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The effect of cigarette smoke on the histopathological changes of the eustachian tube of Wistar rat

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ABSTRACT

Background: Inflammation due to irritation of cigarette smoke causes metaplasia of the squamous epithelium of the Eustachian tube. Data on the prevalence and incidence of Eustachian tube dysfunction are still limited. The study aims to determine the effect of exposure to a stratified dose of cigarette smoke on the histopathological changes of the Eustachian tubes of Wistar rats.

Method: This study was a true experimental study with post-test only control group design using Wistar rats as the subjects. We included 24 Wistar rats in the study and divided them into four groups. The number of Wistar rats in each group was 6. The control group was not exposed to cigarette smoke. The treatment group 1, 2, and 3 were each exposed to four cigarettes, eight cigarettes, twelve cigarettes per day for thirty days, respectively. We assessed the Eustachian tube histopathological changes using four parameters, namely inflammatory cells, cilia, goblet cells, and epithelium. Finally, we analyzed the data using the Kruskal-Wallis test and the Mann-Whitney test.

Result: The Eustachian tube's histopathological changes occurred in inflammatory cells, cilia, goblet cells, and epithelial metaplasia. Group 1 showed grade 1 changes in all parameters assessed. Group 2 showed grade 2 changes in all parameters evaluated. Meanwhile, group 3 showed grade 3 changes in all parameters evaluated compared to other groups ($p<0.05$). There was a significant difference between the treatment group and the control group ($p<0.05$).

Conclusion: Cigarette smoke with stratified doses affects the histopathological changes of the Eustachian tube.

Keywords: cigarette smoke, eustachian tube, Wistar rat.
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INTRODUCTION

The Eustachian tube has three main functions to the ear, namely: (a) Balancing the pressure and ventilation of the middle ear and the mastoid air cells in the middle ear; (b) Drainage of middle ear secretions (mucociliary clearance) (c) Protection of infections originating from the nasopharyngeal area. Eustachian tube obstruction can occur due to several factors, including anatomical, physiological and pathological abnormalities.[1,2]

Exposure to cigarette smoke irritants can cause pathological abnormalities in the form of Eustachian tube obstruction.[3,4] Cigarette smoke can make changes to the Eustachian tube in three ways: 1) direct contact between the smoke and the Eustachian tube mucosa due to its proximity to the upper airway, 2) secondary changes due to Eustachian tube occlusion caused by inflammation and swelling of the nasopharyngeal inlet, and 3) exposure to cigarette smoke can produce mediators that stimulate middle ear disorders due to its association with the upper respiratory tract through the Eustachian tube.[5] Studies on passive smokers with impaired tubal function have not been widely reported. The previous study on the exposure to cigarette smoke in rats for one month disrupted its vascularity because the inflammatory process showed damages to cilia, squamous metaplasia, and goblet cell aplasia, followed by Eustachian tube mucosal hyperplasia.[6]

Previous studies reported that exposure to the same dose and at different times caused insignificant histopathological changes of the Eustachian tube.[7] The purpose of this study was to determine the effect of exposure to a stratified dose of cigarette smoke on the histopathological changes of the Eustachian tubes of Wistar rats.

METHOD

This study was a true experimental study with post-test only control group design using Wistar rats as the subjects. We included 24 Wistar rats in the study. The number of Wistar rats in each group was six. The inclusion criteria in this study were the age of the rats was eight weeks, bodyweight of 150-200 grams, healthy and active movement (visual appearance of the hair is not dull, no hair loss, and no anatomical abnormalities in the ear). Meanwhile, the exclusion criteria were a dramatic weight loss of more than 10%

after adaptation. We divided the subjects into four groups. The control group was not exposed to cigarette smoke. Meanwhile, the treatment group 1, 2, and 3 were each exposed to four cigarettes, eight cigarettes, twelve cigarettes per day for thirty days, respectively.

