

Effects of cigarette smoke and nicotine on cell viability, migration and myofibroblastic differentiation

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Background and Objective: Several studies have analysed the role of nicotine as a prominent agent affecting wound repair in smokers. However, tobacco smoke contains several components that may alter gingival wound healing. The present study aimed to analyse the roles of cigarette smoke condensate (CSC) and nicotine on cell viability, cell migration/invasion and myofibroblastic differentiation using primary cultures of human gingival fibroblasts.

Material and Methods: To compare the effects of CSC and nicotine, gingival fibroblasts were stimulated with CSC (0.4–500 µg/mL) and the corresponding nicotine concentrations (0.025–32 µg/mL) present in research cigarettes (1R3F). Cell viability was evaluated through the MTS assay. Cell migration and invasion were assessed through scratch wound assays, collagen nested matrices and transwell migration. α -Smooth muscle actin production was evaluated by western blotting.

Results: Cigarette smoke condensate at 50 µg/mL induced a moderate increase in cell viability, whereas the corresponding nicotine concentration (3.2 µg/mL) did not produce this response. Cigarette smoke condensate at 250 µg/mL, but not nicotine at 16 µg/mL (the corresponding nicotine concentration), induced cell death. Both nicotine and CSC stimulated cell migration (50 µg/mL CSC; 3.2 µg/mL nicotine). At 150 µg/mL, CSC inhibited cell migration; however, the corresponding concentration of nicotine (9.6 µg/mL), did not have this effect. Although both nicotine and CSC inhibited α -smooth muscle actin production, only the latter induced a statistically significant effect on this response.

Conclusion: Cigarette smoke condensate may stimulate cell survival and migration at low concentrations and inhibit these cell responses at higher levels of exposure. Moreover, CSC may interfere in myofibroblastic differentiation. These results show that cigarette smoke, but not nicotine, may significantly alter cell viability, cell migration and myofibroblastic differentiation in gingival mesenchymal cells.

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The habit of tobacco smoking constitutes a significant risk factor for the initiation and progression of peri-

odontal disease (1,2). The clinical outcome of several periodontal procedures, including nonsurgical and

surgical periodontal therapy (3,4), mucogingival (5) and implant surgery (6), is critically affected by tobacco

smoking. This effect has been documented in human clinical trials (3), animal studies (6) and cell culture experiments (7). Therefore, unraveling the effects of tobacco smoke on gingival cells is critically important in order to understand the mechanisms of action of this risk factor on periodontal tissues.

Tobacco smoke is a complex mixture of up to 6000 components that include nitrosamines, benzenes, aldehydes, carbon monoxide and nicotine (8). Nicotine is a well-studied component of cigarettes that exerts an addictive effect and may also affect the host response (9). Several studies have also considered nicotine to be the main agent affecting several specific responses, such as cell viability (10,11), tissue remodeling (12), cell adhesion and migration (13–17) and myofibroblastic differentiation (18). It is important to clarify that these studies used nicotine to simulate the effects of cigarette smoke exposure on the above-described cellular activities. Although it is relevant to know the effects of nicotine on gingival cells, these responses must be analysed in a physiological context, that is, in the presence of the complete mixture of components present in cigarette smoke. One experimental strategy to evaluate the effects to tobacco smoke on isolated cells is the use of cigarette smoke condensate (CSC), which is generated through the controlled combustion of the 1R3F research cigarette that contains 6.4% nicotine (19). Recent studies have documented that CSC may affect the production of the serine protease urokinase (7) and of MMPs in gingival fibroblasts (20,21). Moreover, whole cigarette smoke may alter fibroblast adhesion and tissue contraction (22).

Wound healing is a highly co-ordinated response, in which mesenchymal cells play a significant role (23,24). After tissue injury, gingival fibroblasts are activated to migrate from the surrounding healthy connective tissue into the temporary wound clot that will mature into granulation tissue (23,24). During this process, cells must remodel their actin cytoskeleton, attach to extracellular matrix (ECM) components through specific receptors and degrade selective molecules present in the ECM to permit an efficient cell

migration into the injured tissue (23). Therefore, factors affecting the migration of mesenchymal cells may alter the outcome of wound repair. During the maturation of granulation tissue, transforming growth factor β 1 (TGF- β 1) plays a key role by stimulating the differentiation of myofibroblasts (25). These cells are characterized by the expression of the actin isoform α -smooth muscle actin (α -SMA), which renders a more contractile phenotype that stimulates tissue remodeling (26,27). Previous studies have explored the role of nicotine, as an isolated compound, in the modulation of cell migration and myofibroblastic differentiation in human gingival fibroblasts (16,18). The present study was designed to evaluate the role of CSC and nicotine on cell viability, cell migration and myofibroblastic differentiation in primary cultures of human gingival fibroblasts.

