Effects of Chronic Nicotine Administration on Salivary Gland in Mice: Immunohistochemical Study

Jung-Woo Jung, Jae-Kap Choi, Jin-Seok Byun

Department of Oral Medicine, School of Dentistry, Kyungpook National University, Daegu, Korea

Purpose: Chronic nicotine administration induce various effects in whole organs of the body; however, little is known about salivary gland. In the present study, we pursued the links between systemic nicotine and the histomorphological changes of the salivary gland in mice.

Methods: Twenty-five C57BL6 mice were allocated into two groups. The control group (n=9) received distilled water only for 8 weeks by gavage. The experimental nicotine group (n=16) was administered nicotine 5 μg/g with distilled water. Animals were sacrificed at 8 weeks; then, submandibular glands were excised and processed for histologic evaluation. Volumetric changes in acinar cells were evaluated by H&E staining. The expression of calponin-positive myoepithelial cells and Ki-67-positive proliferating acinar cells were evaluated by immunohistochemistry.

Results: The nicotine group showed significantly decreased number of calponin-positive myoepithelial cell process compared with the control group. There were no significant differences in average volume of acinar cell and the number of Ki-67-positive acinar cells between both groups.

Conclusions: These findings suggested that chronic nicotine administration may cause decreased function of myoepithelial cells in submandibular glands of mice, and these can partly explain xerostomic conditions in chronic smokers.

Key Words: Acinar cells; Calponin; Myoepithelial cells; Nicotine; Submandibular gland; Xerostomia

INTRODUCTION

The tobacco contains over 4,000 hazardous substances and tobacco smoking is well known a causative factor for respiratory, cardiovascular and musculoskeletal diseases as well as oral complications such as xerostomia, oral mucosal diseases, and even oral cancers. One of important things about the tobacco smoking is addiction which makes it serious health threat all over the world. Of the various hazardous substances, nicotine is well known to a representative substance responsible for the dependence which results in chronic smoking.1-3

Nicotine is a colorless alkaloid found in nightshade family plants and the most abundant volatile alkaloid in tobacco leaf. Basically, nicotine from tobacco products is absorbed into the blood flow through the lung, oral mucosa, skin and intestine, and then moves into cardiovascular, endocrine, musculoskeletal system as well as peripheral and central nervous systems.4 Absorbed nicotine can cause temporal elevation of blood pressure, pulse rate and nausea, xerostomia, and euphoria by activating mesolimbic system. However, repetitive nicotine administration gradually decreases the sensitivity of nicotinic acetylcholine receptor. Thereby it causes the addiction to nicotine which makes the chronicity of smoking.5

There are several studies about the relevance between chronic nicotine intake and secretory impairment such as pancreatitis.6-8 Short-term nicotine administration can induce the elevated Ca2+ influx in pancreatic acinar cell which activates secretion in acinar cell.9 However, chronic
stimulation of acinar cell in pancreas induces physiological overload and develops the pathologic processes in pancreas.\(^7\)

One of main complaints in chronic smokers is a feeling of mouth dryness, called xerostomias. There are several studies to elucidate the relation between smoking and salivation. Rad et al.\(^9\) and Kanwar et al.\(^10\) reported that chronic smoker showed significantly decreased salivation compared with that of non-smoker. However, it is still questionable what specific substance is responsible for the reduced salivation in chronic smokers. Interestingly, Rowell et al.\(^11\) investigated the distribution of nicotine at organ-specific level. According to this study, absorbed nicotine was distributed highly in glands such as adrenal gland and salivary gland. This suggested that the prolonged elevation of nicotine level in salivary gland might be a causative factor for pathologic process of salivary gland. Moreover, other study also showed that chronic stimulation of nicotine could induce the pancreatitis through the following mechanism. Nicotine can elevate Ca\(^{2+}\) influx into pancreatic acinar cells, which temporally activates acinar cells secretion. However, chronic stimulation by nicotine rather induce physiologic overload into acinar cells, and ultimately developed pathologic process in the pancreas.\(^6-8\)

To date, there are a few studies about the role of chronic nicotine on specific organ level. The aim of the study is to elucidate the effects of chronic nicotine administration on salivary gland in mice by histomorphological and immunohistochemical methods. We are specifically focused on the changes of acinar cells and myoepithelial cells in submandibular glands of mice which mainly responsible for the production of unstimulated saliva.

