Effects of sidestream tobacco smoke on P53 expressions in Rattus norvegicus tongue epithelial mucosa

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ABSTRACT

Background: Smoking, both active and passive, has been widely recognised as toxic to the human body, since it induces several forms of cancer, including that affecting the oral cavity. Benzopyrene, the carcinogen contained in tobacco smoke, can even lead to carcinogenesis which potentially affects the regulation of cell apoptosis in both active and passive smokers. Purpose: This study aims to investigate the carcinogenic effects of cigarette smoke on apoptosis of rat tongue mucosae through p53 expression. To determine the risk of malignant transformation through tumor suppressor genes in the apoptotic pathway. Methods: Rattus norvegicus subjects were divided into four groups, namely Treatment Group 1 exposed to sidestream cigarette smoke for four weeks (P1), Treatment Group 2 exposed to sidestream cigarette smoke for eight weeks (P2), Control Group not exposed to sidestream cigarette smoke for four weeks (K2), and Control Group (K) not exposed to sidestream cigarette smoke for eight weeks (K2). The exposure process was conducted using a smoking pump and alternating exposure. Four micron-thick sections of formalin were subsequently fixed together with paraffin embedded biopsy material from tongue mucosa of Rattus norvegicus. The tissue sections from the treatment groups were then analyzed immunohistochemically to compare the expressions of p53 and Bcl-2 proteins with those of the control groups. Results: The T-test results indicated statistically significant differences in the expressions of p53 between the 4-week control group (K1) and the 4-week treatment group (P1) (p=0.01, p<0.05) as well as between the 8-week control group (K2) and the 8-week treatment group (P2) (p=0.03, p<0.05). Conclusion: Exposure to cigarette smoke can induce changes in tumor suppressor genes and also affect the regulation of cell apoptosis, thus changing cell structure and leading to malignancy.

Keywords: apoptosis; carcinogenesis; sidestream cigarette smoke; p53; tongue mucosa

INTRODUCTION

Over the last 50 years, numerous studies have been conducted on the toxic chemicals contained in cigarette smoke which are regarded as carcinogens for humans. Cigarette smoke is one risk factor for oral cancer in both active and passive smokers due to the presence of carcinogenic elements that can potentially induce cancer. Passive smokers inhale the second-hand environmental cigarette smoke exhaled by active smokers.

Smoke inhaled by passive smokers can also cause health problems similar to those experienced by active smokers since it contains approximately 200 toxic substances, 69 of which are carcinogenic. These carcinogens form covalent bonds with DNA (DNA adducts) subsequently inducing carcinogenesis.1,2 The correlation between both active and passive smoking and carcinogenesis has actually been studied and identified to exist in several forms of cancer. A high risk of smoking-related cancers also relates to the head and neck. These include cancer of the oral cavity, pharynx and larynx, in addition to lung cancer.3

The process of carcinogenesis is a somatic event thought to be caused by accumulative genetic and epigenetic changes affecting the normal molecular control settings in cell proliferation. These genetic changes can subsequently deactivate the tumor suppressor gene, thereby triggering tumor formation.4 The tumor suppressor gene (p53 gene) is a transcription factor that activates a large number of
gene expressions involved in cell cycle regulation and apoptosis. The p53 gene is the first tumor suppressor gene identified in cancer cells whose working mechanism is normally in an inactive state. It will become active if the cell experiences stress. Loss of function in the p53 gene due to mutations can affect the apoptotic mechanism involving the bcl-2 and caspase genes. The mechanism of the p53 gene induces apoptosis by stimulating mitochondria through the induction of the Bax gene to release cytochrome c to the cytosol to form caspase cascade.\textsuperscript{5}

In addition, carcinogenesis conditions can induce changes in cells, resulting in malignancy. Therefore, this study aims to examine the carcinogenic effects of cigarette smoke exposure on the mucosa of the rat’s tongue against apoptosis through p53 expressions.

