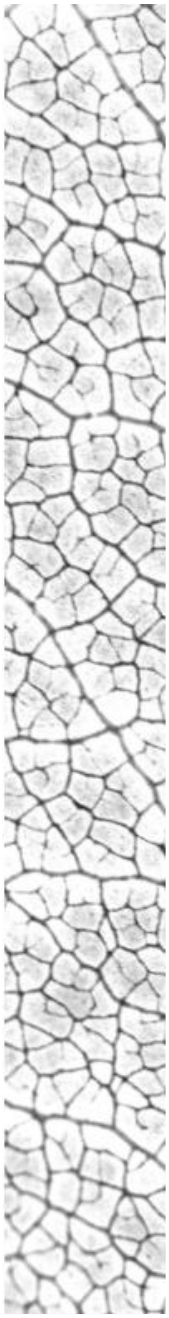

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The increase of dysplasia level in Wistar rats oropharyngeal mucosa exposed by sidestream cigarette smoke

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Abstract

Background: Sidestream cigarette smoke contains several elements that can trigger cancer. The risk of various types of cancer, such as oral cancer, will increase in the number of people exposed to sidestream cigarette smoke. Dysplasia is a histopathological change that shows abnormal activities in the normal epithelium. Examination of oral epithelial dysplasia is vital in predicting the development of malignancy. This study aimed to determine the risk of malignant transformation in the oropharynx of Wistar rats (*Rattus norvegicus*) exposed to sidestream cigarette smoke through the observation of the degree of dysplasia in the oropharyngeal mucous in the 4th and eighth weeks after the exposure began.

Method: Wistar rats were divided into three groups, namely treatment group one, exposed to cigarette smoke for four weeks; treatment group two, exposed to cigarette smoke for eight weeks; and a control group that was not exposed to cigarette smoke. Oropharyngeal mucous of rats from each group were examined histopathologically to find the degree of dysplasia based on 2005 WHO classification. Degrees of dysplasia were treated quantitatively and analyzed statistically.

Results: A significant increase in the degree of dysplasia was found more in the treatment group 2 than in the control group. A significant increase in the degree of dysplasia was also found in treatment group 1 compared to the control group.

Conclusion: The oropharyngeal mucosa of Wistar rats exposed to sidestream cigarette smoke for four weeks and eight weeks experienced an increase in the degree of dysplasia.

Keywords: dysplasia, oropharyngeal mucosa, sidestream cigarette smoke

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INTRODUCTION

Along with the increasing number of smokers, cigarette smoke and smokers have become national and even global problems, mainly supported by the cigarette industry, which is increasingly active in promoting economic activities directly or indirectly (Wati, 2020. Xue, et al. 2014). Moreover, the content of cigarettes consists of polycyclic aromatic hydrocarbons (PAHs), nitrosamines (i.e., NNK, NNN), aromatic amines, aldehydes, phenols, nitro compounds, and other organic and inorganic compounds[2]. Those components often cause potential damage to the body. As a result, poor smoking habits for health have been widely discussed. Referring to Riskedas in 2013, the average number of smokers in Indonesia was 29.3%. The prevalence of men appears 16 times more than women, which is 65.8 %. From the data, 80% of smokers start smoking when they have not reached 19 years old and generally start smoking without knowing the dangers of addictive cigarettes (Wati, 2020). The results showed that approximately 50% of smokers who smoke since adolescence would die from diseases related to smoking. Smoking habits are associated with approximately 25 types of diseases from various organs of the human body (Troy, et al. 2013). The disease, among others: cancer of the mouth, esophagus, pharynx, larynx, lung, pancreas, bladder, and vascular disease (Xue, et al. 2014; El-Gali, 2018).