**MATERIALS AND METHODS**

This experiment was approved by the guideline for the Animal Care and Use Committee of Kyungpook National University (KNU 2014-95). Twenty-five 6-8 weeks C57BL/6J mice weighing 20-22 g were randomly allocated into 2 groups; control and nicotine. Diet and water was to be accessed ad libitum. Mice were raised 4 to 6 per cage and maintained in a room at 21°C±1°C and with 12 hours light/dark cycled. Body weight changes and consumption of water and diet were daily recorded. The control groups (n=9) received 0.2 mL of distilled water by gavage. The nicotine groups (n=16) were administrated nicotine with 5 μg/g (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.2 mL of distilled water. After 8 weeks, mice were sacrificed and submandibular gland were harvested. Submandibular gland were fixed in buffered formaldehyde, embedded in paraffin and cut into 5 μm thick serial sections.

H&E staining was performed for the evaluation of volumetric changes in acinar cells. The expression of the calponin (Abcam, Cambridge, UK) on myoepithelial cells and the Ki-67 (Merck Millipore, Hesse, Germany) for proliferation of acinar cells were evaluated by immunohistochemistry. In detail, secondary antibody was applied using Vectastain Universal Quick kit R.T.U (Vector Laboratories, Burlingame, CA, USA), and finally diaminobenzidine (DAB) substrate-chromogen system (Vector Laboratories) was used for the visualization of positive cells. Slides were examined through a Leica DM2700 M microscope (Leica Microsystems, Wetzlar, Germany) with IMT I-solution software (IMT I-solution Inc., Vancouver, BC, Canada).

In H&E slide, we counted whole number of nuclei and measured total volume of acinar in indicated area under 400× magnification, then calculated average volume of single acinar cell (μm\(^2\)/cell). We next counted whole numbers of Ki-67 positive cells in section of submandibular gland under 400× magnification and measured whole area of submandibular gland under 40× magnification. The data were normalized and represented as total numbers of Ki-67 positive cells per total area of submandibular gland (number/mm\(^2\)). In calponin stained slides, we first selected 10 random sites, then counted whole numbers of calponin positive processes and nuclei of acinar cells. The data were normalized and represented as total numbers of calponin positive process per number of nuclei in indicated acinar (number/number).

The data was analyzed using statistical package for SPSS software version 12.0 (SPSS Inc., Chicago, IL, USA) for Windows. The verification of normal distribution was performed using the Kolmogorov-Smirnov test and the two sample t-test was used to analyze the statistical significance. The data from the study were expressed as mean±standard deviation and p-value below 0.05 was
regarded as statistically significant.

RESULTS

1. No Volumetric Change of Acinar Cells after Chronic Nicotine Administration

Compared with that of control group, there was no significant difference on volumetric changes in acinar cells of submandibular gland after nicotine treatment (Table 1, Fig. 1).

2. No Difference in Numbers of Ki-67 Positive Cells after Nicotine Administration

There was no significant difference in numbers of Ki-67 positive proliferating acinar cells on submandibular glands of mice after nicotine treatment (Table 1, Fig. 2).

<table>
<thead>
<tr>
<th>Group</th>
<th>Control (n=9)</th>
<th>Nicotine (n=16)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinar cell size (μm²/cell)</td>
<td>241.5±16.3</td>
<td>249.0±20.6</td>
<td>0.180</td>
</tr>
<tr>
<td>Ki-67 positive cells (number/mm²)</td>
<td>29.9±6.8</td>
<td>36.2±8.2</td>
<td>0.146</td>
</tr>
<tr>
<td>Calponin positive cells (number/number)</td>
<td>0.48±0.13</td>
<td>0.35±0.11</td>
<td>0.036*</td>
</tr>
</tbody>
</table>

Table 1. Histomorphological and immunohistochemical analysis in submandibular gland of mice

Control group (distilled water only) and nicotine group (5 μg/g concentration in distilled water) were sacrificed at 8 weeks, then submandibular glands were excised, and processed into H&E staining and immunohistochemistry using Ki-67 and calponin antibody. Acinar cell size was calculated from dividing total areas of acinar cells by total numbers of acinar cell’s nuclei. Ki-67 positive cells was calculated from dividing total numbers of Ki-67 positive cells by total area of submandibular gland. Calponin positive cells was calculated from dividing total numbers of calponin positive processes by total numbers of nuclei in indicated acinar.

