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

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ABSTRACT

Background: Hyperplasia, a condition whereby an increasing number of cells are produced due to their uncontrolled division, represents a common symptom of carcinogenesis. Cancer is a physical manifestation of cell malignancy resulting from abnormal proliferation. Globally, oral cancer currently constitutes the sixth largest lethal form of the condition. The most common etiology of oral cancer is tobacco of which cigarettes are the most popular related product. The health risks associated with cigarette smoke not only affect active smokers but also individuals who ingest it passively. Sidestream smoke comes from the lighted end of a burning tobacco product such as a cigarette, pipe or cigar and contains nicotine and many harmful cancer-causing chemicals. Inhaling sidestream smoke increases the risk of lung and other types of cancer. Purpose: The purpose of this study was to understand how sidestream cigarette smoke initiates precancerous changes, in this case hyperplasia, in the oral mucosa epithelium of Wistar rats. Methods: The subjects were divided into three groups, a 4-week treatment group (P1), an 8-week treatment group (P2), and a control group (K), each consisting of ten subjects. The subjects were exposed to a daily two-cigarette dose of smoke. The experiment used a post-test only control group design. All samples were sacrificed during the fourth and eighth weeks. Haematoxylin-eosin staining was performed on the tongues of the Wistar rats to establish the presence of hyperplasia. Data was analyzed using a one-way ANOVA test. Results: After the Wistar rats had been exposed to cigarette smoke, an increased degree of epithelial cell proliferation (hyperplasia) showed a significant difference with a p-value <0.05 during the eighth week. Conclusion: Exposure to cigarette sidestream smoke induces increased epithelial cell proliferation (hyperplasia) in Wistar rats.

Keywords: cigarette smoke; hyperplasia; oral cancer

INTRODUCTION

Body cells undergo a process of proliferation and growth constituting a well-regulated gene regulation phenomenon. When damage occurs, cell growth increases as part of the body’s defense mechanism. The cells surrounding those damaged accelerate their growth to restore normal tissue structure and function. After this has occurred, the tissue cells will die through a process known as apoptosis. The body will subsequently produce replacement cells for those which have died. The process of cell progression and apoptosis does not invariably progress in a uniform manner due to certain risk factors such as stress, chemicals, toxins, bacteria, viruses, parasites, fungi, and genetics. However, cells can survive through a mechanism of adaptation which may occur due to atrophy, hypertrophy, hyperplasia, dysplasia, and metaplasia.1–5

Hyperplasia, also defined as the abnormal proliferation of cells resulting from continuous multiplication, can be regarded as a symptom of the onset of carcinogenesis. Cancer represents a form of malignancy involving abnormal cell proliferation with head and neck cancer constituting the sixth most common form in humans. 48% of cases are located in the oral cavity, with 90% of these being oral squamous cell carcinomas (OSCCs). The common etiology of OSCC is tobacco use in its various forms (22%).6–9
The inhaling of second-hand cigarette smoke by non-smokers is termed passive smoking. For passive smokers, the risk of oral cancer increases by 87% compared to that for non-smokers who are not exposed to cigarette smoke.\textsuperscript{8–11} If the source of smoke inhaled is the cigarette itself then the smoke is called side stream smoke, while the smoke exhaled by the smoker is termed exhaled mainstream smoke.\textsuperscript{6,7,12,13}

Cigarette smoke contains approximately 60 carcinogenic substances. The processes induced by cigarette smoke which have been identified as causing cancer are those of free radical metabolism and DNA damage within the human body which induces gene mutation. In the OSCC, a premalignant lesion develops in the upper aerodigestive tract (UADT). Such lesions experience an increase in epithelial proliferation that can be interpreted as a symptom of the early onset of OSCC.\textsuperscript{5} The purpose of this study was to comprehend the manner in which side-stream cigarette smoke triggers precancerous changes, in this case hyperplasia, in the oral mucosa epithelium of Wistar rats.

**MATERIALS AND METHODS**

This research was received ethical approval from the Ethics Committee of the Faculty of Dental Medicine, Universitas Airlangga, with number 175/HRECC.FODM/IX/2017.

This research represented a laboratory experimental research with post-test only control group design and used a sample of 30 male Wistar rats (\textit{Rattus novergicus}), aged three months, with a body weight of 170 grams (± 10%). The animals were randomly divided into three groups, consisted of two treatment groups (P1 group treated for four weeks and P2 group treated for eight weeks), and one control group (K group), each group consisting of ten rats.

