

## Research

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# Effects of Cotinine on Human Gingival Fibroblast Migration

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## ABSTRACT

Nicotine has a deleterious impact on gingival fibroblast cell viability, adhesion, and migration. Less is known regarding the effect of cotinine, the main metabolite of nicotine, on such processes. The objective of this study was to determine if cotinine affects the adhesion or migration of Human Gingival Fibroblasts (HGF) in culture. HGF were treated with nicotine or cotinine at several concentrations and dose-response cytotoxicity was determined by MTT assay. The effects of nicotine and cotinine on HGF adhesion were measured colorimetrically and cell migration was determined using the scratch wound assay. The number of HGF oriented parallel to the wound edge at 24 hours was counted using phase contrast images. Data were analyzed using ANOVA and Dunnett's multiple comparison post-test. At the highest concentrations of cotinine (640 ng/ml) and nicotine (400 µg/ml) both HGF survival and cell adhesion were significantly inhibited ( $p < 0.01$ ). By scratch wound assay HGF migration from the wound edge at 24 hours was significantly inhibited by 320 ng/ml ( $p < 0.001$ ) and 640 ng/ml ( $p < 0.001$ ) cotinine, and by 400 µg/ml ( $p < 0.01$ ) nicotine. HGF migration was significantly inhibited by 80 ng/ml ( $p < 0.05$ ), 320 ng/ml ( $p < 0.01$ ) and 640 ng/ml ( $p < 0.001$ ) cotinine and by 100 µg/ml ( $p < 0.01$ ), 200 µg/ml ( $p < 0.01$ ) and 400 µg/ml ( $p < 0.001$ ) nicotine at 48 hours. Significantly more HGF were oriented parallel to wound edge with pre treatment of 10 ng/ml cotinine or 50 µg/ml nicotine before wounding ( $p < 0.001$ ). In HGF exposed to nicotine (400 µg/ml) or cotinine (640 ng/ml), cell survival, cell adhesion, and migration were significantly decreased, but cell polarity was not affected. These concentrations are within ranges of serum levels in smokers, providing evidence that multiple cellular aspects of wound healing are compromised in tobacco users.

**KEYWORDS:** Adhesion; Cotinine; Gingival fibroblast; Migration; Nicotine.

## INTRODUCTION

It is well known that tobacco smoking impairs wound healing; a systematic review and meta analysis concluded that smoking cessation in surgical patients was associated with significantly decreased rates of post-surgical complications.<sup>1</sup> Tobacco use is a prime behavioral contributor to periodontal disease with accompanying poor oral wound healing and potential tooth loss.<sup>2</sup> As a phase in the wound healing process, fibroblasts transition to myofibroblasts and form granulation tissue by secreting and remodeling the extracellular matrix.<sup>3</sup> Fibroblasts are located in gingival, lingual, buccal, labial and palatal structures as well as the periodontal ligament fiber groups, and thus are central to production and maintenance of the connective tissue underlying the surface epithelium of the oral mucosa and periodontium. Indeed fibroblasts are the main cell type of the periodontium.

Although cigarettes are a mixture of thousands of carcinogenic compounds, many studies have focused on nicotine as a main deleterious agent in cigarette smoke. With respect to the oral cavity, isolated nicotine has been implicated in affecting gingival fibroblast cell

viability<sup>4</sup> tissue remodeling,<sup>5</sup> cell adhesion and migration<sup>6</sup> and myofibroblast transition.<sup>7</sup> Nicotine at 0.5 mM inhibited human gingival fibroblast migration, potentially through Rac signaling pathways,<sup>6</sup> and altered the response to transforming growth factor-beta 1 by decreasing the morphologic change from fibroblast to myofibroblast. Based on these observations it has been suggested that nicotine interferes with wound closure by changing the ability of oral fibroblasts to contract wounds.<sup>8</sup>