Values are expressed as mean±standard deviation.

*Statistically significant difference among the groups (p<0.05).

Fig. 1. Control group (n=9, distilled water only) and nicotine group (n=16, 5 μg/g concentration in distilled water) were sacrificed at 8 weeks, then submandibular glands were excised and processed into H&E staining. H&E staining (×400) was performed with the submandibular gland of control and nicotine group (A), and average acinar cell size was calculated (B). White dotted lines showed indicated area of acinar cells. Arrows showed nucleus of single acinar cell. Scale bars represent 50 μm in length. Values are expressed as mean±standard deviation.

Fig. 2. Control (n=9, distilled water only) and nicotine treated (n=16, 5 μg/g concentration in distilled water) mice were sacrificed at 8 weeks, then submandibular glands were excised and processed for immunohistochemistry using Ki-67 antibody. Total numbers of the Ki-67 positive proliferating acinar cells were counted under 400× magnification (A) and normalized by whole area of submandibular gland under 40× magnification (B). Arrows indicated Ki-67 positive proliferating acinar cells. Scale bars represent as 50 μm in length. Values are expressed as mean±standard deviation.
area compare to serous cell. Submandibular gland shows mixed patterns of both cell types histologically and is predominantly participated in producing unstimulated saliva. Myoepithelial cells are known to responsible for the secretion of saliva by contraction of acinus and duct. Ductal cell plays a central roles in production of hypotonic saliva by resorption of Na$^+$ and Cl$^-$ ions.\textsuperscript{12-14} As with humans, salivary glands of mice are also composed of three major compartments and each compartment have a similar structures. Even though some unique differences are exist in an aspect of anatomical and histologic view,\textsuperscript{15} salivary glands in both species basically serve similar functions—to help digestion, lubrication, gustations, protection of the teeth and oral cavity.

Oral dryness or xerostomia is one of the chief complaint in chronic smokers, and there were many studies to reveal the relationship between tobacco smoking and reduced salivation.\textsuperscript{9,10} Based on the study of Rowell et al.,\textsuperscript{11} we got a clue for the effects of chronic nicotine not only for the dependence of brain system but also for the possibility on salivary glands. We hypothesized that the prolonged elevation in blood nicotine might induce functional or structural changes directly on salivary gland, especially on acinar cells and myoepithelial cells. Although there were several previous studies using parotid glands of mice, we focused on submandibular glands as following reasons. Most of all, xerostomia was mainly affected by the amounts of unstimulated saliva. Secretion of unstimulated saliva is served 70% from submandibular gland, 20% from parotid gland, and 10% from the other salivary glands.\textsuperscript{12} Therefore, we thought that targeting the submandibular gland is more appropriate to represent clinical significance. In addition, submandibular gland is known to have the experimental feasibility due to its large size while parotid gland is diffusely located and difficult to remove because of surrounding fat cell.

We designed relatively prolonged period of nicotine administration (8 weeks) to establish chronic nicotine model using mice. A previous study by Pekonen et al.\textsuperscript{16} reported that plasmatic nicotine concentration of mouse for 7 weeks administration was similar to those of smoker. There were also several studies to determine the effect of chronic nicotine at 7 weeks.\textsuperscript{17,18} Moreover, regarding the concentration of nicotine, Rowell et al.\textsuperscript{11} reported that 100 μg/mL

### DISCUSSION

Parotid, submandibular, and sublingual glands are three major salivary gland of human, and are mainly composed of acinar cells, myoepithelial cells, and ductal cells. Acinar cells contain various secretory granules inside, and classified into serous and mucous cell depending on the characteristics of containing molecules. Serous cell has round nucleus in center of the cell and produce serous saliva precursor and is prominent in parotid gland. In contrast, sublingual gland is representative mucous gland, and mucous cell is characterized by its flat shaped-nucleus near basal

---

**Fig. 3.** Control (n=9, distilled water only) and nicotine treated (n=16, 5 μg/g concentration in distilled water) mice were sacrificed at 8 weeks, then submandibular glands were excised and processed for immunohistochemistry using calponin antibody. Total numbers of the calponin positive myoepithelial cell process were counted under 400× magnification (A) and normalized by whole number of acinar cell nuclei in 10 randomly selected sites (B). Arrowheads showed calponin positive myoepithelial cell process. Arrows indicated nucleus of acinar cell. Scale bars represent as 50 μm in length. Values are expressed as mean±standard deviation. *p<0.05 is considered as statistically significant.