Material and methods

Cell culture

Primary cultures of human gingival fibroblasts were established by the explant method and were cultured in α -minimal essential medium (α -MEM; Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT, USA) as previously described (28). Tissue explants were obtained from clinically healthy gingiva of nine nonsmoker individuals undergoing extraction of third molars at a private dental practice in Santiago, Chile. Tissue samples were harvested with the informed consent of the patients. The protocol for obtaining tissue was approved by the Ethical Committee, Faculty of Medicine, Pontificia Universidad Católica de Chile. All experiments were performed using cells expanded between passages four and 10.

Cell viability assay [MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay]

The MTSTM cell viability assay (Promega, Madison, WI, USA) was used to

assess cell viability. This is a colorimetric assay, in which the amount of color produced is directly proportional to the number of viable cells. Cells were seeded into 96-well plates (Orange Scientific, Braine-l'Alleud, Belgium) and allowed to attach overnight in the presence of FBS. Cells were then exposed to 0.4, 2.0, 10, 50, 250 or 500 μ g/mL CSC (Murty Pharmaceuticals, Lexington, KY, USA) or to 0.025, 0.12, 0.64, 3.2, 16 or 32 μ g/mL nicotine (Sigma, St Louis, MO, USA) for 24, 48 or 72 h. To make sure that the vehicle of CSC was not affecting cell functions, dimethyl sulfoxide (DMSO) (Sigma) was added to control experiments. Twenty microlitres of MTS labeling reagent was then added to each well and incubated for 4 h, and subsequently read at 492 nm using a microplate reader (ThermoPlate TP-Reader NM; Thermo Fisher Scientific, Rockford, IL, USA).

Wound closure assay

Gingival fibroblasts were seeded on 24-well plates (Orange Scientific). When the cells achieved 90% confluence, cell cultures were scratched with a 10 μ L sterile pipette tip and washed with phosphate-buffered saline to remove detached cells and debris. Cells were then incubated in medium containing 1% FBS and 50, 100 or 150 μ g/mL CSC or 3.2, 6.4 or 9.6 μ g/mL nicotine. Dimethyl sulfoxide was added to control cells. After 16 h, cells were fixed with methanol for 2 min, and incubated with 0.2% crystal violet for 5 min. Images of each wound were captured with a digital camera (Nikon, Coolpix 4500; Nikon, Tokyo, Japan) through an inverted microscope (TMS, Nikon, Tokyo, Japan). Wound closure was determined by counting the average number of migrating cells per photographic field. These assays were performed in three separate sets of experiments. Four images were obtained from different areas of each wound to quantify each experiment.

Cell migration in nested matrices

Cells were cultured within a neutralized collagen solution (1 mg/mL), and

0.2 mL aliquots were placed in 24-well culture plates (Orange Scientific). Collagen was prepared from rat tail tendons as previously described (29). After 60 min, matrices were gently released from the underlying culture dishes and cultured in α -MEM plus 10% FBS to allow contraction. Contracted gels were placed on top of 20 μ L of collagen solution and covered with the remaining 180 μ L. After 1 h at 37°C, α -MEM was supplemented with 1% FBS plus 50, 100 or 150 μ g/mL CSC or 3.2, 6.4 or 9.6 μ g/mL nicotine for 24 h. Dimethyl sulfoxide was added to control cells. Cell migration was evaluated by counting the cells at the periphery of the contracted gels (30).

Cell invasion assay

Cell migration was assayed using transwell chambers (BD Bioscience, Bedford, MA, USA) with 8.0 μ m pore polycarbonate filters (Collaborative Research, Bedford, MA, USA) coated with 10 μ g/mL of Matrigel (Costar, Cambridge, MA, USA). To this end, human gingival fibroblasts were pretreated with different concentrations of CSC or their nicotine equivalents for 24 h. Then, cells were seeded on the top of a transwell cell culture device. Cells were suspended in serum-free medium and seeded on the upper compartment of the chamber. Fetal bovine serum (10 or 1%) and CSC (50 or 150 μ g/mL) or nicotine (3.2 or 9.6 μ g/mL) were added to the lower compartment of the chamber. Dimethyl sulfoxide was added to control cells. Migration was allowed to occur for 16 h. Staining and cell counting were performed as previously described (31).

Myofibroblastic differentiation experiment

Serum-starved human gingival fibroblasts were exposed to 6.4 μ g/mL nicotine, 100 μ g/mL CSC or vehicle for 1 h and then stimulated with 5 ng/mL TGF- β 1 for 72 h. Dimethyl sulfoxide was added to control cells. Stimulated cells were lysed and protein levels detected through western blot as previously described (28). Proteins were

resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis in reducing conditions and transferred to polyvinylidene difluoride transfer membrane (Thermo Scientific, Rockford, IL, USA). Membranes were exposed to primary commercial antibodies against α -SMA, clone 1A4, diluted 1:1000 (Sigma) and β -actin (Sigma), clone AC-74, diluted 1:5000. Afterwards, secondary antibodies coupled to horseradish peroxidase were used (Rockland, Gilbertsville, PA, USA). Finally, films were developed with an enhanced chemiluminescence detection kit (Amersham Corp., Arlington Heights, FL, USA).

