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Research Report

The correlation between exposure to cigarette smoke and the degree of mucosal epithelium-based dysplasia in *Rattus norvegicus* tongues

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ABSTRACT

Background: *Cigarette smoke contains various carcinogenic substances such as polycyclic aromatic hydrocarbons and nitrosamines. These chemicals not only have the potential to damage DNA, but can also induce genetic mutations and activate genes that function during apoptosis. Thus, if the gene is dysregulated, it will cause cells to survive, proliferate and subsequently lead to the development of cancerous ones. Histologically, the carcinogenic process affecting the oral cavity starts with hyperplasia and dysplasia, followed by severe dysplasia then leading to invasive cancer and metastatic processes in other bodies.* ***Purpose:*** *This study aims to reveal the correlation between exposure to cigarette smoke and the degree of epithelial dysplasia evident in research subjects.* ***Methods:*** *This study used 27 samples of Rattus norvegicus tongue, divided into three groups, namely; a control group, a treatment group subjected to four weeks' exposure to cigarette smoke, and a treatment group subjected to exposure lasting eight weeks. Each rat was placed in an individual chamber and exposed to smoke from 20 cigarettes introduced by a pump via a pipe for 7.5 minutes. The degree of epithelial dysplasia in each case was subsequently observed microscopically using HE staining technique.* ***Results:*** *Mild epithelial dysplasia increased by 0.82%, during the fourth week of exposure to cigarette smoke and by 2.99% during the eighth week. Similarly, moderate epithelial dysplasia rose by 5.29% during the fourth week of exposure and 5.99% during the eighth week. Severe epithelial dysplasia also increased by 2.2% during the fourth week of exposure and by 2.66% during the eighth week.* ***Conclusion:*** *The longer the exposure to cigarette smoke, the higher the degree of ensuing dysplasia.*

Keywords: *epithelial dysplasia, exposure to cigarette smoke, oral cancer, Rattus norvegicus*

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INTRODUCTION

Smoking is currently regarded as widespread across Indonesia, indulged in on a daily basis by significant numbers of individuals.[1] Worryingly, the population of passive smokers is reportedly outstripping that of active ones.[2] In fact, both categories of smoker run the risk of developing cancer since cigarettes contain various carcinogenic substances, such as polycyclic aromatic hydrocarbons (PAHs) [3], and Nitrosamines consisting of 4-(methylnitrosamino) -1- (3-pyridyl) -1-butanone (NNK) and N'-nitrosonornicotine (NNN). Each substance is known to have carcinogenic properties in relation to human beings.[4]

Genetic apoptosis is a process of programmed cell death which spontaneously promotes soft tissue growth and plays a regulatory role in the physiological development of cells. Dysregulation in the apoptotic pathway will promote the longevity of cells, while the resulting cell proliferation induces malignancy. Consequently, apoptosis is considered a marker of oral epithelial dysplasia (OED) the presence of which is employed to determine the prognosis of a cancer. In an oral squamous cell carcinoma (OSCC), a lack of apoptosis and an increased degree of dysplasia will usually be detected.[5]

Oral epithelial dysplasia can increase in individuals whose daily consumption of cigarettes can be as high

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as 20.[6] A previous study conducted by Trombitas *et al.* (2016),[7] posited that exposure to cigarette smoke can affect the severity of wounds in rats since epithelial dysplasia was detected in the nasal septum mucosa of those injured and exposed to cigarette smoke for a period of five days. In contrast, orthokeratotic keratinization was diagnosed in those injured but not subsequently exposed to cigarette smoke.[7] Hence, this study aims to examine the correlation between the duration of exposure to cigarette smoke and the degree of dysplasia in the lingual mucosal epithelium of *Rattus norvegicus*.

MATERIALS AND METHODS

This research was approved by the Universitas Airlangga Faculty of Dental Medicine Health Research Ethical Clearance Commission (eligibility number 067/HRECC. FODM/III/2019). The subjects of this study comprised 27 three-month old, male *Rattus norvegicus,* 170g (± 10%) in weight, which were divided into three groups, namely; a control group and two treatment groups. Their respective members were initially required to undergo a one-week period of adaptation, demonstrate excellent health, have clear vision, shiny fur, agility, and excrete solid stools.

