Hyperplasia of Wistar rat’s tongue mucosa due to cigarette side-stream smoke exposure

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ABSTRACT

Background: *Hyperplasia is an increasing amount of cells refers to cell division. Hyperplasia is usually a sign that leads to carcinogenesis. Carcinogenesis is a development process of cancer. Cancer is a manifestation of cell malignancy during abnormal proliferation. Oral cancer is the 6th deadly case of cancer around the world. The most common etiology of oral cancer is tobacco and cigarette is the most well-known tobacco's product. Effect of cigarette smoke not only affects to active smoker but also passive smoker. Sidestream smoke that comes from the lighted end of a burning tobacco product such as a cigarette, pie or cigar. It contains nicotine and many harmful, cancer-causing chemicals. Inhaling sidestream smoke increases the risk of lung cancer and other types of cancer.* ***Purpose:*** *The purpose of this study was to understand how sidestream cigarette smoke initiates precancerous changes, which in this study is hyperplasia, of the oral mucosa epithelium in Wistar rats.* ***Methods:*** *Wistar rats were divided into 3 groups, treatment groups (4 weeks (P1 group) and 8 weeks (P2 group)), and control group (K group), each group consisting of 10 rats. Wistar rats were exposed to cigarette smoke with the following dose which was 2 cigarettes per day. The experiment used the post-test only control group design. All samples were sacrificed in the 4th and 8th weeks. Haematoxylin-eosin staining was performed on Wistar rat's tongue to observe the presence of hyperplasia. Data were analyzed by One-way ANOVA test.* ***Results:*** *After Wistar rats were exposed to cigarette smoke, an increased amount of epithelial cell proliferation (hyperplasia) showed a significant difference with p-value <0.05 at 8th week.* ***Conclusion:*** *There is an increasing amount of epithelial cell proliferation (hyperplasia) in Wistar rats after exposure to cigarette sidestream smoke.*

Keywords: *cigarette smoke; hyperplasia; oral cancer*

INTRODUCTION

Body cells have a process of proliferation and growth of the cells. Body cell growth is a well-regulated gene regulation phenomenon. When damage occurs, cell growth will be increased as part of the body's defense mechanism. The surrounding cells of the damaged cells will increase their growth to restore the normal structure and function of the tissue. After the tissue has functioned normally, the cells will perform the cell death program named apoptosis. Then, the body will produce new cells to replace the dead cells[1]. The process of cell progression and apoptosis is not always going well because there are some risk factors such as stress, chemicals, toxins, bacteria, viruses, parasites, fungi, and genetics. However, the cells will survive through a mechanism of adaptation. Mechanism of cell adaptation may occur through atrophy, hypertrophy, hyperplasia, dysplasia, and metaplasia[1–5].

Hyperplasia is also defined as abnormal proliferation (multiplication) of cells because cell proliferation occurs continuously[1]. Hyperplasia can be used as a sign of the onset of

carcinogenesis. Cancer is a form of cell malignancy that proliferates abnormally. Head and neck cancer is the sixth most common cancer in humans. 48% of the cases are located on the oral cavity, 90% of it are oral squamous cell carcinoma (OSCC). The common etiology of OSCC is tobacco use in various forms (22%)[6–9]. In passive smokers, the risk of oral cancer increases by 87% compared to non-smokers who are not exposed to cigarette smoke[8–11]. Cigarette smoke inhaled by non-smokers is called passive smokers. The inhaled smoke is called secondhand smoke. If the source of smoke inhaled is from the cigarette itself then the smoke called sidestream smoke while smoke released by the smoker called exhaled mainstream smoke[6,7,12,13].

Cigarette smoke contains approximately 60 carcinogenic substances. The mechanism of cigarette smoke that has been recognized to cause cancer is through free radical metabolism production by the human body and DNA damage which results in gene mutation. In the OSCC, the lesion is a premalignant lesion of the upper aerodigestive tract (UADT). UADT lesions show an increase in epithelial proliferation that can be used as a sign of the early stages of OSCC[5]. The purpose of this study was to understand how cigarette side-stream smoke initiates precancerous changes, which in this study is hyperplasia, of the oral mucosa epithelium in Wistar rats.