Oral cancer, furthermore, is the sixth most common malignancy in the world. Mouth cancer, moreover, is a significant concern in Southeast Asia, mainly because of the habit of chewing betel nut, smoking, and alcohol

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consumption (Kumar, et al. 2016). Oral cancer is part of the head and neck cancer group and is defined as the growth of uncontrolled cancer cells that attacks the oral cavity and the throat behind the mouth called oropharynx (Rivera, 2015). Neoplastic transformations occur together with mutations in oncogenes throughout the oral cavity. Inactivation of suppressor tumor genes, such as p53, caused by smoking is an increased risk of oral squamous cell carcinomas. Continuous mutations can also produce changes in DNA repair and apoptosis. These changes are known to increase susceptibility to the transformation of cancer cells (Tanaka, & Watanabe, 2017). Oral Epithelial Dysplasia (OED) is a histopathological diagnosis that is associated with an increased risk of oral cancer (Joseph, 2017).

Dysplasia is a histopathological picture that shows abnormal activity in the normal epithelium (Kumar, et al. 2016). The development of cancer in the oral mucosa begins with precancerous lesions which then develop into cancer. The presence of oral cancer is always preceded by dysplasia or precancerous lesions. An examination of oral epithelial dysplasia, thus, is important in predicting the development of malignancy (Sadiq, et al. 2015).

Tobacco, alcohol, and the tendency for smoking have an impact on the development of oral precancerous epithelial dysplasia shown histologically (Joseph, 2017). Polycyclic aromatic hydrocarbons, aldehydes, aromatic amines, nitrosamines in tobacco are considered carcinogenic and have been shown to cause mutations in oral mucous cells. As an early sign of oral mucosal damage, tobacco often causes the development of precancerous lesions, such as leukoplakia. These lesions can facilitate diagnosis and at the same time, can be considered an indicator of risk of oral cancer (Garg, Raj, & Chandra, 2013).

More than 600,000 people who don't smoke die every year from inhaling sidestream cigarette smoke. When there are people who smoke, they emit sidestream smoke from the tip of the cigarette, and smokers exhale the mainstream smoke. Non-smoking populations exposed to sidestream smoke cause them to become passive smokers. Sidestream smoke, as known, is more toxic than mainstream smoke because the combustion temperature when the cigarette is lit is not as high as when the cigarette is smoked, resulting in incomplete combustion and more complex compounds in sidestream smoke (Ooi, et al. 2014. Schick, et al. 2013). Children and neonates are populations that are more sensitive to the exposure of sidestream smoke, compared to adults. Sidestream smoke exposure in adults can occur in various places, for example, at work, in public places, and at home. In children, sidestream smoke exposure can occur at home, and in some cases, can also occur while still in the womb (Troy, et al. 2013).

There are more than 4000 chemicals in sidestream cigarette smoke which have been shown to be 50 of them known to cause cancer (Reddy, et al. 2015). Sidestream cigarette smoke, additionally, contains several elements that can trigger cancer that can be classified into three different groups, namely nitrosamine, benzopyrene, and aromatic amines. These chemicals are pre-carcinogens, which will bind to oxidative enzymes, resulting in changes in DNA that result in a mutation (Rivera, 2015). Increased levels of free radicals, moreover, are found in precancerous and oral cancers (Choudhari, et al. 2014). Therefore, based on the background above, in this study, the authors are eager to examine the impact of sidestream cigarette smoke exposure to the risk of malignant transformation through the observation of the degree of oropharyngeal mucosal tissue dysplasia in Wistar rats as the experimental animals.

METHODS AND MATERIALS

This study used Wistar rats (*Rattus norvegicus*) as the experiment object. In calculating the number of samples, the Lemeshow formula was applied, resulting in two samples for each group. However, in this study, nine samples were used in each group to anticipate the death of experimental animals. Mice were fed with rats and given water. All samples were grouped into one control group (K) which was given no treatment, and two treatment groups that were exposed to sidestream cigarette smoke for four weeks (P1) and for eight weeks (P2). The exposure was carried out by using a smoking pump with a dose of two cigarettes per mouse every day until the final day of experiment, namely day 30 for the P1 group and day 60 for the K and P2 groups.

At the end of the fourth and eighth week, each Wistar rat underwent the euthanasia procedure by placing it in a glass tube filled with ether in lethal doses. After that, the rat's tongue was cut by using a scalpel and was used to make preparations for histopathological examination by using *Hematoxcylin-Eosin* (HE) staining.