We conducted the study at the Biology Laboratory of the Faculty of Mathematics and Natural Sciences, the University of Semarang for the treatment of experimental animals (necropsy). This study's independent variable was exposure to cigarette smoke, while the dependent variable was the histopathological changes of the Eustachian tube. Two anatomical pathologists carried out the making and interpretation processes of the Eustachian tube histopathological preparations from the Faculty of Medicine of the University of Diponegoro. The kappa compatibility test between two observers was observed in terms of the compatibility of four parameters of the histopathological changes of the Eustachian tube, including inflammation, cilia, goblets, and epithelium, with the degree of the histopathological change was divided into four grades, namely grade 0, 1, 2, and 3. Grade 0 shows no abnormalities or changes (normal), Grade 1 shows mild changes (<30%), Grade 2 shows moderate changes (30%-50%), and Grade 3 shows severe changes (>50%). Statistical hypothesis testing was done using the Kruskal-Wallis test and the Mann-Whitney test.

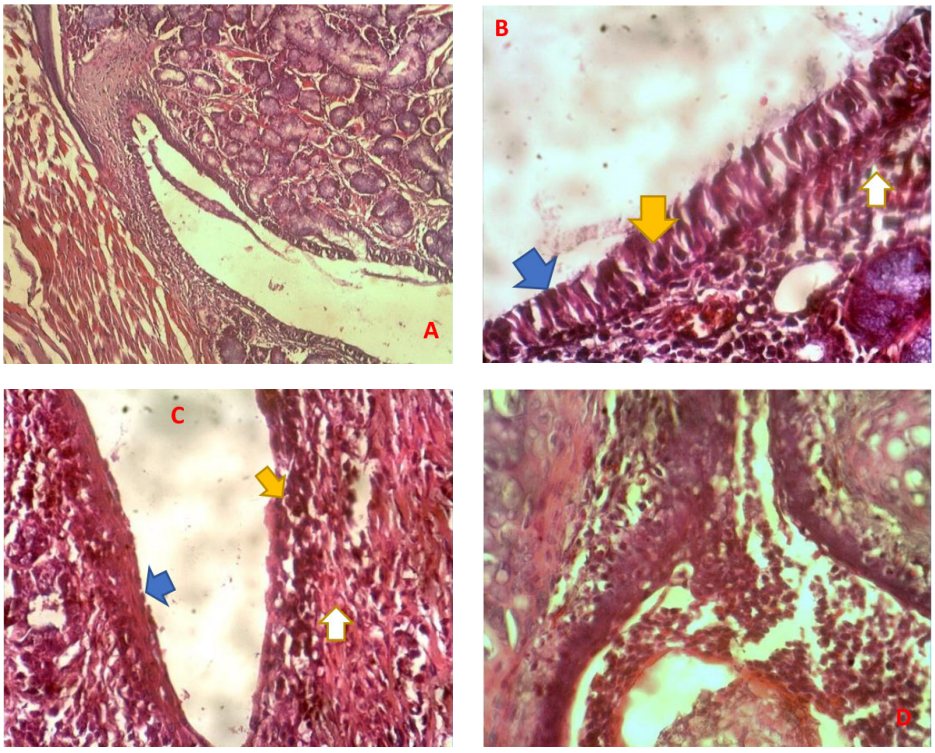

Figure 1. **(A)** Control group (magnification 100x). The eustachian tube of the Wistar rat is shaped like an elongated duct. Eustachian tube mucosa shows no changes or abnormalities. **(B)** Treatment group 1 (magnification 400x). There is inflammation and decreased cilia. Blue arrows: cilia cells, yellow arrows: goblet cells, white arrows: inflammatory cells. **(C)** Treatment group 2 (magnification 400x). There is increased inflammation, reduced cilia, and increased goblets. Squamous epithelial metaplasia is seen. **(D)** Treatment group 3 (magnification 400x). The mucosa has been infiltrated by inflammatory cells in, and outside the eustachian tube mucosa, the mucosal surface looks thickened by edema and fibrosis. Cilia and goblet cells are almost absent. Squamous epithelial metaplasia is seen.