Additional mechanisms to explain nicotine-inhibited cell migration may be through effects on cell adhesion. Nicotine in the range of 5 ng/ml to 10 mg/ml inhibited the attachment and growth of human periodontal ligament fibroblasts.<sup>9</sup> Nicotine-induced decrease of beta1 integrin expression suggests an impairment of human gingival fibroblast ability to adhere to extracellular matrix.<sup>10,11</sup> Conversely whole cigarette smoke inhibited fibroblast migration and correlated with an increased level of focal adhesions, suggesting that nicotine-inhibited cell migration was due to increased cell adhesion.<sup>12</sup> Using nicotine alone, increasing nicotine concentrations correlated to increased human gingival fibroblast adhesion.<sup>13</sup>

In humans 70-80% of nicotine is metabolized to cotinine,<sup>14</sup> which can be assayed in blood, saliva, and urine. Cotinine has a five- to ten-fold longer half-life compared to nicotine, and the literature has documented it to be a specific and sensitive marker for determining exposure to tobacco and nicotine.<sup>15</sup> According to the Foundation for Blood Research ([www.fbr.org](http://www.fbr.org)), serum cotinine levels that are less than 10 ng/ml indicate non-smoking, levels of 10 to 100 ng/ml are indicative of light smoking or passive secondary exposure, and levels greater than 300 ng/ml suggest heavy smoking. Scott et al.<sup>16</sup> reported that in heavy smokers serum cotinine levels are greater than 100 ng/ml, light smoker cotinine levels are less than or equal to 60 ng/ml, and in non-smokers the levels are less than or equal to 10 ng/ml. An analysis of United States National Health and Nutrition Examination Survey (NHANES) data from 1999 to 2010 shows that serum cotinine levels have risen over time for males, females, and whites, and with exposure to second hand smoke at home.<sup>17</sup>

In contrast to cigarette smoke or pure nicotine, there are relatively fewer reports of the effects of cotinine on cell adhesion and migration. In smooth muscle cell culture, nicotine and cotinine increased the expression of several matrix metalloproteinases that are critical in cell migration.<sup>18</sup>

The concentration of serum soluble Intercellular adhesion molecule-1 (ICAM-1)<sup>16</sup> and soluble Vascular Cell Adhesion Molecule-1 (sVCAM-I)<sup>19</sup> correlated directly to serum/plasma cotinine levels in smokers.

Although studies of nicotine and cotinine exist, an electronic database (PubMed) search using the combined terms of "cotinine AND gingiva AND fibroblast" yielded no publications. With the aim of expanding current knowledge on the ef-

fects of cigarette compounds on oral cells, the objective of the present study is to determine if cotinine effects on the adhesion or migration of human gingival fibroblasts in culture is similar to nicotine. Concentrations of nicotine and cotinine analogous to clinically relevant human serum and saliva levels were utilized. It is hypothesized that similarly to nicotine, cotinine will adversely affect human gingival fibroblast adhesion and migration with implications for wound healing in smokers.

## MATERIALS AND METHODS

### Human Gingival Fibroblasts

Human Gingival Fibroblasts (HGF) were obtained from a commercial source (ScienCell, Carlsbad, CA, USA) and were maintained in serum-free fibroblast medium as recommended by the manufacturer. Cells were obtained at passage one and propagated in T-75 flasks. Cells were trypsinized and replated as needed for experiments, and used between passages two and four.

### MTT Assay

HGF at 90% confluence in wells of a 24-well plate were treated with 0, 10, 80, 320, or 640 ng/ml cotinine (Sigma Chemical Co., St. Louis, MO, USA). In an analogous assay HGF were treated with 0, 50, 100, 200, or 400 µg/ml nicotine. Cotinine and nicotine solutions were freshly made for each study and prepared in the fibroblast culture medium. 0.01% chlorhexidine was used as a positive control<sup>20</sup> and culture medium alone as a negative control. After 24 hours a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay was performed according to the protocol of the Sigma Cell Growth Determination Kit MTT Based (Sigma Chemical Co., St. Louis, MO, USA). HGF were incubated with the MTT reagent for 3 hours (n = 4 for all treatments and controls). The resulting formazan crystals were dissolved with MTT solubilization solution, and the absorbance of samples was determined at 570 nm using a SpectraMax M3 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Data were reported as absorbance and as relative cell viability setting the controls of no cotinine or nicotine to 100%.