RESULTS

All preparations in the K group have a score of 0, which indicates the absence of dysplasia, denoted by the normal arrangement of mucosal epithelial cells and the absence of atypia cells or cells that experience mitotic abnormalities.

The sign of dysplasia, however, appears in the P1 preparation group. There were seven preparation samples which showed a sign of mild dysplasia, while two other samples did not show any sign of dysplasia. The description of mild dysplasia, furthermore, is indicated by hyperplasia and pleomorphism of cells in the basal layer.

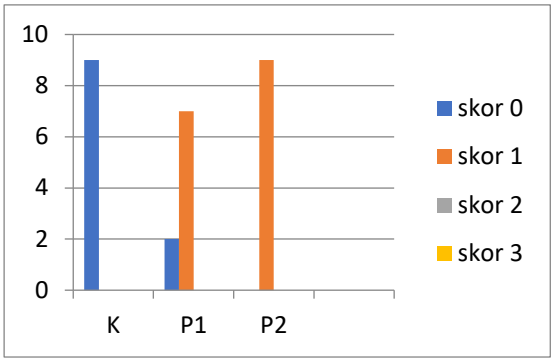

Fig. 1. Distribution of dysplasia scores on the oropharyngeal mucosa

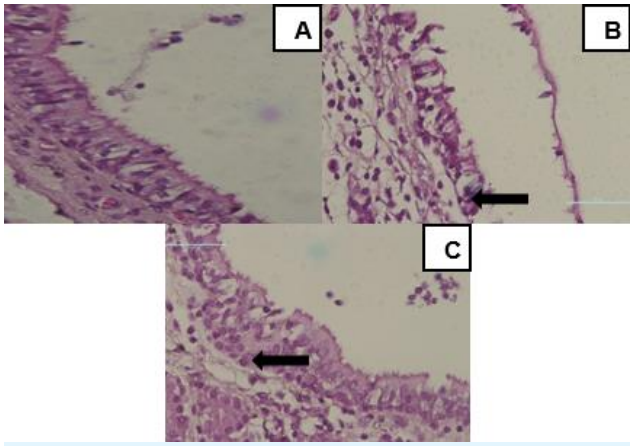

Fig. 2. A. HPA picture of oropharyngeal mucosal preparations in group K where there was no picture of dysplasia indicated by a typical epithelial arrangement in the absence of atypia cells and mitotic abnormalities; B. Description of HPA oropharyngeal mucosal preparations in the P1 group. In the P1 group, moreover, it was found that mild dysplasia was indicated by epithelial arrangement in the form of atypia cells and mitotic abnormalities; C. Description of HPA oropharyngeal mucosal preparations in the P2 group. In the P2 group, additionally, it was found that mild dysplasia was indicated by epithelial arrangement in the form of atypia cells and mitotic abnormalities.

In the P2 group, the appearance of dysplasia was seen in all preparations with degree 1. Moreover, there was a picture of mild dysplasia seen in the P4 group, which was an abnormal mitotic picture 1/3 of the lower layer of the epithelium.

The data analysis of this study began with the Kruskal-Wallis test to determine differences in the three groups. Based on the Kruskal-Wallis test, a significant difference is when the p-value is < 0.05. The results of the Kruskal-Wallis test, however, showed a p-value of < 0.05, which is p=0.000, indicating that there were significant differences in the outcome data between the research groups.

After that, the Mann-Whitney test was conducted to determine the significant differences between the study groups. A value is considered to have a significant difference if p-value < 0.05. In the Mann-Whitney test between P1 and P2 groups, the result was p-value > 0.05, with p=0.145, which showed that there was an increase in the degree of oropharyngeal mucosal dysplasia which was less significant between the P1 group and P2 group. Meanwhile, in the Mann-Whitney test between the K group and P1 group and the K group with the P2 group, the results from showed a p-value of <0.05 with p-value=0.001 and p-value=0.000, respectively. The results, moreover, indicated that there was a significant increase in the degree of oropharyngeal mucosal dysplasia from the K and P1 group and the K group and P2 group.