3. Decreased Numbers of Calponin Positive Process after Nicotine Treatment

Compared with that of control, the numbers of calponin positive myoepithelial cell process were significantly decreased in nicotine group (Table 1, Fig. 3).
of nicotine administration showed chronic effects with no changes in fluid intake or body weight compare with control. That is why we administrate nicotine for 8 weeks at the designated concentration. In addition, in most of previous studies, nicotine was just provided via water bottle ad libitum. However, nicotine administration using water bottle with free access appeared to have the methodological limitation that the exact amount of nicotine intake could not be determined despite of measuring the decreasing amount of nicotine-containing water. Accordingly, our study administrated the nicotine solution directly to mice stomach using oral Zonde Needle (Jungdo Bio & Plant, Seoul, Korea) to determine the exact amount of nicotine intake.

Regarding the effects of nicotine on volumetric change of acinar cell, there were intriguing previous studies using rodents. Maier et al.\[9,20\] reported that chronic nicotine administration induce the swelling of acinar cell and increase of immature secretory granules in the cytoplasm. Based on these reports, we first evaluated volumetric change of acinar cell after nicotine treatment by H&E staining compare with those of control. Contrary to previous finding, our result showed no significant difference in acinar cell volume between both groups. This finding might be due to the difference of animal model and targeting salivary glands. In addition, the number of animal was relatively small to express statistical difference between both groups.

Recently, another interesting studies about the novel role of chronic nicotine were published. Chowdhury and colleagues\[8,23\] reported the nicotine as mitogenic stimulant for proliferation of pancreatic acinar cell. Nicotine activate extracellular signal-regulated kinases pathway, and then stimulate protein synthesis inducing cell proliferation and growth.

To date, however, there was no study regarding the effect of nicotine on the proliferative activity in salivary gland. Thus we examined whether chronic nicotine treatment could affect the proliferation of acinar cells in submandibular gland using Ki-67 immunostaining. Ki-67 is a nuclear protein necessary for cell proliferation that is associated with ribosomal RNA transcription and observed for all active phase of cell cycle.\[22\] Our study showed that chronic nicotine treatment could not change the proliferative ability of acinar cells in submandibular gland of mice. It was not consistent with previous study with pancreatic acinar cells, because change of acinar cell proliferation in pancreas was closely related to the development of oncogenesis. Chronic nicotine itself cannot have influence on proliferation of salivary gland in mice not less than in the present study, and further investigation is needed.

We next focused the effects of nicotine on myoepithelial cells, especially in salivary glands. Myoepithelial cells are found around acinar cells in the secretory units of many exocrine glands such as mammary, sweat, lacrimal and salivary glands.\[22,24\] Myoepithelial cells are primarily known to stabilize the structure of glands and also produce various proteins which is responsible for the suppression of tumors.\[25-27\] Myoepithelial cells of salivary glands are innervated by the muscarinic receptor and alpha-1 adrenergic receptor and play an important role in secretion of saliva in salivary duct by contraction.\[24,26\] There are two types of myoepithelial cells in salivary glands; ductal and acinar. Ductal myoepithelial cells are spindle shaped and oriented parallel to the long axis. Acinar myoepithelial cells are stellate shaped with 4-8 processes, forming basket like network around the luminal cells.\[29\] Myoepithelial cells are known to express several specific contractile proteins such as calponin, actin, myosin, and caldesmon.\[24,30\] We select the calponin antibody in the present study to verify the myoepithelial cells in submandibular gland of mice.

