Effects of nicotine on the attachment and proliferation of periodontal ligament cells, and reversibility of nicotine-induced cytotoxicity

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I. Introduction

Cigarette smoking has been shown to alter an individual's response to various forms of periodontal therapy.¹⁻⁵ In vitro and in vivo studies have indicated that smoking and the use of smokeless tobacco products may significantly contribute to the development and progression of inflammatory periodontal disease. The enhanced periodontal destruction observed in smokers may be caused by an alteration of the host response such as the reduced function of oral and peripheral neutrophils, and the decreased levels of salivary and serum antibodies.⁶⁻⁹ Similar effects have been observed at higher concentrations of various tobacco components such as nicotine, acroleine, and cyanide.¹⁰,¹¹

Another hypothesis regarding the contribution of tobacco, and especially nicotine, to the etiology of periodontitis is that the reparative and regenerative potential of periodontal cells may be affected. It has been documented that nicotine can suppress the growth of osteoblast-like cells in vitro.¹²⁻¹⁴ Additionally, nicotine may be stored in and then released from periodontal fibroblasts. However, most of this cytotoxic substance remains within the fibroblasts, resulting in alterations of the cellular metabolism or various functions.¹⁵

Nicotine is a potent component of tobacco smoke.¹⁶ Nicotinic receptors are widespread and are heterogeneous groups comprised of multiple sub-units. The exact importance of individual receptor sub-units is currently not fully understood.¹⁷ However, this may explain in part the wide variety of physio-

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logical responses produced by nicotine. Sub-unit variability between target organs, cell types, and maybe even between cells at one site may explain the conflicting doseresponses of individual cell types, such as fibroblasts, isolated from different parts of body.\textsuperscript{18}

The major goal of periodontal treatment is the re-establishment of lost connective tissue attachment to the tooth root surface. Clearly this must occur by repopulation of the tooth surface with fibroblasts from the periodontal ligaments. Wound healing is the cumulative result of cell movement, cell division, and the synthesis of a variety of extracellular molecules. An alteration in any facet of these processes could lead to delayed healing.\textsuperscript{19}

Considerable evidence has shown that during periodontal regeneration, the cell type most likely to provide the major contribution is the periodontal ligament cell (PDL).\textsuperscript{19,21} It has been suggested that any factor able to inhibit the function of such cells would also impair tissue repair and regeneration. More recently, biochemical evidence has suggested that nicotine can have direct adverse effects on various functions of the PDL cells.\textsuperscript{22} To further address this hypothesis, we examined the effect of nicotine on the attachment and proliferation of PDL cells, and determined the reversibility of nicotine-induced alteration.

II. Materials and methods

1. Periodontal ligament cell culture procedures and preparation of nicotine solution

To prevent bacterial infection in the early period of culturing, 500 unit/ml of penicillin and 500 g/ml of streptomycin were added in the DMEM\textsuperscript{1}. Periodontal ligament cells were obtained from the periodontal ligament of premolar teeth from patients who had plans for orthodontic treatment and the cells were cultured by the method of Suh et al.\textsuperscript{23} For these experiments, the cells were taken from the fifth and seventh passages.

A nicotine\textsuperscript{5} solution was freshly prepared prior to each experiment in DMEM with 10% FBS\textsuperscript{7} from 1 mg/ml stock solution of pure nicotine in methanol. To study the effects on attachment and proliferation of the periodontal ligament cells, the following concentrations of nicotine, 2, 0.5, 0.1, 0.005, and 0.001 mg/ml were used.

2. Effect of nicotine on the attachment of periodontal ligament cells

Periodontal ligament cells were seeded into a 24-well plate and a 6-well plate, at a cell density of $4 \times 10^4$ cells/ml. The prepared nicotine solution was added to test groups and the same amount of methanol was also added to the control groups. Cultures were

\textsuperscript{5} Gibco, Grand Island, NY
\textsuperscript{1} Sigma Chemical Co., St. Louis, MO
\textsuperscript{2} USB Co., Cleveland, Ohio

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Table 1. Definition and classification of cytotoxic scores based on relative growth rate (RGR)