The research subjects were housed in plastic box cages equipped with effective ventilation and lighting at room temperature. The members of all the control and treatment groups were placed on the same diet consisting of rice husks and water. Treatment Group 1 (P1) was exposed for four weeks to cigarette smoke delivered by means of a pump, while Treatment Group 2 (P2) was exposed for a period of eight weeks. The volume of smoke to which the subjects were exposed was maintained at a consistent daily level equivalent to that of 20 cigarettes. The smoke contained nicotine (1.2-4.5 mg), tar (46.8 mg), and carbon monoxide (28.3 mg).[8]

The Group Control (K1) members were sacrificed on the 29th day before surface tissue was removed from their tongues. Meanwhile, the treatment group subjects were euthanised at weeks four (PI) and eight (P2) by placing them in glass boxes into which a lethal dose of ether was gradually introduced. The tongue mucosa was subsequently removed by means of a 2 cm scalpel incision before being placed in 10% formalin. Tissue preparation was then completed. The degree of dysplasia was examined using HE staining and a Nikon e100 light microscope at 400x magnification.

The data obtained was analysed with a Statistical Package for the Social Sciences (SPSS) 19 (IBM, New York, US). A MANOVA test was conducted to determine the respective effects of 4- and 8-week exposure to cigarette smoke on each degree of dysplasia in the mucosal epithelium of the research subjects' tongues. However, prerequisite tests were initially performed, namely; variant homogeneity tests using Levene's test for Equivalence of Error Variances and Box's Test for Equivalence of Covariance Matrices. The MANOVA test results were then subjected to a range of analyses including; Pillai's Trace, Wilks' Lambda distribution, Hotelling's Trace, and Roy's Largest Root. Finally, in order to determine the correlation between exposure to cigarette smoke and each degree of dysplasia, a Between Subject Effect analysis was carried out. Meanwhile, a post hoc Bonferroni test was conducted to identify differences in the degree of dysplasia demonstrated by the group exposed to cigarette smoke.

RESULTS

In the control group (K1) not exposed to cigarette smoke (Figure 1), the condition of the squamous epithelial cells was found to be normal. Meanwhile, in the four-week exposure to smoke group (P1), mild, moderate and severe epithelial dysplasia (Figure 2) was detected, as was the case with the group exposed to smoke for eight weeks (P2), (Figure 3).

At this point, a number of pre-tests for homogeneity of variance and homogeneity of variance-covariance matrices were conducted, the results of which indicated significance values > 0.05. Therefore, a MANOVA test was performed whose results, as illustrated by the contents of Table 1, showed that F values for Pillai's Trace, Wilks' Lambda distribution, Hotelling's Trace, and Roy's Largest Root had a significance value of 0.000 <0.05. These findings confirmed the significant effect of 4- and 8-week exposure to cigarette smoke on each degree of epithelial dysplasia in Groups P1 and P2.

Moreover, based on the Between Subject Effect test results contained in Table 2, a correlation existed between exposure to cigarette smoke and the degree of dysplasia,

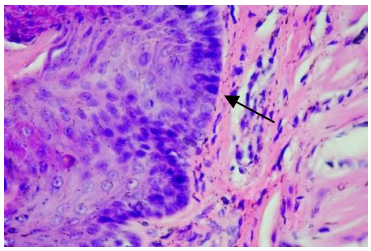

Figure 1. A histopathological image of the control group. Normal squamous epithelial cells (HE 400x).

Table 1. The effects of 4- and 8-week exposure to cigarette smoke on each degree of dysplasia in the mucosal epithelium of *Rattus norvegicus* tongue

Cigarette smoke exposure - degree of dysplasia	F	Significance
Pillai's Trace	15.284	0.000
Wilks' Lambda	31.589	0.000
Hotelling's Trace	58.874	0.000
Roy's Largest Root	124.058	0.000