Data analysis

All experiments were performed at least five times on separate occasions. Data are represented in each graph as means and standard errors. Statistical analysis was by one-way ANOVA and Dunnett's test. Statistical significance was set at $p < 0.05$. All the analyses were performed using SPSS software for Windows (version 16.0.2; SPSS Inc., Chicago, IL, USA).

Results

Effects of CSC and nicotine on cell viability

We first evaluated the viability of primary cultures of gingival fibroblasts exposed to different concentrations of CSC and the corresponding concentrations of nicotine (6.4%) present in the 1R3F research cigarettes. Gingival fibroblasts were stimulated separately with 0.4, 2.0, 10, 50, 250 or 500 μ g/mL CSC or with 0.025, 0.12, 0.64, 3.2, 16 or 32 μ g/mL nicotine in α -MEM without FBS. Control cells were stimulated with DMSO diluted in α -MEM without FBS. As a positive control of cell proliferation, cells were stimulated with α -MEM supplemented with 10% FBS. As a positive control of cell death, cells were stimulated with 50, 100, 250 or 500 μ g/mL tert-butylhydrogen peroxide (t-b-peroxide). Cell viability was evaluated through the MTS assay. At 24 h, 250 and 500 μ g/mL CSC decreased cell viability up to

61 and 52% (of the original 100%) ($p = 0.001$ and $p = 0.0008$, respectively), and 500 μ g/mL t-b-peroxide altered this cell response in a similar manner, reducing cell viability up to 47.2% ($p = 0.00004$), suggesting a toxic effect for these agents at these concentrations (Fig. 1A). In accordance with the 24 h experiment, at 48 and 72 h, 250 and 500 μ g/mL CSC and 100, 250 and 500 μ g/mL t-b-peroxide induced a significant reduction in cell viability when compared with vehicle-stimulated cells (Fig. 1B and C). It was interesting to note that at 48 h, 50 μ g/mL CSC slightly induced cell viability without reaching statistical significance. Moreover, at 72 h 10 μ g/mL CSC stimulated cell viability at significant levels ($p = 0.02$). Moreover, none of the nicotine concentrations used in this experiment induced changes in cell viability (Fig. 1A, B and C).

Effects of CSC and nicotine on wound closure

Cell migration was also evaluated using a scratch wound closure experiment. Wounds were created in gingival fibroblast monolayers, and cell migration was followed for 16 h in the presence of α -MEM plus 1% FBS, supplemented, or not, with 50, 100 or 150 μ g/mL CSC or with 3.2, 6.4 or 9.6 μ g/mL nicotine. Again, 10% FBS was used as a positive control. Cell migration was stimulated by 50 and 100 μ g/mL CSC, with an increase of 25 and 29% when compared with the control ($p = 0.01$ and $p = 0.03$, respectively), and even more potently stimulated with 3.2, 6.4 and 9.6 μ g/mL nicotine (Fig. 2) representing an increase in cell migration of 110, 112 and 120% ($p = 0.006$, $p = 0.002$ and $p = 0.002$, respectively). Only 150 μ g/mL CSC inhibited cell migration when compared with 1% FBS alone (Fig. 2), with a reduction of up to 26% ($p = 0.01$).

Effects of CSC and nicotine on nested cell migration

Cell migration was evaluated using an experimental model in which gingival fibroblasts are cultured within a

