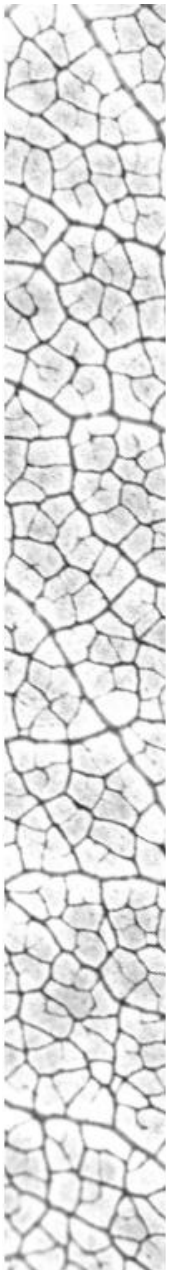

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Buccal and labial mucosa dysplasia in Wistar rats exposed to sidestream cigarette smoke as early detection of precancerous lesions

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Abstract

Background: Sidestream cigarette smoke contains cancer-causing substances. Risks of cancer, especially oral cancer, will most likely increase in people who are exposed to sidestream cigarette smoke. Dysplasia is a histopathological picture that indicates abnormal activities in the normal epithelium. The assessment of epithelial dysplasia in the oral cavity is vital to predicting malignancy development. This study aims to identify the existence of buccal and labial mucosa dysplasia in Wistar rats (*Rattus norvegicus*) that are exposed to sidestream cigarette smoke by observing the level of dysplasia in both buccal and labial mucosa in the fourth and eight weeks after the initial exposure.
Method: The Wistar rats were divided into three groups: group 1 that was exposed to the smoke for four weeks; group 2 that was exposed to the smoke for eight weeks; and the control group that was not exposed to any smoke. The buccal and labial mucosa of the rats were then examined histopathologically to identify the level of dysplasia, following the 2005 WHO classification. After that, the dysplasia levels were processed quantitatively and were analyzed statistically.
Results: There was a significant result of dysplasia in both group 1 and group 2 if compared to the control group.
Conclusion: The exposure of side stream cigarette smoke to Wistar rats resulted in the existence of buccal and labial mucosa dysplasia.

Keywords: buccal mucosa, dysplasia, labial mucosa, side stream cigarette smoke

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INTRODUCTION

The use of tobacco is one of the factors that can cause cancers (Siegel, et al. 2019; Kujan, 2017; Manoharan, et al. 2016). Cigarettes are a tobacco product that contains addictive psychoactive ingredients, including nicotine (Schick, et al. 2013; de Oliveira Semenzati, et al. 2012; Gupta, et al. 2018). The widespread of tobacco is one of the biggest public health threats the world has ever faced, killing more than seven million people every year. More than six million of the death cases were due to the direct use of tobacco, while approximately 890,000 of the death were passive smokers exposed to cigarette smoke (Siegel, et al. 2019; Gupta, et al. 2018; Patra, Sharma, & Behera, 2012; Reddy, 2015).

There are more than 4,000 chemical substances contained in cigarette smoke, which 250 of them are known as harmful ingredients and more than fifty of them are cancer-causing substances (Siegel, et al. 2019; Reddy, 2015; Viroonudomphol, et al. 2018; Cheng, 2016). Some of the main substances contained in cigarettes are nicotine, carbon monoxide, nitrosamine specific tobacco metabolite, volatile organic compounds or polycyclic aromatic hydrocarbon (PAHs), and carcinogenic heavy metals (Cheng, 2016).

Tobacco, furthermore, contains carcinogenic substances that can trigger the change in oral epithelial dysplasia (OED) to oral cancer (Joseph, 2017; Widyanti, Ardiaria, & Widyastuti, 2015; Abdul Hamid, Baom, 2017). The development of cancer in the oral mucosa is step by step, starts from the precancerous lesion to cancer itself (Kujan, 2017; Tanaka, & Watanabe, 2017). Oral cancer, in other words, is always initiated by

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dysplasia or precancerous lesion. Thus, the examination of epithelial dysplasia in the oral cavity is vital in predicting the malignancy development (Sadiq, et al. 2015; Pervez, & Abro, 2017; Awadallah, Idle, Patel, & Kademani, 2018; El-Hamady, 2017).