Table 1. Comparison of the oropharyngeal mucosal treatment groups

Comparison of Treatment Groups	p-value	Information
P1 with P2	0.145	p> 0.05
P1 with Control	0.001	p <0.05
P2 with Control	0.000	p <0.05

DISCUSSION

Wistar rats (*Rattus norvegicus*) were sued as the sample of this study because there were genetic and biological similarities in neoplastic development besides the histopathological features between humans and the sample (Balmain, & Harris, 2000). The Wistar rats used as the samples were at least three months old because, at that age, Wistar rats had achieved biological and social maturity (Sengupta, 2013). Male rats are chosen because they are not influenced by hormonal conditions, such as menstrual cycles and pregnancy like female mice (Beery, & Zucker, 2011). Hormonal fluctuations, besides, can affect the state of body immunity of the mice so that it can have an impact on the inclusion of the research results (Khan, & Ansar Ahmed, 2016).

Twenty-seven Wistar rats were divided into three groups: treatment one who received sidestream cigarette smoke exposure for four weeks (P1), treatment two who received sidestream cigarette smoke exposure for eight weeks (P2), and the control that was not exposed to any sidestream cigarette smoke (K). The treatment groups, besides, were given constant exposure to sidestream cigarette smoke every day, while the control group was not given any exposure. One cigarette contains 2.1 milligrams of nicotine. In other words, the exposure given is equal to twenty cigarettes per day for ten rats in each treatment group.

This study applied the whole-body exposure method since this method provides sufficient space for experimental animals when being treated, with the exposure dose of the sidestream cigarette smoke becomes not centered on the oral cavity but throughout the body. Even so, the histological results obtained from this method remain significant. In addition, other methods, for instance, the nose inhalation method, is

noticeably ineffective because it requires a tool such as an oxygen mask. Since rats are rodents, chances are the masks are quickly damaged. Other than that, the results of the hyperplasia histology of the method are also not very significant (de Oliveira Semenzati, et al. 2012).

After the treatment, the oropharyngeal tissue of the rats was examined by employing the HPA examination to observe the degree of the after-treatment dysplasia tissue. The oropharynx, in addition, was chosen because it is part of the aerodigestive tract for which the majority of HNSCC cases are found (Kumar, et al. 2014). Additionally, people who are exposed to sidestream cigarette smoke are faced to the risk of upper aerodigestive tract cancer, for example, s doropharynx (Troy, et al. 2013). The determination of the degree of dysplasia, moreover, referred to 2005 WHO classification, which divided dysplasia into four categories: mild dysplasia, moderate dysplasia, severe dysplasia, and carcinoma in situ. The degree of dysplasia was then converted to a dysplasia score with a range of 0-4 according to the 2005 WHO classification: 0 (no dysplasia), 1 (mild dysplasia), 2 (moderate dysplasia), 3 (severe dysplasia), and 4 (carcinoma in situ) (Ranganathan, & Kavitha, 2019).

The results of the examination showed a significant increase in the degree of dysplasia in the P1 group ($p<0.05$) when compared to the K group. The description of mild dysplasia in the form of cell changes in 1/3 of the epithelial base layer, additionally, was found in seven samples and two samples of normal mucosal images. The description indicated that the effect of sidestream cigarette smoke exposure for four weeks had a risk of causing dysplasia. Although the result denoted such outcome, further research is still needed because the treatment results of P1 group showed that several samples did not suffer from dysplasia, besides the fact that within twenty-five days of exposure, Wistar rats' hyperplasia, metaplasia, and dysplasia were in the mucosal tissue area of the vocal cords (Duarte, et al. 2006).

In the P2 group, there was a significant increase in the degree of dysplasia ($p<0.05$) when compared to the K group. The description of mild dysplasia, furthermore, occurred in all P2 group samples in the form of cell changes in 1/3 the epithelial base layer. A similar finding was also found, for instance, epithelial hyperplasia and mild dysplasia, in Wistar rats' mucosal tissue after being exposed to sidestream cigarette smoke for 60 days (de Oliveira Semenzati, et al. 2012). The initial stages of carcinogenesis in aerodigestive mucosal tissue for less than 75 days, nonetheless, is required. The signs of malignancy in the form of severe dysplasia in the rats' tissues, however, were discovered after being exposed to sidestream cigarette smoke for 260 days (Garcia Martins, et al. 2012).