RESULT

We included twenty-four Wistar rats as the study subjects. The microscopic examinations in the control group did not show any histopathological changes of the Eustachian tube. In the treatment group 1, there were two Wistar rats with grade 2 changes regarding the epithelial metaplasia parameters, while the other four rats of group 1 showed no changes. There were two rats with grade 2 cilia changes regarding cilia parameters, while the other four rats of group 2 showed grade 1 changes. In the treatment group 2, there were grade 2 changes in all the parameters assessed, while in the treatment group 3, there were the most severe histopathological changes of the Eustachian tube (grade 3) in all parameters. All study histological slide could be seen in Figure 1 and comparison of mean in all parameters could be seen in Figure 2.

The results of the 'compatibility' test between the two observers were: inflammatory cells (κ: 0.889), cilia (κ: 0.949), goblet cells (κ: 0.987), and epithelial metaplasia (κ: 0.826). The results of the histopathological preparations carried out by two anatomical pathologists showed a very good 'compatibility' between the two examiners.

Table 1, shows the results of significant histopathological changes in inflammatory cells of the Eustachian tube between the treatment and the control groups.

Table 2, proves that the inflammatory cells between the treatment group 1 and 2 were not significant (p=1.000), but for the other groups, the results were significant (p<0.05)

Table 3, shows the results of significant histopathological changes in the Eustachian tube epithelium between the treatment and the control groups. Table 4, proves that the epithelial changes between the control group and treatment group 1 were not significant (p=0.138), but for the other groups, there were significant results (p<0.05)

Table 5, shows histopathological changes in cilia of the Eustachian tube between the treatment and the control groups. Table 6 proves a significant grade of cilia changes in treatment group 3 (p<0.05).

Table 7, shows the results of the histopathological changes in goblet cells of the Eustachian tube between the treatment and the control groups. Meanwhile, table 8

Table 1. **The test results of inflammatory cell between the treatment and control groups**

Group	Inflammation								p
	Grade0		Grade 1		Grade2		Grade3		
	n	%	n	%	n	%	n	%	
Control	6	100	0	0	0	0	0	0	<0.001*
Tr.1	0	0	0	0	6	100	0	0	
Tr.2	0	0	0	0	6	100	0	0	
Tr.3	0	0	0	0	0	0	6	100	

Tr.1: Group treatment 1
Tr.2: Group treatment 2
Tr.3: Group treatment 3

Table 2. **The result of difference test in the inflammation between the groups**

Inflammation	Tr. 1	Tr.2	Tr.3
Control	0.001*	0.001*	0.001*
Treatment 1	–	1.000	0.001*
Treatment 2		–	0.001*

Tr.1: Group treatment 1
Tr.2: Group treatment 2
Tr.3: Group treatment 3
*Mann-Whitney test: Significant (p<0.05)

Table 3. **The test results of the epithelial change between the treatment and the control groups**

Group	Epithelial changes								p
	Grade 0		Grade 1		Grade 2		Grade 3		
	N	%	N	%	n	%	N	%	
Control	6	100	0	0	0	0	0	0	<0.001*
Tr.1	4	66.7	0	0	2	33.3	0	0	
Tr.2	0	0	0	0	6	100	0	0	
Tr.3	0	0	0	0	0	0	6	100	

Tr.1: Group treatment 1
Tr.2: Group treatment 2
Tr.3: Group treatment 3
*Kruskal-Wallis test: Significant (p <0.05)

Table 4. **The test results of the epithelial metaplasia change between the treatment groups**

Epithelial change	Tr. 1	Tr. 2	Tr. 3
Control	0.138	0.001*	0.001*
Treatment 1	–	0.019*	0.002*
Treatment 2		–	0.001*

Tr.1: Group treatment 1
Tr.2: Group treatment 2
Tr.3: Group treatment 3
*Mann-Whitney: Significant (p<0.05)

proves that the changes in goblet cells in the control group and treatment group 1 were not significant (p=0.138), but for the other groups, the results were significant (p<0.05).