### Cell Adhesion Assay

HGF at 90% confluence were treated with 0, 10, 80, 320, or 640 ng/ml cotinine, or in a separate experiment with 0, 50, 100, 200, or 400 µg/ml nicotine for 24 hours. HGF were then trypsinized and pelleted by centrifugation in trypsin-neutralizing solution. Cell pellets were washed three times in fibroblast medium and resuspended in the same medium. HGF were plated onto non-treated flat-bottom tissue culture 96-well plates at  $2 \times 10^4$  cells/well and incubated for 1 hour at 37 °C in 5% CO<sub>2</sub>. The wells were washed with medium to remove unattached cells, and attached cells were fixed in 3.7% formalin, stained with 2% crystal violet, and lysed in 2% SDS (200 µl of each solution per well). The absorbance of the lysate was measured at

570 nm wavelength. Each adhesion assay was repeated three times, with six wells of each treatment and control assayed each time. The results were reported as absorbance and as the percentage of adhesion compared with controls of no cotinine or nicotine set at 100%.

### Cell Migration Assay

HGF were cultured in wells of a 24-well plate until confluent and then pre-treated with 0, 10, 80, 320, or 640 ng/ml cotinine, or in a separate experiment with 0, 50, 100, 200, or 400  $\mu\text{g/ml}$  nicotine for 24 hours. Scratch wounds were created in each well by dragging a sterile tip for an automatic pipettor down the middle of the well several times. At 24 and 48 hours following the wounding procedure, the extent of cell migration from the wound edge was measured and calculated in mm using the NIH ImageJ image processing program. In addition, non-pretreated confluent HGF were wounded and then treated with 0, 10, 80, 320, or 640 ng/ml cotinine. Cell migration from the wound edge was monitored for 24 hours. Experiments were repeated three times. Data were analyzed using one way ANOVA with Dunnet's multiple comparison post-test and probability at  $p < 0.05$ .

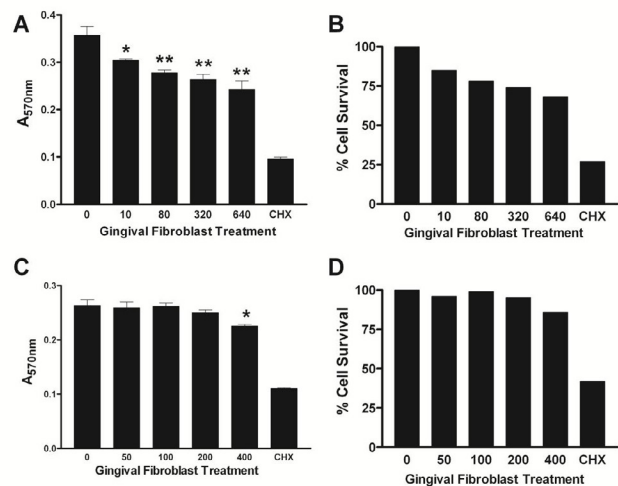
### Image Analysis of Cell Orientation

The orientation of migrating cells with respect to the wound edge 24 hours following wounding were analyzed using phase contrast images of HGF treated with 0, 10, 80, 320, or 640 ng/ml cotinine or 0, 50, 100, 200, or 400  $\mu\text{g/ml}$  nicotine. The number of cells with an orientation parallel to the defined wound edge was counted for each treatment. Data are reported as the mean of ten images and were analyzed using one way ANOVA with Dunnet's multiple comparison post-test and probability at  $p < 0.05$ .