MATERIALS AND METHODS

This research is a laboratory experimental research with post-test only control group design. This research used a sample of 30 male Wistar rats (Rattus novergicus), age 3 months, body weight 170 (± 10%) grams. The material used in this research were clove cigarettes. The animals were randomly divided into 3 groups, consisted of 2 treatment groups (P1 group treated for 4 weeks and P2 group treated for 8 weeks), and 1 control group (K group), each group consisting of 10 rats.

The exposure of cigarette smoke was performed by using a device called the smoking pump. Each rat received exposure to cigarette smoke as much as 2 cigarettes per day which was performed 1 time a day[14]. This amount is maintained until the rat's sacrifice time. Rats from the control group were placed on different tubes to obtain simultaneous air exposure. To control the difference smoke exposure between the tubes in the smoking pump, the placement of rats in each tube is rotated (Figure 1).

At the end of the 4th and 8th weeks, each rat was sacrificed. Then the rat's tongue is collected and histopathological examination was performed by Hematoxylin-Eosin (HE)

staining. The microscopic assessment used a light microscope with 1000x magnification at random 5 different visual fields and compared between the K, P1, and P2 groups.

Kolmogorov-Smirnov test was performed to discover whether the data obtained has been normally distributed (value p>0.05). Then the data of the research treatment groups tested with the homogeneity test using Levene's test followed by One-way ANOVA test. Normally distributed data is forwarded to Tukey HSD to determine differences between groups (value p<0.05).

RESULTS

The contents of Table 1 show that there is an increase in the thickness of the epithelium and stratum corneum of the rat's tongue. Epithelial and stratum corneum thickness increases, observed from the K group compared to the P1 and the P2 groups. Epithelium thickness served as hyperplasia and stratum corneum thickness served as hyperkeratosis.

The Tukey HSD test result, showed a significant increase in terms of hyperplasia on the K group compare to the P2 group with p-value p=0,000 (p<0.05). While hyperplasia on the K group compare to the P1 group and the P1 group compare to the P2 group showed an increase by p-value (p=0.067) and (p=0.076) respectively. While in terms of hyperkeratosis on the K group compare to the P2 group with p value p=0.014 (p<0.05). While hyperplasia on the K group compare to the P1 and the P1 group compare to the P2 group showed an increase by p-value (p=0.566) and (p=0.127) respectively.

Figure 2 shows the histopathological features of the rat's tongue mucosa (one representative field of view), it can be observed that there is increased hyperplasia and hyperkeratosis in the K group compare to the P groups.

DISCUSSION

Cancer is a form of cell malignancy that loses its proliferation control. Cancer occurs through the process of carcinogenesis. The early stage of carcinogenesis is characterized by hyperplasia. Hyperplasia is often accompanied by hyperkeratosis. Oral cancer is the sixth deadly cancer in the world. The most common etiology of oral cancer is tobacco and cigarettes, these products most commonly found in the community environment[1,3–5].

The study was conducted based on a simple hypothesis about the possibility of hyperplasia occurrence in Wistar rats exposed to cigarette smoke in a certain period of time. In this study, we used male Wistar rats (*Rattus norvegicus*) because they were not affected by hormonal conditions their body weight is maintained during a week adaptation process to be ideal at 170

grams (± 10%). Wistar rats were chosen because of the similarity of its immune system and oral mucosa with humans. Wistar rats also respond to tumor antigens with T lymphocytes and have lymphatic drainage which is similar to humans. The oral epithelium on the inferior surface of the rat tongue is much thinner (8-12 layers) than humans (20-30 layers) with the same grade of rete ridge[3,15].

Previous cigarette smoke research using Wistar rats, which has been carried out through three treatment methods, namely inhalation exposure via the respiratory tract, whole body exposure, and nicotine injection. Nasal inhalation methods require oxygen masks, but the masks may be damaged quickly because rats belong to rodent species. Besides, the results of the hyperplasia histology occurrence with the nasal inhalation method are not significant. Based on these considerations, most researchers chose the whole-body exposure method. The whole body exposure method provides enough room for the animal while being treated but the exposure dose of cigarette smoke is not centered on the oral cavity but throughout the body. Even so, the histology results obtained from the last mentioned method remain significant. Based on that, this study chooses the whole-body exposure method with modication[16].