The research material consisted of clove cigarettes. Exposure to their smoke was effected using a device referred to as a smoking pump. Each subject underwent a single daily exposure to the smoke derived from a maximum of two cigarettes.\textsuperscript{14} This level of exposure was maintained up to the time at which the subject was sacrificed. The control group subjects were placed in separate tubes to ensure their simultaneous exposure to the air. In order to ensure that the difference in smoke exposure between the tubes in the smoking pump was consistent, the placement of subjects in each tube was rotated (Figure 1).

At the end of the fourth and eighth weeks, each subject was sacrificed, its tongue being removed and subjected to histopathological examination by means of Hematoxylin-Eosin (HE) staining. Microscopic assessment comparing the K, P1, and P2 groups was conducted with a light microscope at 1000x magnification across five random visual fields.

**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 experiment subjects exposed to cigarette smoke by means of a full-body exposure device.

**Figure 2.** Histopathological features of rat 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 (at 1000x magnification). *The yellow line indicates hyperkeratosis, while the red line denotes hyperplasia.
A Kolmogorov-Smirnov test was performed to discover whether the data obtained was normally distributed (value p>0.05). The data of the research treatment groups was then subjected to a Levene’s homogeneity test followed by a one-way ANOVA test. Normally distributed data subsequently underwent a Tukey HSD to determine the differences between groups (value p<0.05).

RESULTS

The contents of Table 1 show that an increase in the thickness of the epithelium and stratum corneum of the subject’s tongue occurred. Increases in the epithelial and stratum corneum thickness were observed in the K group compared to the P1 and the P2 groups. Thickening of the epithelium indicated hyperplasia, while stratum corneum thickness constituted a symptom of hyperkeratosis.

The Tukey HSD test result indicated a significant increase in terms of hyperplasia in the K group compared to the P2 group with a p-value of 0.000 (p<0.05). Meanwhile, the increases in hyperplasia in the K group compared to the P1 group and in the P1 group compared to the P2 group as indicated by p-values were 0.067 and 0.076 respectively. In terms of hyperkeratosis, there was significant difference in the K group compared to the P2 group with a p value of p=0.014 (p<0.05). With regard to hyperplasia, a significant increase occurred in the K group in comparison to the P1 and in the P1 group compared to the P2 group as indicated by their respective p-values of p=0.566 and p=0.127.

Figure 2 shows the histopathological features of the tongue mucosa of the subjects. From the one representative field of view, it can be observed that there is increased hyperplasia and hyperkeratosis in the K group compared to the P groups.

DISCUSSION

Cancer constitutes a form of cell malignancy in which proliferation occurring through the process of carcinogenesis is uncontrolled. The early stage of the condition is characterized by hyperplasia which is often accompanied by hyperkeratosis. Oral cancer is the sixth most common fatal form of the condition in the world. Its most common etiology consists of tobacco and cigarettes, products most commonly found in the community environment.1,3–5

The study conducted was based on a simple hypothesis about the potential for hyperplasia to occur in Wistar rats exposed to cigarette smoke over a certain period of time. In this study, male Wistar rats (Rattus norvegicus) were used due to their being unaffected by hormonal conditions. Their body weight was maintained at an ideal level of 170 grams (+ 10%) during a week-long process of adaptation. Wistar rats were selected because of the similarity of their immune system, oral mucosa, and lymphatic drainage system with that of humans. Their response to tumor antigens with T-lymphocytes is also comparable. The oral epithelium on the inferior tongue surface of rats is much thinner (8-12 layers) than that of humans (20-30 layers) with the same degree of rete ridge.3,15

Previous research into cigarette smoke involving the use of Wistar rats has been carried out using three treatment methods, namely; inhalation exposure via the respiratory tract, whole body exposure, and nicotine injection. Nasal inhalation methods require oxygen masks, but these may be easily damaged by rats which belong to the rodent species. Moreover, the hyperplasia histology involving nasal inhalation method results are not significant. Based on these considerations, most researchers opt for the whole-body exposure method which provides sufficient space for the subject during treatment. However, the exposure dose of cigarette smoke is not administered via the oral cavity but, rather, throughout the entire body. Nevertheless, the histology results produced by the later method remain significant. Accordingly, this study adopted the whole-body exposure method with certain modification.16

The research material selected for this study consisted of clove cigarettes on the basis of their nicotine level being twice as high as the standard cigarettes commonly consumed by the public. The nicotine content of the latter is 1.1 mg, while that of clove cigarettes, which contain 60% tobacco and 40% cloves, amounts to 2 mg. There are several different ingredients of such cigarettes compared to white varieties. Clove cigarettes contain five additional ingredients, namely; eugenol, acetyl eugenol, β-caryophyllene, α-humulene, and caryophyllene epoxide6,16,17

In a previous study, the oral cavity of in vivo male Wistar rats exposed to cigarette smoke for 60 days presented premalignant lesions. Due to the absence of p53 protein expression, their time period proved of insufficient duration to cause cancer. However, a Ki-67 analysis showed an increase in epithelial proliferation resulting from the damage response.16 Therefore, the researchers decided to adopt point of exposures of 4 and 8 weeks duration.