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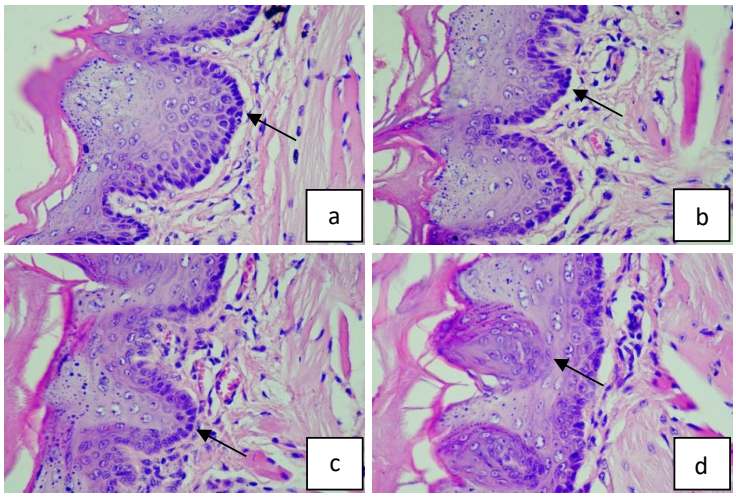

Figure 2. Pictures of epithelial dysplasia in Group P1. a) Mild dysplasia, b) Moderate dysplasia, c) Severe dysplasia, d) Hyperplasia (microscope H&E 400x light).

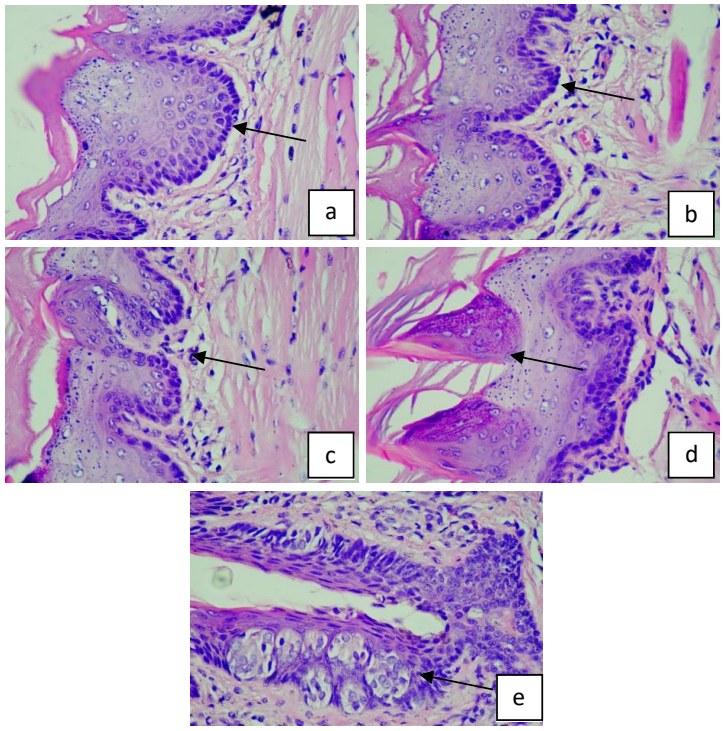

Figure 3. Histopathological pictures in Group P2. a) Mild dysplasia, b) Moderate dysplasia, c) Severe dysplasia, d) Hyperplasia, e) carcinoma in situ. (microscope H&E 400x light).

Table 2. The correlation between exposure cigarette smoke and each dysplasia degree in the mucosal epithelium of *Rattus norvegicus* tongue

Degrees of dysplasia	F	Significance	M
Mild	47.073	0.000	0.780
Moderate	43.317	0.000	0.765
Severe	20.574	0.000	0.601

Table 3. The results of a *Post Hoc Bonferroni* test on the degrees of dysplasia in Group K1, Group P2, and Group P3

Degrees of dysplasia	Smoke exposure	*Mean*	Significance
Mild	Control – 4 weeks	-3.11	0.050
	Control – 8 weeks	-11.33	0.000
	4 weeks – 8 weeks	-8.22	0.000
Moderate	Control – 4 weeks	-10.00	0.000
	Control – 8 weeks	-11.33	0.000
	4 weeks – 8 weeks	-1.33	0.979
Severe	Control – 4 weeks	-5.89	0.000
	Control – 8 weeks	-7.11	0.000
	4 weeks – 8 weeks	-1.22	0.938

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such as mild, moderate, and severe with a significance value of 0.00 <0.05. This indicates that each degree of dysplasia was caused by an increase in the duration of exposure to cigarette smoke.