The Squamous cell carcinoma (SCC) in the buccal mucosa has aggressive characteristics – it can grow fast and metastasize in tissues with a high recurrence rate (Kim, & Myoung, 2017). Hence, the SCC in the buccal mucosa needs thorough treatment since its early stage (Kim, & Myoung, 2017; Brand, & Isselhard, 2014). The SCC in both buccal mucosa and labial mucosa, however, are rarely to occur, unlike the SCC in the tongue that contributes around 10% of all cases of oral cancer (Padma, Paulraj, & Sundaresan, 2017). Based on the elucidation, this study aims to investigate the impacts of sidestream cigarette smoke exposure, especially to the risk of malignant transformation. The investigation is carried out by further observing the buccal and labial mucosa dysplasia in Wistar rats as the experimental animals in vivo.

METHODS AND MATERIALS

This study applied in vivo experimental laboratory research to Wistar rats as the experimental animals. Moreover, this study used a randomized posttest-only control group design research design. The Wistar rats (*Rattus norvegicus*) used as the experimental animals were taken from the Biochemistry Laboratory of the Faculty of Medicine, Universitas Airlangga and the Research Center of the Faculty of Dentistry, Universitas Airlangga.

Wistar rats were chosen as the carcinogenic models that were chemically induced due to the dynamic changes in the gene expressions that undergo oral carcinogenesis as in humans, which leads to the development of histological changes, such as hyperplasia and dysplasia, followed by the development of the SCC (Sengupta, 2013; Sharp, & Villano, 2012) .In brief, rats are one of the best organism models for cancer research due to several characteristics, for instance, its small size, its tendency to reproduce in captivity, its 2-3 years of lifespan, and a quite significant number of physiological and molecular similarities with humans (Ishida, et al. 2017; Manzoor, Raza, & Chaudhry, 2013).

Several specifications become the inclusion criteria for the Wistar rats used in this study: Male Wistar rats, since the male ones are not influenced by hormonal situations, such as menstruation and pregnancy, as undergone by female rats; have lived for more than three months; weight 170 grams; have good health during one week of the adaptation period, with the characteristics of clear-eyed, shiny fur, agile movements, and non-soft feces. The Wistar rats used as experimental animals must be older than three months since, at that age, the rats have reached the biological maturity (Sharp, & Villano, 2012; Sengupta, 2013). As stated, male rats are chosen instead of the female ones since male rats are not influenced by hormonal fluctuation (Beery, & Zucker, 2011). given hormonal fluctuation can affect the immunity state that may have impacts on the inconsistency of the results (Manzoor, Raza, & Chaudhry, 2013; Khan, & Ansar Ahmed, 2016). After being counted by using the Lemeshow formula, the number of the samples was obtained as many as nine rats in each group.

There were three research groups in this study, namely the control group, the P4 group with four weeks of exposure, and the P8 group with eight weeks of exposure. Different from the other two groups, the control group was not given any sidestream cigarette smoke exposure. The exposure, additionally, was given once a day with 20 cigarettes containing 2.1 milligrams of nicotine and 34 milligrams of tar by using a smoking pump.

The cigarettes used, furthermore, were *kretek* cigarettes, or the cigarettes containing chopped cloves in brand “Istana”, where every cigarette contained 2.1 milligrams of nicotine. The exposure of the smoke was using a smoking pump that could flow the combustion of cigarette smoke into a tube with Wistar rats in it. Once a day, the rats were exposed to the smoke of 20 cigarettes per exposure for ten rats in total; thus, each rat was exposed to two cigarettes per day. The amount of exposure was maintained until the time to sacrifice the rats. The exposure, furthermore, was done for four and eight weeks for the P4 group and P8 group, respectively.

The measurement of dysplasia level was carried out by observation to the architectural and cellular characteristics of epithelial cells in both buccal and labial mucosa preparation of the rats with hematoxylin-eosin (HE) staining (Masthan, et al. 2016). The observation, moreover, was performed by utilizing a light microscope with a magnification of 200 times for atypical cell proliferation and architectural changes and a magnification of 400 times for cellular changes (Ishida, et al. 2017).