## RESULTS

The current study utilized nicotine concentrations within the ranges of those reported by other investigators using culture models.<sup>21-23</sup> However to determine appropriate concentrations of both nicotine and cotinine specifically for the HGF used in this study dose-response curves were generated. Cotinine concentrations in ng/ml indicative of non-smoking, light smoking, or heavy smoking were used to treat HGF prior to cytotoxicity testing using a colorimetric MTT assay. Both absorbance values and calculated percentage of cell survival as shown in figure 1A and 1B demonstrate that all cotinine concentrations tested significantly suppressed cell proliferation and proved cytotoxic (specific  $p$  values provided in Figure 1 legend). Compared to control, 85% of fibroblasts survived following 24 hours of treatment with 10 ng/ml cotinine, 78% survived following 80 ng/ml, 74% survived following 320 ng/ml, and 68% survived following 640 ng/ml cotinine exposure (Figure 1B). Using the colorimetric MTT assay, the cytotoxicity of nicotine was evaluated us-

ing HGF. Only the highest concentration of nicotine used, 400  $\mu\text{g/ml}$ , resulted in significant cytotoxicity (Figure 1C,  $p < 0.01$ ). Compared to control 86% of fibroblasts survived a 24 hour exposure to 400  $\mu\text{g/ml}$  nicotine (Figure 1D).

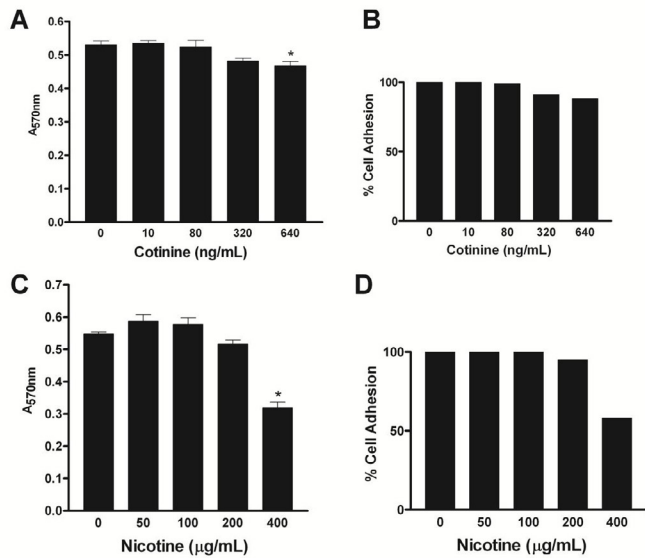


**Figure 1:** Effects of cotinine and nicotine on HGF cell survival. Cells were treated with the indicated concentrations of cotinine (A, B) or nicotine (C, D) for 24 h prior to assay for cytotoxic effects. Both the absorbance values (A, C) and relative cell survival compared to negative control set to 100% (B, D) are shown. All concentrations of cotinine were toxic to HGF (\* in A  $p < 0.01$ , \*\*  $p < 0.001$ ). For nicotine only the highest concentration tested was cytotoxic to HGF (\* in C  $p < 0.01$ ).

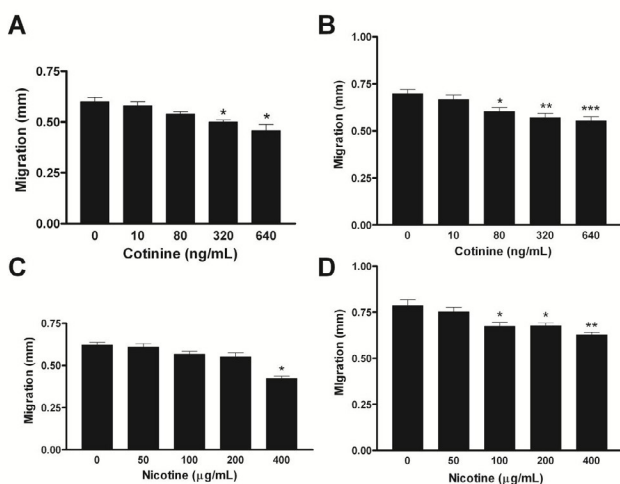
A colorimetric assay was used to determine rapid HGF adhesion to uncoated tissue culture substrate. Cytotoxic effects of cotinine and nicotine as shown in figure 1 would not have an effect on the results of this assay as adhesion was determined after 60 minutes, a time frame that is too early to detect cytotoxic effects. Of the cotinine concentrations tested, only the 640 ng/ml value significantly suppressed rapid cell adhesion (Figure 2A,  $p < 0.01$ ) with 88% of cells adherent compared to control (Figure 2B). Similarly, only the highest concentration of nicotine tested, 400  $\mu\text{g/ml}$ , resulted in a significant decrease in cell adhesion (Figure 2C,  $p < 0.001$ ) with 58% of cells adherent compared to control (Figure 2D).