<table>
<thead>
<tr>
<th>RGR (%)</th>
<th>Score</th>
<th>Classification</th>
</tr>
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<tbody>
<tr>
<td>100</td>
<td>-</td>
<td>None</td>
</tr>
<tr>
<td>75-99</td>
<td>+</td>
<td>Weak</td>
</tr>
<tr>
<td>50-74</td>
<td>++</td>
<td>Moderate</td>
</tr>
<tr>
<td>25-49</td>
<td>+++</td>
<td>Marked</td>
</tr>
<tr>
<td>1-24</td>
<td>++++</td>
<td>Strong</td>
</tr>
<tr>
<td>0</td>
<td>++++</td>
<td>Extreme</td>
</tr>
</tbody>
</table>

incubated for 1, 3, 6, 12 and 24 hours at 37°C in 100% humidity and 5% CO₂. The attached cells in the 24-well plate were detached with 0.05% trypsin¹/0.02% EDTA² solution and counted with a hemocytometer. The attached cells in the 6-well plate were washed with phosphate buffered saline, fixed with 4% PFA¹ solution at 4°C for 2 hours, stained by the Hematoxylin and Eosin(H&E) staining method, and observed with phase-contrast microscope.

3. Effect of nicotine on the proliferation of periodontal ligament cells

Periodontal ligament cells were seeded into a 24-well plate and a 6-well plate at a cell density of 2×10⁴ cells/ml. Cultures were incubated for 24 hours to permit cell attachment at 37°C in 100% humidity and 5% CO₂. The cultures were then exchanged with a new medium and a prepared nicotine solution was added to the test groups and the same amount of methanol was added to the control groups. Cells were incubated for 1, 4, 7, 11, and 14 days at 37°C in 100% humidity and 5% CO₂. The proliferated cells in the 24-well plate were counted. The cells in the 6-well plate were stained by the H&E staining method and observed with a phase-contrast microscope.

4. Evaluation of cytotoxicity

The relative growth rate(RGR) of the test group over the control group was calculated by the formula below and the data was interpreted by the cytotoxicity index in Table 1.²⁴)

\[
\text{Relative growth rate(RGR)} = \frac{\text{Mean number of fibroblast in respective condition}}{\text{Mean number of fibroblast in control group}} \times 100
\]
5. Evaluation of the reversibility of nicotine-induced cytotoxicity

Periodontal ligament cells were seeded in the same manner as the experiment for proliferation: the cells were allowed to attach for 24 hours, changed with a new medium, and added to 2, 0.5, 0.1 mg/ml of a nicotine solution in the test group and the same amount of methanol was added to the control group. The cells were incubated for 1, 4, 7, and 11 days at 37°C in 100% humidity and 5% CO₂, washed with PBS. The cells were then allowed to regenerate by changing with a new medium without nicotine or methanol or allowed a toxic effect of nicotine with no change by a new medium for 2 days. The proliferated cells in the 24-well plate were counted. The cells in the 6-well plate were stained by the H&E staining method and observed with phase-contrast microscope.

6. Statistical Evaluation

All data were described in terms of mean ± standard deviation and analyzed with a one-way ANOVA.

III. Results

1. Effects of Nicotine on Attachment of PDL Cells

Maximal attachment of control cells in the absence of nicotine was observed after incubation for 6 hours (Figure 1). No effect was found in the presence of 1 and 5 g/ml nicotine at all of the incubation periods. With higher concentrations (100g/ml to 2mg/ml) there was a dose-dependent inhibition of the attachment, which was even more evident after 6-hour incubation. With a nicotine concentration of 500g/ml, the percentage of cells attached at 1, 3, 6 hours was 57, 56, and 58% respectively, whereas the respective values for control cells were 86, 78, and 100%.

Morphological observation of the control cells after H&E staining revealed round or irregular shaped cells at 1 hour, elongated and extended cells after 3 and 6 hours, and more elongated and linear shaped cells after 12 and 24 hours. The cells of the 2mg/ml nicotine-treated group were exhibited cytoplasmic constriction and cell necrosis. Partial cytoplasmic constriction and cell necrosis was observed in the cells of 0.5mg/ml nicotine-treated group. Cytoplasmic extension slowly progressed in the 0.1mg/ml nicotine-treated group (Figure 1).