Interestingly, in our study, the average number of myoepithelial cell process in submandibular glands were significantly decreased after nicotine treatment. Even though the previous studies which demonstrated the role of nicotine on myoepithelial cells of mammary gland or pancreas were reported, it is difficult to apply these results directly to our study because of the difference of disease models. They investigated the role of myoepithelial cells only about the tumor suppression with tumor models. In the view of our result so far achieved, it is hard to conclude the role of nicotine as a salivary tumor enhancer. Nevertheless, decreased numbers of myoepithelial cell process in nicotine group could be an interesting finding in an aspect of xerostomias. The effects of nicotine on salivary gland are closely related to the development of oncogenesis. Chronic nicotine itself cannot have influence on proliferation of salivary gland in mice not less than in the present study, and further investigation is needed.

We next focused the effects of nicotine on myoepithelial cells, especially in salivary glands. Myoepithelial cells are found around acinar cells in the secretory units of many exocrine glands such as mammary, sweat, lacrimal and salivary glands.\[22,24\] Myoepithelial cells are primarily known to stabilize the structure of glands and also produce various proteins which is responsible for the suppression of tumors.\[25-27\] Myoepithelial cells of salivary glands are innervated by the muscarinic receptor and alpha-1 adrenergic receptor and play an important role in secretion of saliva in salivary duct by contraction.\[24,26\] There are two types of myoepithelial cells in salivary glands; ductal and acinar. Ductal myoepithelial cells are spindle shaped and oriented parallel to the long axis. Acinar myoepithelial cells are stellate shaped with 4-8 processes, forming basket like network around the luminal cells.\[29\] Myoepithelial cells are known to express several specific contractile proteins such as calponin, actin, myosin, and caldesmon.\[24,30\] We select the calponin antibody in the present study to verify the myoepithelial cells in submandibular gland of mice.

Interestingly, in our study, the average number of myoepithelial cell process in submandibular glands were significantly decreased after nicotine treatment. Even though the previous studies which demonstrated the role of nicotine on myoepithelial cells of mammary gland or pancreas were reported, it is difficult to apply these results directly to our study because of the difference of disease models. They investigated the role of myoepithelial cells only about the tumor suppression with tumor models. In the view of our result so far achieved, it is hard to conclude the role of nicotine as a salivary tumor enhancer. Nevertheless, decreased numbers of myoepithelial cell process in nicotine group could be an interesting finding in an aspect of xerostomias. The effects of nicotine on salivary gland are closely related to the development of oncogenesis. Chronic nicotine itself cannot have influence on proliferation of salivary gland in mice not less than in the present study, and further investigation is needed.
which dealt with chronic smoking and salivation showed decreased salivation by chronic smoking. Although they reported interesting phenomenon about the role of smoking on salivation, they failed to suggest underlying mechanisms for that. Based on our findings, the decreased number of myoepithelial cell process might lead to the impaired discharge capacity in submandibular gland which was primarily responsible for the unstimulated saliva, and subsequent disturbance in salivary secretion. Even though we do not know what are the specific components or mechanisms mainly responsible for the decreased salivation in previous reports, our findings might provide a reasonable support for the previous studies via direct nicotine-dependent manners.

The purpose of this study was to investigate any “direct” effects of chronic nicotine on submandibular gland of mice via histomorphological methods. Of many hypotheses we explored, the number of myoepithelial cell process was significantly decreased compared with those of control in chronic nicotine groups. Although the results from this study are not conclusive in explaining the effect of chronic nicotine exposure on salivary gland, our results showed a dim light on the field of xerostomia by suggesting intriguing findings of morphological changes. We confirmed long-term administration of nicotine directly induced the morphological change on myoepithelial cells, and consequently these might caused the impaired function of salivary glands in advance. Further investigation to reveal the direct effect of chronic nicotine in “organ level” not restricted only “dependence” on brain system are strongly warranted.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

ACKNOWLEDGEMENTS

We deeply appreciated professor Jae-Kwang Jung for his insightful comments on our manuscript. Professor Jae-Young Kim and Dr. Wern-Joo Sohn provided critical comments for the staining. We also thank Dr. Choon-Ik Kwon, In-Kyung Ki, Ki-Mi Kim, and So-Yeon Lee for their devoted supports during the study.

REFERENCES