MATERIALS AND METHODS

This study was received ethical approval from the Ethics Committee of the Faculty of Dental Medicine, Universitas Airlangga, (No: 769/HRECC.FODM/XII/2019). This study constitutes experimental laboratory research involving 24 male Wistar strain rats (\textit{Rattus norvegicus}) aged 3-week old and 170 grams in weight which had been obtained from the Biochemical Laboratory of Universitas Airlangga, Surabaya. The inclusion criteria for these Wistar rats comprised; a good state of health, a body weight of 160-180 grams, and a 1-week adaptation period during which they had to satisfy the requirements of being clear-eyed, having a shiny coat, being agile, and passing firm stools.

This study was conducted at the Biochemistry Laboratory of Universitas Airlangga, Surabaya and the Biomolecular Laboratory of Universitas Brawijaya, Malang. The Wistar rats were divided into four groups, namely two control groups consisting of a 4-week control group and an 8-week control group, as well as two treatment groups composed of a 4-week treatment group and an 8-week treatment group. Each group contained 6 members. The subjects in the treatment groups were exposed to unfiltered clove cigarette smoke with a tar content of 34 mg and a nicotine content of 2.1 mg via a smoking pump which ensured constant exposure to a dose equivalent to that of 20 cigarettes per day.\textsuperscript{6} Meanwhile, the subjects in the control groups received their usual rations of food and drink and were placed in an open space.

After completion of the planned treatment, the subjects were sacrificed by means of inhalation of lethal doses of ether. Those in Treatment Group 1 and Control Group 1 were sacrificed at the end of the 4\textsuperscript{th} week, while their counterparts in Treatment Group 2 and Control Group 2 were sacrificed at the end of the 8\textsuperscript{th} week. Following excision of their tongue mucosa, histological preparations were produced. The tissue sections were immunohistochemically analyzed for the expression of p53 gene.

The preparations were examined through a Nikon e100 light microscope at 400x magnification in order to calculate their p53 expressions. The results of each parameter were then analyzed statistically. A Shapiro-Wilk normality test was carried out to determine the distribution of research data, followed by a Levene’s variance homogeneity test. If the results showed normally distributed data, a paired two-sample T test was subsequently performed. The analysis was carried out using SPSS for Windows version 16 (IBM, Armonk, New York, USA).

RESULTS

The mean and standard deviation of p53 expressions in the control groups and the treatment groups after exposure to cigarette smoke for four weeks and eight weeks are presented in Table 1 and Figure 1. The p53 immunohistochemical staining process results can be seen in Figure 2. The T test

Table 1. The expressions of p53 in the control groups and the treatment groups after exposure to cigarette smoke for 4 and 8 weeks

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean ± SD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>K1</td>
<td>13.17 ± 2.9</td>
<td>0.01*</td>
</tr>
<tr>
<td>P1</td>
<td>7.17 ± 3.8</td>
<td></td>
</tr>
<tr>
<td>K2</td>
<td>14.17 ± 3.8</td>
<td>0.03*</td>
</tr>
<tr>
<td>P2</td>
<td>7.5 ± 1.9</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. The graph of the average p53 expressions in the 4-week control group and the 4-week treatment group as well as between the 8-week control group and the 8-week treatment group.
results indicated a significant difference in p53 expression between the control and treatment groups after cigarette smoke exposure for four weeks (p=0.01, p<0.05). Similarly, there was also a significant difference in p53 expression between the control and treatment groups after cigarette smoke exposure for eight weeks (p=0.03, p<0.01).