2. Effects of Nicotine on Proliferation of PDL cells

The direct effects of nicotine on the growth of cells from periodontal ligament were determined over 14 days. Proliferation of cells in the presence or absence of nicotine is shown in Figure 2. All concentrations above 100g/ml produced a statistically significant dose-dependent inhibition of proliferation at 1, 4, and 7 days. Nicotine inhibited the growth of cells at the 3 highest concentrations studied 2, 0.5, and 0.1mg/ml, throughout the 14 days.
Morphological observation after H&E staining revealed cytoplasmic constriction and unclear cell shape after the one day incubation period in the 2mg/ml nicotine-treated group. There was no difference observed after the one day incubation period but there were many vacuolizations in the cytoplasm and there was a reduction in the cytoplasmic process after four days incubation in 0.1mg/ml nicotine treated-group(Figure 2).

3. Evaluation of cellular toxicity of PDL cells

After a one-day incubation period, extreme cytotoxicity was evaluated by a cytotoxicity index in the 2mg/ml nicotine-treated group, marked cytotoxicity was found in the 0.5mg/ml nicotine-treated group, moderated cytotoxicity was discovered in the 0.1mg/ml nicotine-treated group and weak cytotoxicity was observed in the 0.005 and the 0.001 mg/ml nicotine-treated groups. After a 14-day incubation period, the following determinations were made by cytotoxicity index:

<table>
<thead>
<tr>
<th>Incubation Period(day)</th>
<th>Concentration of nicotine(mg/ml)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>
| 1                     | 100    | 0.66(++++) | 48.34(+++)
|                       | 57.6(++) | 77.48(+) | 88.08(+)
| 4                     | 100    | 0.68(++++) | 1.02(++++)
|                       | 63.95(++) | 108.84(-) | 100.68(-)
| 7                     | 100    | 0(++++)   | 0.15(++++)
|                       | 11.92(+++) | 97.17(+) | 96.27(+)
| 11                    | 100    | 0(++)    | 0(++)
|                       | 12.13(+++) | 95.3(+) | 96.29(+)
| 14                    | 100    | 0(++++)  | 0(++)
|                       | 1.64(+++) | 101.74(-) | 99.59(-)

Scores were made according to Table 1.

4. Evaluation of the reversibility of cell damage of PDL cells

Cells were incubated with DMEM culture medium for 24 hours then added with nicotine for various periods. Reversibility of cellular alterations was found at nicotine concentrations of 0.1 mg/ml during 7 days (Figure 3). After application of 0.1 mM of nicotine, the PDL cells were vacuolized (Figure 4). This process was reversed after nicotine withdrawal(Figure 4). At concentrations of 0.5 and 2.0 mg/ml, the effects of nicotine became irreversible over the incubation period of 4, 7, and 11 days. After incubation with nicotine at 2.0mg/ml, no viable cells were detected at the end of the reversibility studies.

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IV. Discussion

The present study investigated the effects of nicotine and its role of in periodontal disease via its direct effects on PDL cells. We found that nicotine had a direct biologic and cytotoxic effect on PDL cells such as inhibiting cell proliferation and attachment and stimulating of cellular damage in a concentration- and time-dependent manner. Previous studies have suggested that the cell growth, proliferation, and matrix synthesis of PDL cells are necessary for regeneration of a connective tissue attachment which is the ultimate goal in periodontal therapy. Therefore, a direct toxic effect on host cells can retard periodontal wound healing during periodontal therapy. This may impair the reparative and regenerative potential of periodontal tissues of smokers.

There are conflicting results on the effects of nicotine on proliferation and DNA synthesis, depending on the type of cell studied. For example, nicotine caused a decreased DNA synthesis in brain cells of rat, leukemia cells of human, and cardiac fibroblasts but had no effect on HeLa cells and fetal fibroblasts of human. Giannopoulou et al. found a dose-dependent inhibition of proliferation and morphological alterations such as the vacuolization and disruption of orientation at a concentration which was lower than that of the present experiment. James et al. observed an inhibitory response at a concentration which had caused a significant inhibition in the present study. These discrepancies in the range of inhibitory concentrations may be due to differences in age, strain, and race of the donor of the PDL cells. The difference of the constitution of culture medium might have influenced the results as our experiment used 10% FBS as a control medium while James et al. used 1% fetal calf serum.