The mean differences in the degree of dysplasia in Group K1, Group P1, and Group P2 indicated an increase in the average degree of dysplasia in the mucosal epithelium of *Rattus norvegicus* tongue along with the greater duration of exposure to cigarette smoke as can be seen from the contents of Table 3. The degree of mild epithelial dysplasia increased in Group P1 by 0.82% and by 2.99% in Group P2. Similarly, the degree of moderate epithelial dysplasia increased by up to 5.29% in Group P1 and 5.99% in Group P2. The degree of severe epithelial dysplasia also increased by 2.2% in Group P1 and 2.66% in Group P2.

DISCUSSION

In this study, the subjects were exposed to cigarette smoke for either 29 days or 59 days with the objective of rendering them more likely to become passive smokers, also referred to as second-hand smokers (SHS) or environmental tobacco smokers (ETS).[9] Actually, both active and passive smokers are suspected of possessing one of the predisposing factors for oral squamous cell carcinoma.[10]

According to the results of this study, those subjects exposed to cigarette smoke for 29 days presented mild dysplasia up to a level of 0.82%. 5.29%, also presented moderate dysplasia, while 2.2% suffered from severe dysplasia. On the other hand, 2.99% of those subjects exposed to cigarette smoke for 59 days presented mild dysplasia, while 5.99% had moderate dysplasia and 2.66% severe dysplasia. There was even a subject in Group P2 which presented carcinoma *in situ*.

A previous study conducted by Martins *et al.* (2012)[11] produced similar findings to those of this analysis, namely; that 60% of subjects exposed to cigarette smoke for 260 days presented lingual dysplasia, while a further 20% suffered from dysplasia of the pharynx and one subject had severe dysplasia or carcinoma *in situ*. Similarly, another previous study conducted by Martins *et al.* (2012) indicated that exposure to cigarette smoke carries the potential risk of carcinoma, although the treatment applied differed from that employed in this study.[11] In the research conducted by Martins *et al.* referred to above, the subjects were exposed to the smoke produced by ten cigarettes per day, while being provided with food and drink ad libitium. Unlike Martins' study, this research involved the burning of 20 clove cigarettes (*kreteks*) per day which contained higher levels of nicotine (1.2-4.5 mg), tar (46.8 mg) and carbon monoxide (28.3 mg). Moreover, the subjects were provided with food and drink devoid of additional substances. When a *kretek* is burned the temperature of a mixture of tobacco and cloves is raised with the result that the level of carbon dioxide and nicotine can be as much as three times as high, while that of tar may increase fivefold.[12]

Moreover, another previous study performed by Radwan *et al.* (2016),[13] produced similar results. This previous study asserted that male albino rats passively exposed three times a day to the smoke produced by four cigarettes during a period of 40 days (Group 2) or 60 days (Group 3) would present symptoms of epithelial dysplasia. These results indicated that a lingual dorsum epithelium developed under normal conditions in the control group that had not been exposed to cigarette smoke (Group 1), while the subjects in Group 2 experienced a change in their epithelial cell architecture accompanied by desquamation and hyperkeratosis. In addition, the Group 3 subjects presented symptoms of hyperkeratosis accompanied by macrophages and lymphocytes.

According to Xue *et al.* (2014), Second Hand Smoke (SHS)[4] can increase the risk of nasopharyngeal cancer, nasal cavity cancer, breast cancer, leukemia, lymphoma, and brain tumors in children. SHS contains a variety of toxic chemicals such as formaldehyde which can irritate the eyes, nose and throat, in addition to hydrogen cyanide, carbon monoxide, and ammonia which can weaken the natural airbourne cleaning mechanism which eradicates toxins. Moreover, SHS contains polycyclic aromatic hydrocarbons and nitrosamines potentially damaging to DNA. Arsenic, Benzene, Cadmium and Tar constitute other chemicals also contained in tobacco.[9]