The tongues of Wistar rats were selected because previous studies had confirmed these areas to have frequently presented significant symptoms of hyperplasia and hyperkeratosis. Moreover, 40% of OSCC cases affect the tongue. The three groups presented differences in the effect of cigarette smoke on the thickness of the epithelium and stratum corneum.3,16,18 Based on the results of histopathological examination of the K, P1, and P2 groups, a significant difference was identified in the epithelial thickness of the epithelium and stratum corneum. As indicated by the contents of Table 1, the K group which was not exposed to cigarette smoke presented an average hyperplasia value of 179.89μm and an average hyperkeratosis value of 64.38μm. When compared with the P1 and P2 groups, the K group was the thinnest, while the P2 group was the thickest. The P1 group recorded a hyperplasia value as high as 206.01μm and a hyperkeratosis value of 68.97μm, while the P2 group registered hyperplasia thickness.
and hyperkeratosis values of 231.46μm and 77.97μm, respectively.

Based on these results, it can be argued that clove cigarette smoke affects the proliferation occurring in the epithelium and stratum corneum. When the oral mucosal epithelium is exposed continuously to clove cigarette smoke its structure and function may be negatively impacted. The nicotine in cigarette smoke contains tobacco-specific N-nitrosamines (TSNA) derivatives which, in turn, contain carcinogenic substances in the form of 4-[(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N-nitrosonornicotine (NNN). Cigarette smoke molecules can induce epidermal growth factor receptor (EGFR) phosphorylation which mediates cytoplasmic tail (CT) mucin-1 (MUC1) phosphorylation. This, in turn, induces the cleavage of β-catenin from E-cadherin to form the β-catenin/MUC1-CT complex of the Wnt/β-catenin canonical pathway. The resulting complex translocates from the cytosol to the nucleus and joins the family of T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) and transcription factors leading to the transactivation of genes that drive cell cycles (e.g., C-Myc, cyclin-D).19–21

EGFR, which continuously binds to the molecular components of cigarette smoke, causes an increase in epithelial cell proliferation and decreased attachment between cells which will subsequently 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 the activation of EGFR and reduction of E-cadherin leading to the development of cancer.19,21

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 others ERK1/2, P38, JNK, and NF-kB. 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 crucial to cell cycle progression an increase of which leads to higher cell proliferation. Judging from the level of cell proliferation following exposure to cigarette smoke, a mechanism of resistance to apoptosis operates. Oral epithelial cells repeatedly exposed to cigarette smoke show 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 have an effect on keratin protein expression. Cigarette smoke reduces the expression of Keratin1, Keratin5, Keratin10, Keratin16 proteins and stimulates that of their Keratin6 and Keratin14 counterparts. 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 demonstrates 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 epithelial cells which experience increased proliferation and migration. Continuously activated EGFR will result in greater uncontrolled proliferation and migration of cells without being offset by apoptosis. Consequently, cells will metastasize and become malignant. The process of proliferation enhancement that occurs is one of pathological hyperplasia and the subsequent involvement of keratin cytoskeleton protein facilitates hyperkeratosis.25,26

The research results reported here were in line with those of previous studies. Exposing mice to cigarette smoke for one month, a period considered to constitute long-lasting, chronic exposure, may induce a thickening of the airway epithelium as the body’s defenses against cigarette smoke compounds intensify.21 Against this background, the ratio of K to P1 and of P1 to P2 become insignificant in the course of 28 days because of the ability of the subjects to tolerate cigarette smoke compounds. However, a significant comparison between the K and P2 groups occurred because the P2 group demonstrated mucosal defense against cigarette smoke and simultaneously adapted to exposure to it over a period of eight weeks. This was due to proliferation enhancement resulting from a combination of pathological hyperplasia and hyperkeratosis. Thus, the statistical results confirmed that the K group showed significant differences to the P2 group, while the K group showed no significant difference to the P1 group and the P1 group showed no significant difference to the P2 group.

Since hyperplasia is a reversible condition, further experiments are required to observe any histopathological changes due to extended exposure to sidestream cigarette smoke which support a hypothesis of hyperplasia, characterized by increased proliferation of cells resulting from chronic irritation as a symptom of the onset of carcinogenesis.13–5,26-Taken together, it can be concluded that sidestream cigarette smoke exposure induces chronic irritation which may lead to prolonged inflammation and precancerous changes in the oral mucosa of the tongue of a Wistar rat, thereby inducing a response of pathological hyperplasia together with hyperkeratosis.

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