In the present investigation, concentrations of nicotine higher than 2 mg/ml but lower than 5 g/ml, caused a dose-dependent inhibition of the proliferation of PDL cells. Peacock et al. found that in vitro exposure of human gingival fibroblasts to low concentrations of the drug caused a significant increase in fibroblast number over period of 48 h. Higher concentrations of nicotine appeared to transiently impair metabolic activity, but the cells recovered and proliferation was ultimately increased. In contrast, Tipton and Dabbous showed that at nicotine concentrations lower than those causing cell death(0.075%), there was a dose-dependent inhibition of proliferation. The different responses of fibroblasts to nicotine may be due to the different concentrations used or to the heterogeneity among subpopulations of fibroblasts.

Concerning the attachment of cells on a surface, Peacock et al. demonstrated a positive effect of nicotine on gingival fibroblast attachment to a plastic surface and showed that this enhanced effect was concentration-dependent. However, Raulin et al. found that exposure of such cells to nicotine resulted in structural alterations that prevented them from becoming firmly attached to the plastic or root surfaces. Our results seem to confirm those of Raulin et al.
Ginannopoulou et al.\textsuperscript{22} observed that the attachment of the PDL cells was inhibited from 100ng/ml to 2g/ml of nicotine which in the present experiment had no effect on the attachment of the PDL cells. James et al\textsuperscript{7} observed that the attachment of the PDL cells was not inhibited at a concentration which in the present study caused a significant inhibition of the attachment. Discrepancies among various results could be explained by differences in the concentration of drug used and/or differences of fibroblast strains.

Vacuolization of human fibroblasts due to nicotine and other components of tobacco smoke have also been documented by several researchers.\textsuperscript{38,37,40,41} A number of vacuolizations were found prior to cell necrosis in the present experiment. Hanes et al\textsuperscript{15} suggested that the number of vacuolizations is the result of the releasing of process a nicotine in the form of a vesicle. Tipton and Dabbos\textsuperscript{39} noted that the vacuolization was temporary, and that the cells returned to normal conditions after three day exposure to nicotine. In the current study, vacuolization disappeared when PDL cells were refreshed with the control medium after incubation at nicotine concentrations of 0.1mg/ml for 1, 4, and 7 days. This result confirmed Tipton and Dabbos's observation\textsuperscript{39} that the cytotoxicity was temporary. The cells, however, still had vacuoles after incubation period of 11 days. These results suggest that the reversible condition may turn out to be permanently irreversible after long-time exposure to nicotine.

Our studies revealed that these cytotoxic effects are reversible after nicotine has been removed from the cell cultures. However, reversibility was observed to be dose- and time-dependent. At concentrations ranging between 0.5 and 0.1mg/ml, fibroblast functions were restored, resulting in regeneration of the altered cells, but at higher nicotine concentrations cellular damage became irreversible. Therefore, exposing the cell to nicotine for an extended time may cause permanent cell damage. A relationship has been reported between the severity of inflammatory periodontal disease, the number of cigarettes smoked per day, and the number of years of smoking.\textsuperscript{42,43} These epidemiological data may be at least partially explained by our results. The present data show that periodontal fibroblasts affected by nicotine treatment may be repaired and regenerated, depending upon the dose and duration of exposure to nicotine.

In conclusion, nicotine inhibits the attachment and proliferation of periodontal ligament cells in a dose-dependent fashion. The cytotoxicity of the nicotine was irreversible at higher concentrations and at later periods.

V. References

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34. Hassell TM, Stanek EJ. Evidence that healthy human gingival contains func-


FIGURE LEGENDS

Figure 1. Effect of nicotine on the attachment of PDL cells. A. Changes in percentage of the attached PDL cells in the presence of the various concentration of nicotine incubated for 1, 3, 6, 12, and 24 hours. B. Morphological images of attachment of PDL cells cultured for 24 hours in the presence of 2 mg/ml(a): 0.5 mg/ml(b): 0.1 mg/ml(c): 0.005 mg/ml(d): 0.001 mg/ml nicotine(e): and no nicotine(f) (H&E stain: magnification 100).

Figure 2. Effect of nicotine on the proliferation of PDL cells. A. Changes in percentage of the attached PDL cells in the presence of the various concentration of nicotine incubated for 1, 4, 7, 11, and 14 days. B. Morphological images of proliferation of PDL cells cultured for 14 days in the presence of 2 mg/ml(a): 0.5 mg/ml(b): 0.1 mg/ml(c): 0.005 mg/ml(d): 0.001 mg/ml nicotine(e): and no nicotine(f) (H&E stain: magnification 100).