Cell adhesion from a wound edge using an *in vitro* scratch wounding model was measured 24 and 48 hours following the initiation of the wound. Figure 3 depicts results when HGF were pre-treated with the agents and then wounded. Compared to control, 24 hours following wounding cotinine at 320 ( $p < 0.001$ ) and 640 ( $p < 0.001$ ) ng/ml significantly inhibited migration (Figure 3A). At 48 hours following wounding (Figure 3B), cotinine concentrations of 80 ( $p < 0.05$ ), 320 ( $p < 0.01$ ) and 640 ( $p < 0.001$ ) ng/ml significantly inhibited migration (Figure 3B). In nicotine experiments, a concentration of 400  $\mu\text{g/ml}$  significantly inhibited migration at 24 hours (Figure 3C,  $p < 0.01$ ). In contrast 48 hours following wounding (Figure 3D) 100 ( $p < 0.01$ ), 200 ( $p < 0.01$ ), and 400 ( $p < 0.001$ )  $\mu\text{g/ml}$  nicotine significantly inhibited migration from the wound edge. Similar inhibitory effects of cotinine were also noted if untreated HGF were first wounded, then treated with cotinine for 24 hours following

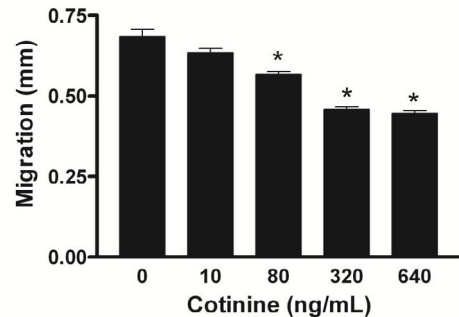
wounding (Figure 4). However the deleterious effects were noted at lower concentrations at the 24 hour time point when cotinine was present during the migratory phase. Cell migration was significantly inhibited by the presence of 80, 320 and 640 ng/ml cotinine ( $p < 0.001$ ).



**Figure 2:** Rapid cell adhesion of HGF to uncoated tissue culture plastic substrate. HGF were treated with the indicated concentrations of cotinine (A, B) or nicotine (C, D) for 24 h. Trypsinized fibroblasts were incubated on plastic for 60 min prior to assay for adhesion. Both absorbance values (A, C) and relative cell adhesion compared to negative control set at 100% (B, D) are shown. Only the highest concentrations of cotinine (\* in A,  $p < 0.01$ ) and nicotine (\* in C,  $p < 0.001$ ) significantly inhibited rapid cell adhesion of HGF.