The cigarettes selected for this study were clove cigarettes. Clove cigarettes are chosen because their nicotine level is two times higher than cigarettes commonly consumed by the public. Nicotine in cigarettes commonly consumed by the public is 1.1 mg while nicotine content in clove cigarettes is 2 mg. Clove cigarettes contain 40% clove and 60% tobacco. This cigarette has several different compositions compare to white cigarettes. Clove cigarettes contain 5 additional compositions namely eugenol, acetyl eugenol, β-caryophyllene, α-humulene, and caryophyllene epoxide[6,16,17].

In a previous study, in vivo oral cavity (male Wistar rats) exposed cigarette smoke for 60 days showed premalignant lesions, 60 days were not enough to cause cancer, based on the absence of p53 protein expression. However, the Ki-67 analysis showed an increase in epithelial proliferation as the damage response occurred[16]. So that we choose 4 weeks and 8 weeks time point-exposure based on the previous study.

Wistar rat tongue was chosen because, in previous studies, these areas often showed significant features of hyperplasia and hyperkeratosis. In addition, 40% of OSCC cases are found on the tongue. The three groups showed differences in the effect of cigarette smoke on changes in epithelium and stratum corneum thickness[3,16,18]. Based on the results of histopathological examination of the K, P1, and P2 groups, there was a significant difference in the epithelial thickness of epithelium and stratum corneum. Based on Table 1, the K group which did not expose to cigarette smoke showed an average hyperplasia value of 179.89μm

and an average hyperkeratosis value of 64.38μm. When compared with the P1group and the P2 group, the K group becomes the thinnest while the P2 group becomes the thickest. The P1 group showed hyperplasia value as high as 206.01μm and hyperkeratosis value 68.97μm while the P2 group showed hyperplasia and hyperkeratosis value of 231.46μm and 77.97μm, respectively.

Based on these results, clove cigarette smoke affects the proliferation that occurs in the epithelium and stratum corneum. Clove cigarette smoke which exposed continuously to the oral mucosal epithelium may damage the structure and function of the epithelium. Nicotine in cigarette smoke contains *tobacco-specific N-nitrosamines* (TSNA) derivatives. TSNA has carcinogen substances in the form of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N-nitrosonornicotine (NNN). The cigarette smoke molecule can induce *epidermal growth factor receptor* (EGFR) phosphorylation, mediating cytoplasmic tail (CT) mucin-1 (MUC1) phosphorylation. This then causes the cleavage of β-catenin from E-cadherin to form the β-catenin/MUC1-CT complex of the Wnt/β-catenin canonical pathway. Resulting in complex translocation from the cytosol to the nucleus and joins the family of *T-cell factor/lymphoid enhancer-binding factor* (TCF/LEF) transcription factors, leading to the transactivation of genes that drive cell cycles (eg C-Myc, cyclin-D)[19–21].

EGFR which continuously binds to the molecular components of cigarette smoke causes an increase in epithelial cell proliferation, decreased attachment between cells, and cells will experience abnormal migration. This occurs because the activation of EGFR by cigarette smoke causes the loss of the E-cadherin/β-catenin complex which mediates adherence between cells. Decreased levels of E-cadherin cause metastatic cancer. Thus, cigarette smoke can increase epithelial proliferation and migration through activation of EGFR and reduction of E-cadherin, which causes cancer development[19,21].

In addition, cigarette smoke also contains free radicals such as *reactive oxygen species* (ROS) and *reactive nitrogen species* (RNS) which increase oxidative stress in the body. Excessive oxidative cells can trigger epithelial cell activity through various signaling pathways. Among them are ERK1/2, P38, JNK, and NF-κβ. The pathway will lead to the secretion of proinflammatory cytokines. Exposure to cigarette smoke will also stimulate the expression of Ki67 in the mucosal tissues of the oral cavity. Ki67 is a protein that is crucial in cell cycle progression and its increase leads to higher cell proliferation. Judging from the level of cell proliferation exposed to cigarette smoke, there is a mechanism of resistance to apoptosis. Cigarette smoke exposure repeatedly given to oral epithelial cells shows a decrease in Bax expression (proapoptotic protein) and an increase in Bcl-2 (antiapoptotic protein)[22–26].

Exposure to cigarette smoke that modulates epithelial cell proliferation may affect on keratin protein expression. Cigarette smoke reduces the expression of proteins Keratin1, Keratin5, Keratin10, Keratin16 and stimulates the expression of Keratin6 and Keratin14 proteins. Cigarette smoke that modulates gene expression related to keratin is often associated with protein production. Increasing Keratin14 protein, indicates an increase in stratum basal cell proliferation. Conversely, a decrease in Keratin10 expression due to cigarette smoke which can increase cell proliferation shows the Keratin10 function, as a negative modulator in the cell progression cycle[18,25].

