al*, 2021b). There are two types of macrophages that can appear in the lungs, between the alveolar and interstitial macrophages. Both conditions reflect two conditions that are different from one another. In particular, alveolar macrophages are found in alveolar cells, both type I and II, indicating damage that causes a decrease in the quality of respiration. Meanwhile, interstitial macrophages show damage to the lung parenchyma (Hussell and Bell, 2014). In normal conditions, alveolar macrophage functions by secreting inflammatory cytokines at a lower level, maintaining phagocytosis capacity, and reducing the inflammatory response and adaptive immune system (Hu and Christman, 2019).

The alveolar macrophage is the first immune system to fight various pollutants and pathogen bacteria in the lungs. There were two types of macrophages identified which are macrophage type 1, which improves the phagocytosis process and stimulates the release of pro-inflammatory cytokines. While macrophage type II functions by stimulating apoptosis cell phagocytosis, anti-inflammatory cytokine secretion, collagen disposition, and tissue injury recovery (Viola *et al*, 2019). Free radicals entering the respiratory tract due to exposure to tobacco smoke will induce alveolar macrophages to increase by 4-5 times compared to the number of alveolar

macrophages in the normal lungs. The increased number of alveolar macrophages is strongly related to chemotactic factors produced in the lungs due to the activation of pro-inflammatory transcription activation factor, nuclear factor kappa B (NF- κ B), and interleukin-8. Bronchi epithelial cells stimulating the release of interleukin-8 functions as stimulating the transfer of macrophages and neutrophils to the lungs (Suryadinata and Wirjatmadi, 2020; Lugg *et al*, 2022).

Exposure to tobacco smoke also influences sperm production which then influences its number. It is related to the imbalanced number of free radicals and antioxidants in the body which leads to oxidative stress. In normal conditions, free radicals deal with sperm ovulation, spermatozoa fertilization, motility, hyperactivity, and sperm capacity. The excessive production of reactive oxygen species (ROS) as free radicals will decrease the quality and function of sperms and their number. Oxygen, thus, plays an important role in this process through mitochondria oxidative related to adenosine-5-triphosphate (ATP) (Lushchak, 2014). The medium increase in the number of free radicals will contribute to the immune response and homeostasis of cells, while the high increase may result in cell damage due to the protein, lipid, and nucleate acid (DNA or RNA) peroxidation (Kruk *et al*, 2019). Besides, factors such as environmental factors, physical exercises, lack of antioxidants, and immune system dysfunction may cause oxidative stress. Tissue injury and cell death lead to changes in the reproductive system.

Free radicals stimulate cyclic adenosine monophosphate (cAMP) in the sperm which then stimulates tyrosine phosphorylase through tyrosine phosphatase inhibition. Spermatozoa will experience changes in their cellular, morphological, and dynamicity characteristics, thus it results in decreasing fertility (Wagner *et al*, 2018). Lipid peroxidation caused by free radicals is related to fluidity and changes in membrane permeability, enzyme and membrane-bound receptor inhibition, and apoptosis cascade activation which further contributes to oxidative stress causing motile and

morphological disorders in the sperms (Mannucci *et al*, 2021). The lack of an antioxidant system may make spermatozoa vulnerable to DNA oxidation (Aitken *et al*, 2014). Besides, spermatogenesis is influenced by the Leydig cell which functions as secreting androgen home, and the secretion is influenced by luteinizing hormone (LH). The nicotine level in the tobacco leaves may influence testosterone hormone secretion, and it then disrupts the spermatogenesis process through apoptosis in the Leydig cell (Suryadinata *et al*, 2021a)

In summary, various types of tobacco in Indonesia are used as the main cigarette material, and they can cause negative health impacts. One of them is the transfer of free radicals to the lungs, increasing the number of alveolar macrophages and decreasing the number of sperms.

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CONFLICT OF INTEREST DISCLOSURE

The authors declare no conflict of interest.

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