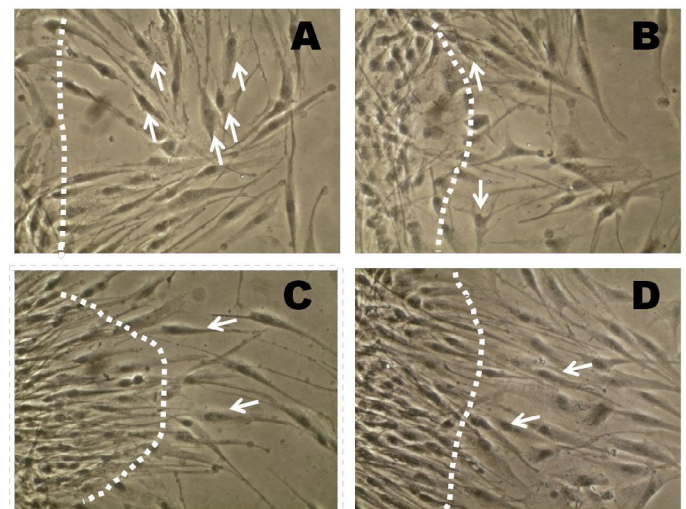


**Figure 3:** Effect of pre-treating HGF with cotinine and nicotine on migration. Cells were treated with the indicated concentrations of cotinine or nicotine for 24 h prior to wounding. Cell migration from the wound edge was calculated in mm 24 h (A, C) and 48 h (B, D) after the wound. At 24 h post-wound cotinine at 320 and 640 ng/ml significantly inhibited cell migration (\* in A  $p < 0.001$ ). In addition at 48 h post-wound 80 ng/ml cotinine inhibited migration (\* in B  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ). 24 h post-wound only the highest concentration of nicotine significantly delayed migration (\* in C  $p < 0.01$ ), while 48 h post-wound 100, 200, and 400 µg/ml significantly inhibited migration (\* in D  $p < 0.01$ , \*\*  $p < 0.001$ ).



**Figure 4:** Effect of cotinine on HGF migration. Cells were wounded then treated with the indicated concentration of cotinine for 24 h. Cell migration was significantly inhibited by the presence of 80, 320 and 640 ng/ml cotinine (\*  $p < 0.001$ ).

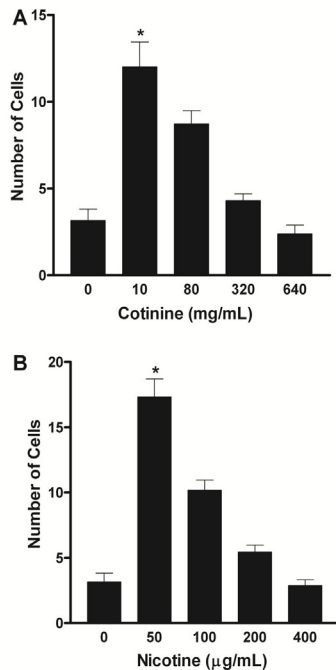
Cell migration based on morphological orientation of cells at the wound edge was assessed by inspection of phase-contrast images. 24 hours following wounding, cells treated with 10 ng/ml cotinine showed multiple cells oriented parallel to the wound edge (Figure 5A). With increasing concentration of cotinine however fibroblasts took on an elongated spindle shape oriented perpendicular to the wound edge. In many of these cells the nucleus was polarized towards the wound edge (Figure 5 B, C, D).



**Figure 5:** Effect of cotinine on orientation of HGF at the wound edge. Phase contrast images of cells adjacent to the wound edge (dotted line) 24 h after wounding were captured. Prior to wounding cells were treated with 10 (A), 80 (B), 320 (C), or 640 (D) ng/ml cotinine. Arrows in (A) and (B) show HGF oriented parallel to the wound edge. Arrows in (C) and (D) show HGF oriented perpendicular to the wound edge.

The number of HGF oriented parallel to the defined wound edge was counted for both cotinine and nicotine treated cells. A dose-response relationship was observed. Fewer cells oriented to the wound edge were observed as both cotinine and nicotine concentrations increased. The lowest concentrations of both cotinine and nicotine were correlated with significantly higher numbers of cells oriented parallel to the wound edge

compared to control or HGF treated with higher concentrations of cotinine (Figure 6A  $p < 0.001$ ) or nicotine (Figure 6B  $p < 0.001$ ).



**Figure 6:** Effect of cotinine and nicotine on orientation of HGF with respect to the wound edge. Phase-contrast images of cells treated with the indicated concentrations of cotinine (A) or nicotine (B) were analyzed to determine the numbers of cells oriented parallel to the wound edge. For both cotinine and nicotine the lowest concentrations used resulted in the highest number of cells with parallel orientation compared to controls or to other concentrations (\* in A and B  $p < 0.001$ ).

## DISCUSSION

Numerous studies have investigated the effects of nicotine on oral cells, however the correlative data for cotinine is not as abundant. In this study the effects of biologically relevant concentrations of nicotine and cotinine on human gingival fibroblast cytotoxicity, adhesion and migration were examined and compared. Alpar et al.<sup>4</sup> reported that concentrations of 6 mM (973 µg/ml), 8 mM (1298 µg/ml) and 10 mM (1620 µg/ml) nicotine decreased the cell viability of human periodontal ligament and gingival fibroblasts. The data from this study shows that a nicotine concentration of 400 µg/ml (2.46 mM) is cytotoxic to HGF, demonstrating that an even lower concentration than that reported by Alpar, et al. can kill oral cells. Cytotoxicity was not detected at lower concentrations so taking the data together it appears that nicotine levels above approximately 2 mM (300 to 400 µg/ml) are toxic to human oral cells in culture. This is well within the range of saliva nicotine concentrations of 96 ng/ml to 1.6 mg/ml measured in tobacco users.<sup>24</sup>