Figure 3. Reversibility of cell damage of PDL cells. The numbers of the attached PDL cells were counted after preincubation with nicotine for 1 day(A); 4 days(B); 7 days(C); and 11 days(D). One portion of cells was treated with nicotine for an additional 2 days(black columns), the other cultures were incubated with fresh culture medium(gray columns)(* P<0.05).

Figure 4. Morphological changes of PDL cells after the withdrawal of nicotine. PDL cells were preincubated with 2 mg/ml(a, b): 0.5 mg/ml(c, d): 0.1 mg/ml nicotine(e, f) and no nicotine(g, h) for 1 day and then one portion of cells was treated with nicotine for an additional 2 days(left column), the other cultures were incubated with fresh culture medium(right column)(H&E stain: magnification 100).
Fig. 1.
Figure 2.
Figure 3.
사진부도 (IV)

Figure 4.
니코틴이 치주인대세포의 부착과 증식에 미치는 영향 및 니코틴에 의해 야기된 세포독성의 가역성에 대한 연구

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본 실험은 흡연이 치주인대세포의 기능에 미치는 영향을 알아보기 위하여 담배 부산물 중 하나인 니코틴이 치주인대세포의 부착과 성장 및 세포의 가역성에 미치는 영향을 관찰하였다. 니코틴이 치주인대세포의 부착에 미치는 영향을 관찰하기 위하여 치주인대세포에 니코틴을 투여한 후 1, 3, 6, 12, 24시간에 trypan blue 염색 후 부착된 세포수의 측정과 H&E stain 후 형태학적 관찰을 하였으나 니코틴이 치주인대세포의 성장에 미치는 영향을 관찰하기 위하여 치주인대세포에 니코틴을 투여한 후 1, 4, 7, 11, 14일에 trypan blue 염색 후 부착된 세포수의 측정과 H&E stain 후 형태학적 관찰을 하였다. 또 니코틴의 세포독성효과의 가역성 평가를 위하여 니코틴을 투여하고 1, 4, 7, 11일째 니코틴이 없는 새로운 배지로 갈아 주어 2일 더 배양한 후 trypan blue로 염색하여 세포수를 측정, 비교하였고 H&E 염색 후 형태학적 관찰을 하였다.

실험결과 니코틴 농도가 증가함에 따라 치주인대세포의 부착율은 감소되었고 24시간 배양 후 2mg/ml, 0.5mg/ml, 0.1mg/ml 니코틴 투여군에서 통계적으로 유의성 있는 부착억제가 관찰되었고(P<0.01) 형태학적 관찰결과 2mg/ml, 0.5mg/ml 니코틴 투여군에서는 대조군에서 관찰되는 세포질의 확장이 관찰되지 않았다. 니코틴이 치주인대세포의 성장에 미치는 효과를 살펴본 결과 2mg/ml, 0.5mg/ml, 0.1mg/ml 니코틴 투여군에서 중독억제가 관찰되었고(P<0.01) 0.005mg/ml이하의 니코틴 투여군에서는 아무런 변화도 관찰되지 않았다. 14일 배양 후 형태학적 관찰결과 0.1mg/ml 니코틴 투여군에서 세포질 내 공포를 관찰할 수 있었고 2mg/ml, 0.5mg/ml 니코틴 투여군에서는 세포의 해사가 나타났다.

니코틴의 세포독성효과의 가역성평가결과 2mg/ml 이상의 니코틴은 세포에 비가역적인 손상을 일으키며 0.5mg/ml, 0.1mg/ml 니코틴에 의해 세포독성은 초기에는 가역적이며 장기간 세포에 노출시키면 비가역적인 손상을 야기하는 것으로 나타났다(P<0.05).

본 실험결과 및 배양의 구성성분 중 니코틴은 농도 의존적으로 치주인대세포의 부착과 증식에 영향을 주었고 니코틴의 치주인대세포에 대한 독성효과는 농도가 증가함수록, 시간이 지남수록 비가역적으로 나타났다. 따라서 흡연은 치주조직세포에 영향을 미칠 것으로 생각되며 흡연의 기간과 정도가 치주질환의 심도 및 치주치료 후 불량한 치유반응과 관계가 있을 것으로 사료된다.

주요어: 니코틴, 치주인대세포, 부착, 증식, 가역성, 세포독성