The determination of dysplasia level, moreover, referred to the 2005 WHO classification of histopathological changes that indicate abnormal activities of atypical cells in the oral mucosa of the Wistar rats. The classification is further divided into four categories: mild dysplasia, moderate dysplasia, and severe dysplasia. The levels were then converted into a scoring system with a range of 0-3, according to 2005 WHO classification, where: 0 (no dysplasia), 1 (mild dysplasia), 2 (moderate dysplasia), and 3 (severe dysplasia)(Sadiq, et al. 2015). The data obtained from the observation were analyzed using the non-parametric test. The significance test employed, in addition, was the Kruskal-Wallis test before the Mann-Whitney test for a more in-depth analysis.

RESULTS

The data were first analyzed by using the Kruskal-Wallis test to identify the differences among the three groups. The analysis on the variable of dysplasia level of buccal mucosa cells showed a significant result, which indicated that there were differences of treatments given (p-value=0.000). The same result was also found in the second analysis, the Kruskal-Wallis analysis to the labial mucosa cells (p-value=0.000).

The Mann-Whitney test was carried out to identify the significant differences among the research groups. From **Table 1**, it can be seen that the test performed in the buccal mucosa between the P4 group and P8 group obtained a result of p>0.05, or p=0.065, which signified that there was no increase in the number of cells undergoing dysplasia or that a less significant dysplasia between the P4 group and P8 group. On the other hand, the test performed in the buccal mucosa between the K group and P4 group and between the K group and P8 group showed an identical p-value (p<0.05), with p=0.000. The results denoted that there was a significant increase in the number of the cells undergoing dysplasia in the buccal mucosa from the K group and P4 group and the K group and P8 group.

Table 1. Data Analysis

Mann-Whitney Test	Comparison of Treatments among Groups	p-value	Information
Buccal Mucosa	P4 with P8	0.065	p > 0.05
	P4 with K	0.004	p < 0.05
	P8 with K	0.000	p < 0.05
Labial Mucosa	P4 with P8	0.317	p > 0.05
	P4 with K	0.000	p < 0.05
	P8 with K	0.000	p < 0.05

Similar to the buccal mucosa, the Mann-Whitney test was also carried out in the labial mucosa. Between the P4 and P8 groups, the result obtained was p>0.05, with p=0.317, which implied that there was no significant increase in the cell numbers undergoing dysplasia between the two groups. The result of the test performed to the K and P4 groups and the K and P8 group, on the contrary, showed the amount of p<0.05, with p=0.000. The result suggested that there was a significant increase in the number of cells undergoing dysplasia in the labial mucosa of both research groups.

From **Table 2**, it can be noticed that from the observation in the buccal mucosa, all preparations in the K group had a score of 0, which denoted the absence of dysplasia. Furthermore, the absence of dysplasia was indicated by the normal formation of mucosal epithelial cells besides the absence of atypical cells nor the cells experiencing mitotic abnormalities. The indication of dysplasia, however, started to show in the P4 group's preparations, where there were six samples of preparation that indicated mild dysplasia. The observation of labial mucosa in all preparations of the K group, in contrast, had a score of 0, which meant that there was no dysplasia, indicated by the normal formation of mucosal epithelial cells and the absence of atypical cells nor the cells undergoing mitotic abnormalities.

Table 2. Sample Observation

Number of Dysplasia Sample	K group	P4 group	P8 group
Buccal Mucosa	0	6	9
Labial Mucosa	0	8	9

In **Figure 1**, mild dysplasia was indicated by hyperplasia and pleomorphism of cells in the basal layer. In the P8 group, dysplasia was seen in all preparations with a level of 1. In other words, there was a mild dysplasia as seen in the P4 group, that is a picture of abnormal mitosis in 1/3 of the lower layer of the epithelium.

In **Figure 2**, dysplasia started to be seen in the P4 group preparation. Furthermore, eight preparations showed the indication of mild dysplasia, signified by hyperplasia and pleomorphism of cells in the basal layer. In the P8 group, dysplasia was seen in all preparations with a level of 1. Similar to the P4 group, mild dysplasia was also revealed and indicated by abnormal mitosis in 1/3 of the lower layer of the epithelium.