DISCUSSION

In general, unfiltered clove cigarettes are more dangerous than their filtered counterparts due to a higher tar, nicotine and carbon monoxide (CO) content. The cloves contained in such cigarettes actually constitute an additive whose effect is that the mixture of tobacco and cloves can increase the temperature of a lighted cigarette. As a result, the CO and nicotine levels of clove cigarettes are three times higher than those of tobacco-only cigarettes, while their tar levels are as much as to five times higher.6

Cigarette smoke can potentially induce cancer due to the presence of carcinogenic elements. Consequently, it is considered a risk factor of oral cancer in both active and passive smokers, influenced by the duration and dose of exposure to it. A previous study by Kushihashi et al. (2012),3 assessed the impact of the number of cigarettes smoked per day and the duration of smoking among active smokers or that of cigarette exposure among passive smokers on the occurrence of head and neck cancer. The study argued that smoking plays a significant role in the development of squamous cell carcinoma (p = 0.0338). The development of oral cancer is a complex process that can be observed through the use of animal models. Their use facilitates accurate and representative descriptions of cellular and molecular changes which have been analyzed histopathologically and which arise from the initiation and development of oral cancer due to a change from normal to pathological conditions.8

Employing in vivo animal models as research subjects renders the initiation, promotion, development and metastasis of cancer, including oral cancer, observable.9 Therefore, this study was conducted using 3-month-old, male, Wistar rats (Rattus norvegicus) which represent the most widely employed animal subjects for laboratory research and which, at the age of three months, have reached biological maturity.10 Male Wistar rats were chosen as research subjects because their conditions are not affected by hormonal factors, such as the menstrual cycle and pregnancy.11 Hormonal changes can affect the immunity of the subjects which will, in turn, affect the results of the study.12 p53 constitutes a group of tumor suppressor gene proteins playing a role in cell growth control in the nucleus, particularly with regard to the cell division cycle.

This study produced significant decreases in the level of p53 expressions in the 4-week treatment group (p = 0.01, p<0.05) and the 8-week treatment group (p=0.03, p<0.05) after exposure to cigarette smoke for 4 and 8 weeks. Similarly, a previous study conducted by Husgafvel et al. (2000),13 focused on non-smokers to ascertain the relationship between exposure to environmental tobacco smoke (ETS) and lung cancer in non-smokers and employed both frequency biomarkers and p53 mutation types as carcinogenetic biomarkers associated with tobacco use. It concluded that ETS directly exhaled into the air can negatively affect the health of non-smokers without their even inhaling it since ETS contains carcinogenic substances.

The increase in mutant p53 in cases of lung cancer is potentially experienced to a greater extent by non-smokers who are in the immediate vicinity of smokers and, therefore, exposed to ETS. This indicates that prolonged exposure to second-hand smoke increases the risk of neoplasia in non-smokers.13 Other oral cancer-related studies have been carried out by inducing rats through exposure to carcinogens in the oral cavity such as those found in tobacco
cigarettes. One such investigation was conducted by Pfeifer et al. (2002), which found a strong correlation between smoking and oral cancer through analysis of the p53 mutation spectrum.

The increased amount of carcinogenic material absorbed by mucosal epithelial cells will heighten the risk of oncogene, tumor suppressor gene (TSG) and deoxyribonucleic acid (DNA) mutations that play a role in cell division. 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosonornicotine (NNN), together with cigarette smoke content, for example polycyclic aromatic hydrocarbons (HAP), are able to modify DNA nitrogen bases through methylation and hydroxylation. Other ingredients, such as nitric oxide (NO) and free radical compounds, can induce the accumulation of free radicals in the oral mucosa. The excessive activity of free radicals and other reactive elements can subsequently lead to oxidative stress resulting in genomic instability, such as modification of DNA nitrogen bases potentially leading to gene mutations. The modification of the DNA nitrogen base can promote DNA adduction potentially leading to damage to the DNA of oral mucosal epithelial cells. DNA damage, in turn, triggers the oncogene, TSG and DNArg mutations.

The gene mutations can indicate whether changes in malignant epithelial cells of the oral mucosa have been initiated. Similarly, in this study, the decreased expression of p53 due to exposure to cigarette smoke shows that gene mutations can normally affect the cell cycle and initiate the risk of malignancy. In conclusion, this study argues that exposure to cigarette smoke can cause decreased expression of wild p53 leading to the cells becoming malignant.

REFERENCES