DISCUSSION

Cigarette smoke is formed by mainstream smoke and sidestream smoke. The mainstream smoke is tobacco smoke inhaled directly by smokers, while the sidestream smoke is tobacco smoke spread into the air. In other words, the sidestream smoke is the smoke inhaled by other people or passive smokers. The chemical content in the sidestream smoke is higher than the chemical content in the mainstream smoke, partly because tobacco burns higher at low temperatures when the cigarette is not being smoked. The result of this process is that the combustion is less complete so that it releases more chemicals.[6,8,9]

Cigarette smoke exposure was shown to influence the histopathological changes in the mucosal lining of the Eustachian tube and the middle ear. One of the damages caused by cigarette smoke is oxidative stress. Oxidative stress caused by cigarette smoke will induce an inflammatory response that can cause changes in the eustachian tube histopathology.[10] Exposure to cigarette smoke in experimental animals caused anatomical and physiological lesions in the Eustachian tube, namely inflammation, reduced cilia and goblet cells, as well as epithelial changes, all of which can eventually cause ear symptoms. Previous studies used the parameters of inflammatory cells, cilia, goblet cells and epithelial metaplasia. The subjects of the study were exposed to 5 cigarettes consecutively during a 2.5 hours cycle. Several groups of rats were observed based on the differences in exposure time. The results of histopathological changes at certain times were not significant.[7]

The results of this study showed that the control group did not show histopathological changes in the Eustachian tube. Furthermore, there were no abnormalities of cilia and goblet cells, no inflammation and squamous epithelial metaplasia. There were similar results of the inflammation changes between treatment groups 1 and 2 (grade 2). These

Table 5. The test results of the changes in cilia between the treatment and the control groups

Group	Cilia								P
	Grade 0		Grade 1		Grade 2		Grade 3		
	n	%	n	%	n	%	n	%	
Control	6	100	0	0	0	0	0	0	<0.001*
Tr.1	0	0	4	66.7	2	33.3	0	0	
Tr.2	0	0	0	0	6	100	0	0	
Tr.3	0	0	0	0	0	0	6	100	

Tr.1: Group treatment 1
Tr.2: Group treatment 2
Tr.3: Group treatment 3
*Kruskal-Wallis test: Significant (p<0.05)

Table 6. The test result of the changes in cilia between the treatment groups

Cilia	Tr. 1	Tr. 2	Tr. 3
Control	0.002*	0.001*	0.001*
Treatment 1	–	0.019*	0.002*
Treatment 2		–	0.001*

Tr.1: Group treatment 1
Tr.2: Group treatment 2
Tr.3: Group treatment 3
*Mann-Whitney test: Significant (p<0.05)

Table 7. The test result of the changes in goblet cells between the treatment and the control groups

Group	Goblet cells								P
	Grade 0		Grade 1		Grade 2		Grade 3		
	N	%	n	%	n	%	n	%	
Control	6	100	0	0	0	0	0	0	<0.001*
Tr.1	0	0	0	0	4	66.7	2	33.3	
Tr.2	0	0	0	0	6	100	0	0	
Tr.3	0	0	0	0	0	0	6	100	

Tr.1: Group treatment 1
Tr.2: Group treatment 2
Tr.3: Group treatment 3
*Kruskal-Wallis test: Significant (p<0.05)

Table 8. The test results of the changes in goblet cells between the treatment groups

Goblet Cells	Tr. 1	Tr. 2	Tr. 3
Control	0.138	0.001*	0.001*
Treatment 1	–	0.019*	0.002*
Treatment 2		–	0.001*

*Mann-Whitney test: Significant (p<0.05)

results indicated that exposure to cigarette smoke with a dose of at least four cigarettes gave the same results with a larger dose.