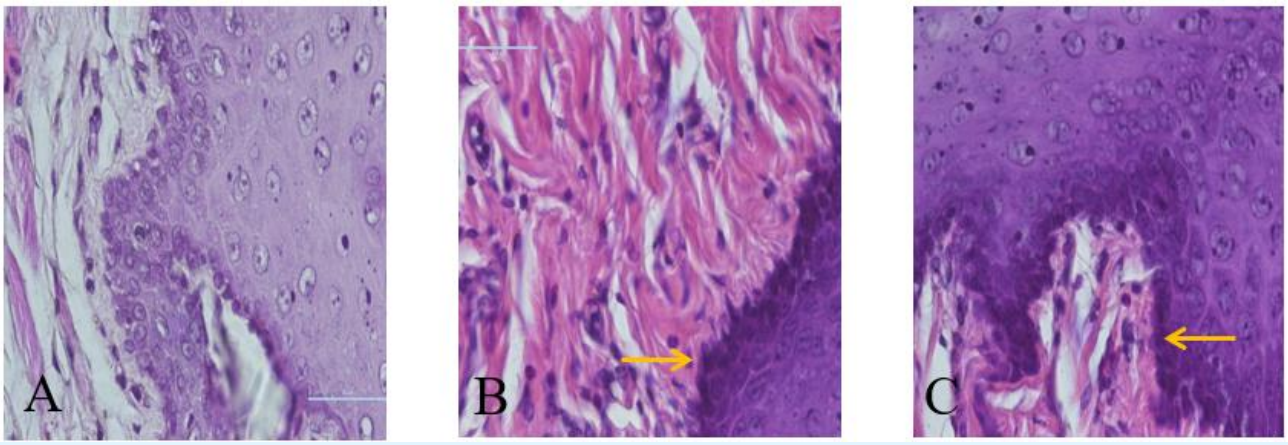

Fig. 1. Preview of HPA preparations for the buccal mucosa of the K group (A). Preview of HPA preparations for the buccal mucosa of the P4 group (B). Preview of HPA preparations for the buccal mucosa of the P8 group (C)

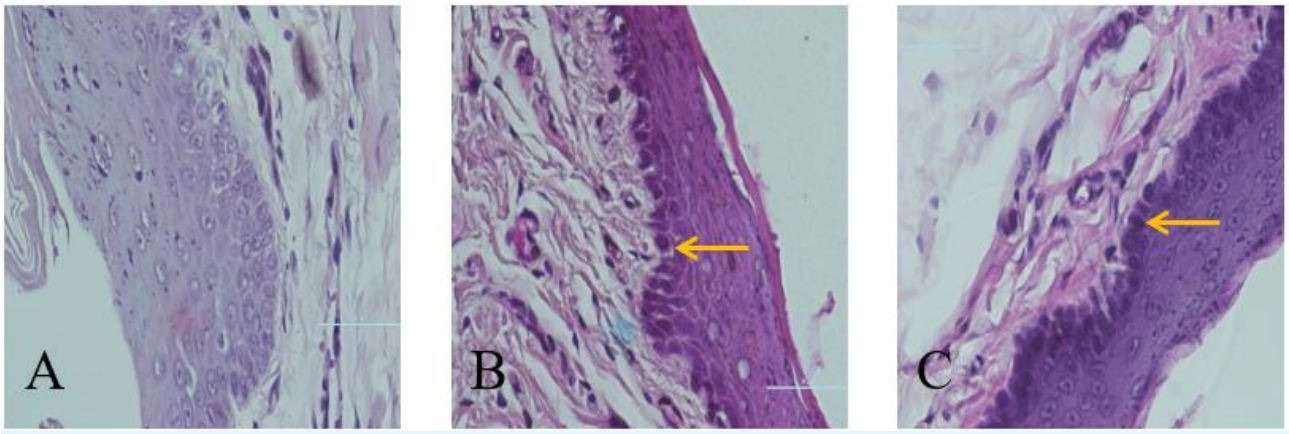

Fig. 2. Preview of HPA preparations for the labial mucosa of the K group (A). Preview of HPA preparations for the labial mucosa of the P4 group (B). Preview of HPA preparations for the labial mucosa of the P8 group (C)