Testing cell lines by neutral red cytotoxicity assay, Babich and Borenfreund<sup>25</sup> concluded that nicotine was more cytotoxic than cotinine, which was more cytotoxic than nicotinic

acid or nicotinamide. The cell growth of MA-10 Leydig tumor cells was significantly inhibited by 1 mM cotinine, or 176 µg/ml.<sup>26</sup> This study revealed that HGF cell growth was significantly inhibited by 10 ng/ml cotinine (57 nM), substantially less than previous reports, and in contrast to the study concluding that nicotine is more cytotoxic than cotinine if such a comparison is based solely on concentration.

James et al.<sup>9</sup> reported that nicotine and cotinine inhibited the attachment and growth of human periodontal ligament fibroblasts. In agreement, this study showed that both nicotine and cotinine significantly inhibited rapid HGF adhesion to plastic tissue culture substrate. In contrast increased vinculin expression and focal adhesions in fibroblasts exposed to mainstream whole smoke suggest that nicotine-exposed cells have increased adhesion.<sup>12</sup> Fully functional focal adhesions would likely not be formed during the 60 minute rapid adhesion assay and would explain the different outcomes in the effects of nicotine and cotinine on adhesion. During wound healing, cells must be adherent to a substrate *in vitro* or to extracellular matrix *in vivo* to allow for subsequent migration. The role of nicotine in extracellular matrix modeling is not clear, as it has been reported that 2.4 µg/ml nicotine did not affect collagen degradation<sup>27</sup> but 25 to 500 µg/ml nicotine increased collagen degradation in HGF.<sup>28</sup> There is essentially no information about the effects of cotinine on extracellular matrix. To explain discrepancies in the effects of nicotine and cotinine on cell adhesion it is certainly plausible that different cell types respond differently to any agent in culture. Although many of these studies used human gingival fibroblasts, the concentrations of nicotine and cotinine that were used varied. Standard concentrations of these agents for use in *in vitro* analyses would allow for better comparisons between individual studies.

Migration is an essential component of wound healing and it is well documented that tobacco use correlates to poor wound healing.<sup>29,30</sup> A previous study demonstrated that nicotine inhibits cell migration between 16 and 162 µg/ml.<sup>6</sup> The results of the present study agree that nicotine can be detrimental to cell migration at similar concentrations, and extend previous reports by suggesting that cells become more sensitive to lower concentrations of nicotine with extended wound healing time. Cotinine affected cell migration in a similar manner as for nicotine, with HGF showing greater sensitivity to lower cotinine concentrations with increased wound healing time. Based solely on the concentration needed to affect HGF migration, cotinine is more detrimental to the migration phase of wound healing compared to nicotine.

Maninova et al.<sup>31</sup> reported that cell polarity is an essential step in the process of cell migration. These authors showed that in polarized cells the axis of migration was aligned perpendicular to the wound edge. The present study showed that as the concentration of nicotine and cotinine increased, relatively fewer HGF were oriented parallel to the wound edge and

relative more cells were oriented perpendicular to the wound edge. This suggests that nicotine and cotinine may facilitate the polarization of HGF at the wound edge. While this seems counter-intuitive it may be that polarity, adhesion, and cell movement are not linked in cells exposed to higher concentration of tobacco components.

In summary, in HGF exposed to nicotine (400 µg/ml) or cotinine (640 ng/ml), cell survival, rapid cell adhesion, and migration were significantly decreased, but cell polarity with respect to the wound edge was not affected. These concentrations are within ranges of serum levels in smokers, providing evidence that multiple cellular aspects of wound healing are compromised in tobacco users.

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#### CONFLICTS OF INTEREST

We have no conflicts of interest to declare.

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