Differences in results found in different rat samples that belong in one group may occur because of the differences between an individual's molecular level. Moreover, likely, the sample did not come from the same parent. Thus, the gene differences that are influential in cell divisions, such as p53, may relate to the susceptibility to genetic mutations differences between individuals (Greenblatt, 1994).

The results, moreover, displayed that the duration of exposure to sidestream cigarette smoke for eight weeks (60 days) for P2 group and four weeks (30 days) for P1 group could significantly increase the risk of malignancy, compared to K group that was not exposed at all. In more details, however, the group with eight-week of exposure showed a less significant increase malignancy than the four-week of exposure group. In some cases, this may occur due to the prolonged (chronic) cigarette exposure that increases the thickness of the airway epithelium, alveolar septum, mucous hypersecretion, and ciliostasis that make the body defenses more against the sidestream cigarette smoke compounds (Smoke, & Smoking, 2004). The ability of mice that had adapted to sidestream cigarette smoke for 30 days, furthermore, brought significant results in both K group with P1 group and K group with P2 group.

The varied results of this study and other studies that investigated the effect of sidestream cigarette smoke exposure on mucosal tissue of the oropharynx of Wistar rats could be influenced by several factors (Osimitz, Droege, & Finch, 2007). Factors such as duration of treatment, number of cigarettes per exposure, duration per exposure, and carcinogenic content in each cigarette may affect the determination of the total number of carcinogens, especially nicotine, received by each mouse (Garcia Martins, et al. 2012)., which is directly proportional to the mentioned factors. In addition to these factors, the number of samples can also influence the results because the increase in the number of samples is inversely proportional to the number of carcinogens received (Garcia Martins, et al. 2012. Choudhari, et al. 2014).

Metabolic NNK and NNN are activated by cytochrome P450 (CYPs) and induce DNA addiction. Further unresolved DNA induction leads to mutations in the oncogenes and tumor suppressor genes, which consists of the first steps of NNK and specific NNN carcinogenesis. NNK and NNN can further bind to nAChRs (nicotinic acetylcholine), which can promote tumor growth by increasing and deregulating cell proliferation, cell survival, and cell migration and cell invasion, as the second step in NNK-induced cancer and NNN. The combination of these two aspects of the biological reaction of NNK and NNN, later, provides conditions for tumor development in smokers (Xue, Yang, & Seng, 2014. Ghani, et al. 2019).

Sidestream cigarette smoke contains free radicals, such as nitric oxide and a mixture of hydroquinones,

semiquinones, and quinones, which can induce redox cycles that can cause oxidative damage (Xue, Yang, & Seng, 2014. Sah, et al. 2018). ROS causes mutations that can damage DNA and cell division. The majority of mutations caused by ROS further appear to involve modification of guanine, causing transversion of G → T (Guanin-Tenol), damage to a single strand, and instability that is formed directly or with the repair process. In human tumors, transverse G → T is the most common mutation in the suppressor gene p53 (Katakwar, et al. 2016). The p53 tumor suppressor gene plays a role in the early stages of carcinogenesis. Therefore, it can be stated that there is a correlation between the increase in p53 and the increase in the degree of dysplasia (Sadiq, et al. 2015).

CONCLUSION

After experimenting, it can be disclosed that the increase in the duration of sidestream cigarette smoke exposure is related to the increase in the degree of dysplasia of the mucous tissue of the oropharynx of Wistar rats. The results are most likely due to an increase in the duration of exposure, which causes an increase in the number of carcinogens received by mucous tissue. In other words, the higher the number of carcinogenic materials that expose epithelial cells, the higher the number of cells that experience gene mutations. As a consequence, the number of cells that experience mitotic abnormalities increases. However, the increase can also lead to increased changes in architectural and cellular characteristics that occur so that it can be diagnosed as an increase in the degree of 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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