Nitrosamines consist of 4- (methylnitrosamino) -1- (3-pyridyil) -1-butanone (NNK), and N 'nitrosonornicotine (NNN) which contain carbon monoxide, hydrogen cyanide, and oxygen radicals that induce an increase in ROS (Reactive Oxygen Species). As a result, the activation of ROS has a pathological effect culminating in DNA damage[4,9] which will, in turn, stimulate P53 to activate the Bcl-2 family consisting of anti-apoptosis proteins (bcl-2, Bcl-xl and MCL1), pro-apoptotic protein (BAX, BAK), and the third protein (BH3), usually referred to as BH3-only protein (Bad, Bid). Bax is a pro-apoptotic protein which, if activated or increased, will induce porosity in the mitochondria culminating in the release of cytochrome c. This chain of events activates not only caspase 3 as the executor, but also caspase 9, while also causing apoptosis.[14] In other words, if the anti-apoptotic protein is activated, the cytochrome release can activate caspase 9, resulting in the absence of apoptosis, thereby enabling cells to survive. Nevertheless, if this condition occurs continuously, then abnormal cell proliferation will ensue.

Such abnormal cell proliferation can lead to hyperplasia, mild dysplasia, moderate dysplasia, severe dysplasia, and carcinoma *in situ*. Similarly, this condition was found to have occurred by the research reported here following controlled exposure to cigarette smoke for 29 days and 59 days. The incidence of epithelial dysplasia was detected since Oral Epithelial Dysplasia (OED) is histopathologically associated with an increased risk of oral cancer[6] and can be considered one of the markers employed to detect early oral squamous cell carcinoma (OSCC).[15] Jain (2019)[16] even argues that, histologically, the process of

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oral carcinogenesis commences with hyperplasia, moving through dysplasia to severe dysplasia before, finally, culminating in invasive and metastatic conditions which reflect the combined changes transforming normal cells into cancerous ones. Dysplasia in the epithelium is even considered to be the initial stage of cellular morphological change which precedes malignancy.[15]

In addition, epithelial dysplasia constitutes a developmental abnormality which indicates histological cellular and architectural changes in the epithelium. Characteristic features of cell architecture in epithelial dysplasia can be manifested by irregular layers of epithelium, loss of basal cell polarity, proliferation of mitotic features, abnormalities of mitotic and keratinous surfaces without the presence of a rete peg.[17] However, in this study, the different degrees of dysplasia in each treatment group can be due to gene variations resulting in cell changes in a single group. According to Ge *et al.* (2016),[18] approximately 600 genes influence the process of normal cell changes culminating in malignancy, including transcription factors, oncogenes, differentiation markers, tumour suppressors, and metastatic proteins.

Genetically, changes that occur during carcinogenesis can be due to mutations, amplifications, rearrangements, and deletions.[16] This carcinogenic stage may be preceded by initiation, promotion, progression, and metastasis. The initiation stage involves structural changes, gene mutations occurring either spontaneously or because of stimulation resulting from exposure to carcinogens. Such genetic changes subsequently cause dysregulation of cell proliferation pathways, survival, and differentiation which can be influenced by several factors, including the size and type of carcinogenic metabolism, as well as the response of DNA repair functions. The subsequent promotion stage is considered a relatively protracted and reversible process in which active cells undergoing preneoplastic proliferation can accumulate. This period can be changed by chemo-preventive materials which will affect the growth rate. The subsequent progression stage is considered to be that between premalignant lesions and progression to invasive cancer. Progression is also considered the final stage in neoplastic transformation, where genetic changes, phenotypes, and proliferation occur. It involves the rapid growth in tumor size in which cells undergo further mutations that will potentially become invasive and metastatic. Furthermore, the metastastic stage is the one in which cancer cells spread from their original location to other sites around the body through the bloodstream or lymphatic system.[19]

Finally, the different degrees of dysplasia in each treatment group in this study could also be caused by the nicotine and other substances passing into the subjects' body differing from each other, despite the equal duration of exposure to cigarette smoke. In other words, the degree of dysplasia could be influenced by several factors, such as genetic and carcinogenic, which affect the body. In conclusion, the more protracted the exposure of *Rattus norvegicus* to cigarette smoke, the higher the degree of epithelial dysplasia, indicating that such exposure plays an active role in the carcinogenic process affecting the oral mucosa.

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