Previous studies showed the results of the analysis of the Eustachian tube function of active smokers with a mild degree of tubal function disorders, which was proven by the present study. There were significant histopathological changes in cilia of the Eustachian tube in the treatment and the control groups. The smallest doses of cigarette smoke can cause damages to cilia. Cigarette smoke can reduce the function of the cilia due to its location in the superficial layer. This condition makes them easily damaged by exposure to cigarette smoke. Exposure to cigarette smoke in only a month is enough to reduce the function of the cilia.[11,12]

In this study, the treatment group 1 and 2 showed similar grades of histopathological changes in goblet cells. In treatment group 1, four Wistar rats had moderate histopathological changes. These results were identical to those of treatment group 2, with a total of six rats having moderate histopathological changes. Cilia and goblet cells of the Eustachian tube have an important role as a mucociliary transport system, namely the mechanical defense of the tubal mucosa consisting of a mucous blanket. Cigarette smoke affects the activity of cilia and goblet cells in moving the mucous blanket to the proximal side of the Eustachian tube, which is one of the most important parts in the mucociliary clearance system.[13,14]

Changes in squamous epithelial metaplasia between the control group and treatment group 1 were not significantly different. Treatment group 3 showed a higher grade of histopathological change (grade 3). The mucosal epithelial layer appeared to be dominated by stratified squamous epithelial cells; this suggested that there was a differentiation of the ciliated columnar epithelium into stratified squamous epithelium or squamous metaplasia. This metaplasia occurred as a cellular response to injury or persistent irritation by the exposed cigarette smoke. The ciliated columnar epithelium could not survive the irritating chemicals contained by cigarette smoke, causing a differentiation into the stratified squamous epithelium.[15,16]

CONCLUSION

This study indicated that exposure to a stratified dose of cigarette smoke affected the histopathology changes of the Eustachian tube of Wistar rats. The greater the amount of exposure to cigarette smoke, the more severe the histopathological

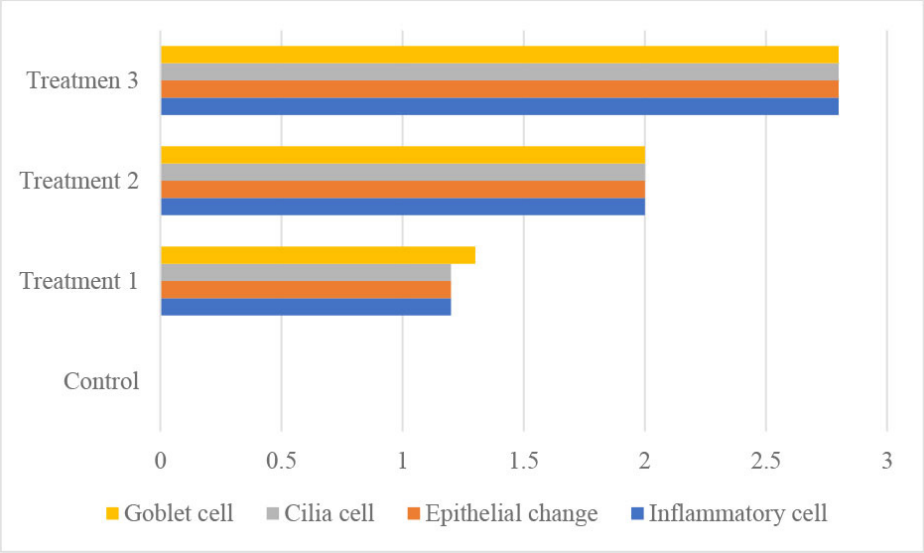

Figure 2. The comparison of the mean parameters between the treatment groups

changes occurred.

ETHICAL CONSIDERATION

We obtained the ethical approval from the Health Research Ethics Committee (KEPK), Faculty of Medicine, Universitas Diponegoro, Semarang, Indonesia with ethical clearance reference number No. 21/EC/H/FK-UNDIP/III/2020.

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None.

CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTION

All authors had contributed equally in the manuscript preparation and review. All author had agreed for the final form of manuscript for publication

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