DISCUSSION

The results of the study denoted that the duration of the exposure to sidestream cigarette smoke for eight weeks (60 days) for the P8 group and four weeks (30 days) for the P4 group could increase the risk of malignancy significantly if compared to the K group that was not exposed to any kind of smoke. However, the different duration of exposure, namely between eight weeks and four weeks, did not significantly show a different increase of malignancy. The result may occur because of long-term (chronic) cigarette smoke exposure increases the thickness of the airways epithelium, alveolar septum, and mucous hypersecretion due to hyper-keratinization of the epithelial layer that makes the body becomes more in a defense state against cigarette smoke compounds. As a consequence, the ability of the rats to adapt to 30-day of sidestream cigarette smoke exposure brought a significantly different result between the K group and the P4 group and between the K group and P8 group.

During the experiment, the rats received sidestream cigarette smoke that contains two types of ingredients, namely in the form of a particle (tar) and gas (free radicals, benzene, vinyl chloride, acrolein, toxicants, nicotine, etc.). Tar, moreover, comprises various types of ingredients, such as alkaloid, nicotine, aromatic amine, polycyclic aromatic hydrocarbons (PAH), etc. (Gupta, et al. 2018; Abdul Hamid, Baom, 2017; Besaratinia, & Pfeifer, 2008).

Sidestream cigarette smoke has a high level of nicotine, which is an addictive material. The presence of nicotine can help the process of carcinogenesis by causing the promotion of cancer, facilitating cancer growth, such as the process of angiogenesis, migration, and invasion. Nicotine, additionally, undergoes the process of nitrosation and can change into a carcinogenic chemical substance, for instance, NNK and NNN when smoked. NNK and NNN, furthermore, trigger carcinogenic by induction and DNA mutation along with tumor proliferation. As a result, the body responses to balance the increase of free radicals, other chemical substances, and nicotine by increasing antioxidant. However, persistent exposure, as done in this study causes ongoing oxidative stress and buildup in the body (Xue, Yang, & Seng, 2014).

Oxidative stress, furthermore, has an effect on the DNA, protein, and fat in the body of passive smokers, which can trigger cancer. When normal cells receive the process of DNA mutation, and DNA synthesis become initiated cells, it means that the cancer initiation process has begun. ROS, additionally, will support the existence of DNA modification. ROS will also expand cell expansions by temporarily regulating the process of cell proliferation and death (apoptosis) besides regulating the transcription factors in DNA. The involvement of ROS in those processes, consequently, leads to DNA base damage and triggers DNA mutations (Schieber, & Chandel, 2014).

The majority of the mutation caused by ROS includes guanine modification, which causes the transversion of G → T (Guanine-Tylenol), single-strand damages, and instability that is directly formed or through a repair process. In a human tumor, G → T transversion is the most-frequent mutation in the p53 suppressor gene (Katakwar, et al. 2016). The p53 tumor suppressor gene, furthermore, is influential in the initial process of carcinogenesis. Therefore, it can be implied that there is a correlation between the increase in p53 and the increase in the level of dysplasia (Sadiq, et al. 2015).

CONCLUSION

After the observation and the analysis, it can be concluded that the exposure of sidestream cigarette smoke is correlated with the increase in dysplasia level in buccal and labial mucosa of Wistar rats. The result is most likely caused by the increase in the exposure duration, which causes an increase in the amount of carcinogen received by both buccal and labial mucosa. In other words, the higher the number of carcinogenic

substances exposed to epithelial cells, the higher the number of cells experiencing gene mutation that the number of cells with mitosis abnormalities increases. In consequences, the increase in the change of architectural and cellular characteristics occurs and is diagnosed as leveling up the dysplasia.

Conflict of Interest: There is no conflict of interest.

Source of Funding: This study is self-funded.

Ethical Clearance: This study was approved by Ethical Commission of Health Research, Faculty of Dental Medicine, Universitas Airlangga.

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