The disrupted cell cycle and cytoskeleton protein result in the abnormal orientation of basal and suprabasal cells. Basal and suprabasal epithelial cells will experience increased proliferation and migration. Continuously activated EGFR will result in increased uncontrolled proliferation and migration of cells without being offset by apoptosis so that cells will metastasize and become malignant cells. The process of proliferation enhancement that occurs was pathological hyperplasia and subsequent involvement of keratin cytoskeleton protein allows hyperkeratosis[25,26].

Results obtained in this study were in line with previous studies, mice exposed to cigarette smoke for one month, considered as long-lasting (chronic) cigarette exposure may increases the thickness of the airway epithelium as the body's defenses higher against cigarette smoke compounds[21]. Based on this, the ratio of K to P1 and P1 to P2 becomes insignificant because of the ability of mice to tolerate cigarette smoke compounds, in 28 days. However, a significant comparison between K and P2 occurred because the P2 group has carried out the mucosal defense against cigarette smoke and simultaneously adapted to exposure to cigarette smoke in a period of 8 weeks by proliferation enhancement which is pathological hyperplasia along with hyperkeratosis. Thus, the statistical results obtained are K group to P2 group showed significant difference while K group to P1 group and P1 group to P2 group showed non significant difference.

Since hyperplasia is a reversible condition, further experiment needs to be done, to observe any histopathological changes due to extended exposure of side stream cigarette smoke. To support hypothesis of hyperplasia, in terms of abnormal proliferation (multiplication) of cells caused by chronic irritation, as a sign on the onset of carcinogenesis since cancer is a form of cell malignancy that proliferates abnormally[1,3–5,26]. Taken together, it can be concluded that sidestream cigarette smoke exposure initiates chronic irritation which may leads to prolonged inflammation and precancerous changes in oral mucosa, which is in

this study showed by Wistar rat's tongue mucosal's response of pathological hyperplasia along with hyperkeratosis.

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Table 1. Average and standard deviations of increased thickness of the epithelium and stratum corneum

Layer	K (μm)	P1 (μm)	P2 (μm)
Epithelium	179.9±23.15	206.02±28.55	231.46±22.79
Stratum Corneum	64.38±11.33	68.97±8.94	77.97±9.48

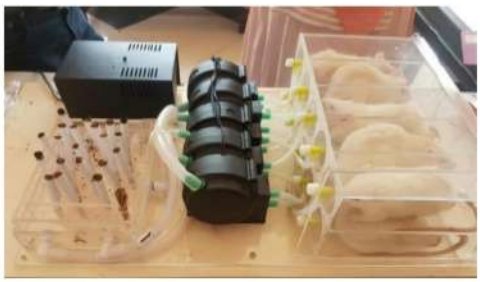

Figure 1. The process of handling experimental animals exposed to cigarette smoke with a device of full-body exposure.

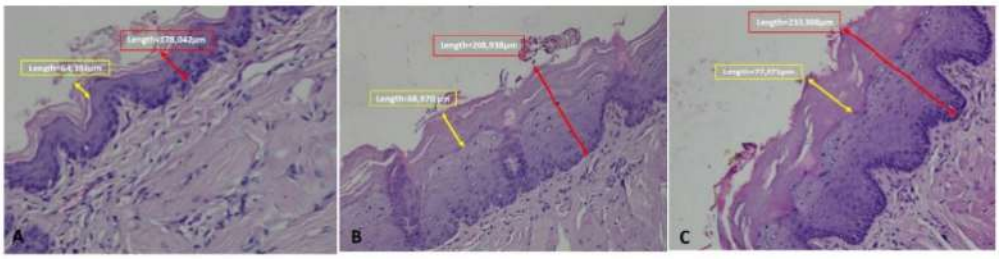

Figure 2. Histopathological features of the rat's tongue mucosa (one field of view). (A) The thickness of the epithelium and stratum corneum in the K group; (B) Epithelial and stratum corneum thickening in the P1 group; (C) Thickening of the epithelium and stratum corneum in the P2 group (1000x). *The yellow line shows hyperkeratosis while the red line shows hyperplasia.

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