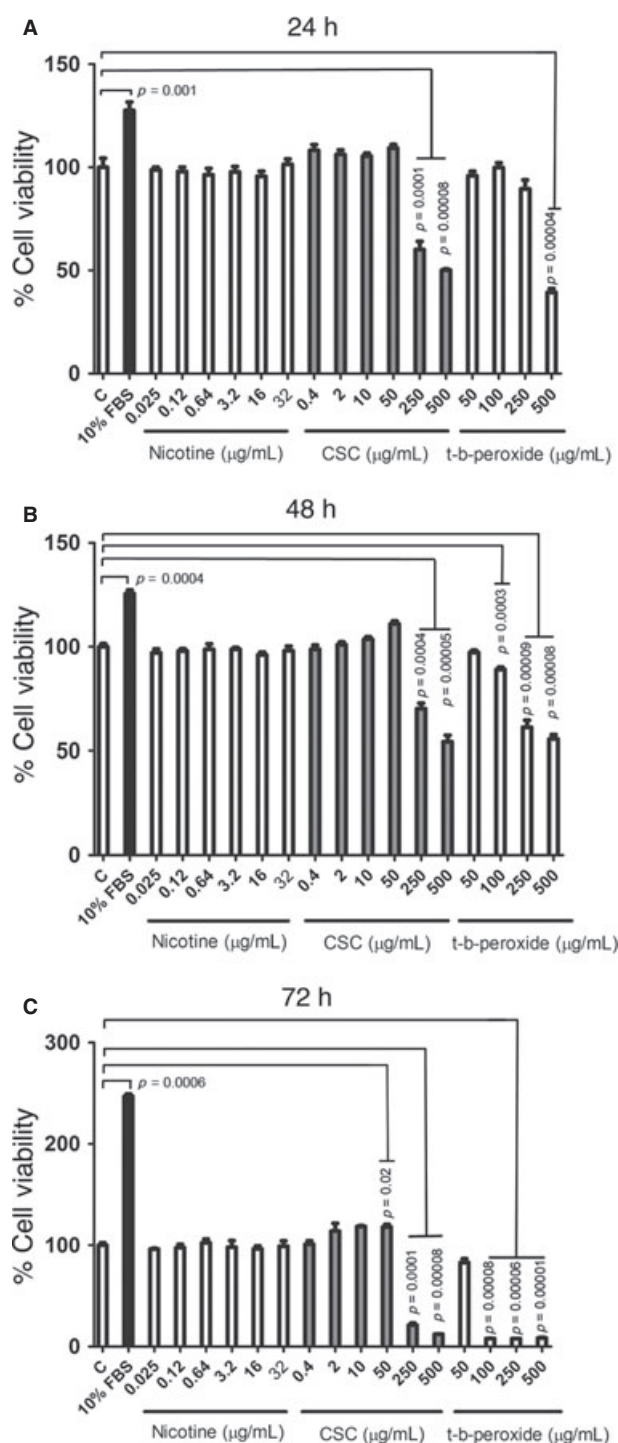


Fig. 1. Modulation of cell viability by cigarette smoke condensate (CSC) and nicotine. Effect of increasing concentrations of CSC, and its nicotine equivalent, on the viability of serum-starved human gingival fibroblasts at 24, 48 and 72 h. tert-Butyl-hydrogen peroxide (t-b-peroxide) was used as a positive control of oxidative stress-induced cell damage. Data represent the percentage of cell viability \pm SEM determined through the MTS assay. Values of $p \leq 0.05$ are indicated above each bar of the graph (ANOVA followed by Dunnett's *post hoc* test).

collagen gel. This first gel was included in a second cell-free collagen gel, and cell migration was stimulated with FBS

in association with different nicotine or CSC concentrations. After 24 h, cell migration was significantly stimulated

with 100 $\mu\text{g/mL}$ CSC from an average of 2.41 to 3.14 cells per field ($p = 0.03$; Fig. 3). On the contrary, 150 $\mu\text{g/mL}$ CSC significantly inhibited cell migration (average of 1.79 cells per field; $p = 0.02$). Interestingly, exposure of cells to 9.6 $\mu\text{g/mL}$ nicotine significantly stimulated cell migration (average of 2.86 cells per field; $p = 0.02$). Stimulation with 3.2 or 6.4 $\mu\text{g/mL}$ nicotine did not affect cell migration (Fig. 3).

Effect of CSC and 1% FBS on cell viability

Considering that the above-mentioned cell migration assays (wound closure and nested cell migration) were performed in the presence of 1% FBS and that CSC induced a modest but statistically significant increase in cell viability, we performed a cell viability assay in which cells were stimulated with 1% FBS plus 10–250 $\mu\text{g/mL}$ CSC for a total period of 24 h. This experiment showed that 10–100 $\mu\text{g/mL}$ CSC plus 1% FBS did not modify cell viability. Moreover, 125, 150 and 175 $\mu\text{g/mL}$ CSC + 1% FBS induced a modest but significant increase in cell viability that reached 120, 115 and 114% ($p = 0.02$, $p = 0.03$ and $p = 0.03$, respectively; Fig. 4). At concentrations of 200 $\mu\text{g/mL}$ CSC and above, we observed a statistically significant decrease in cell viability, with an average of 91% ($p = 0.04$; Fig. 4). Therefore, this experiment demonstrated that cell viability or even cell proliferation was not affecting the cell migration experiments performed in transwell cell culture systems or wound closure assays.

Effects of CSC and nicotine on transwell cell invasion

To confirm the general trend in the modulation of cell migration by CSC, we performed a transwell cell invasion assay. A bicameral cell culture system was used to evaluate the migration and invasion of gingival fibroblasts through a reconstituted ECM. On the lower compartment of the chamber, α -MEM was supplemented with 10% FBS (as a positive control) or 1% FBS plus CSC or nicotine. After 24 h of exposure to

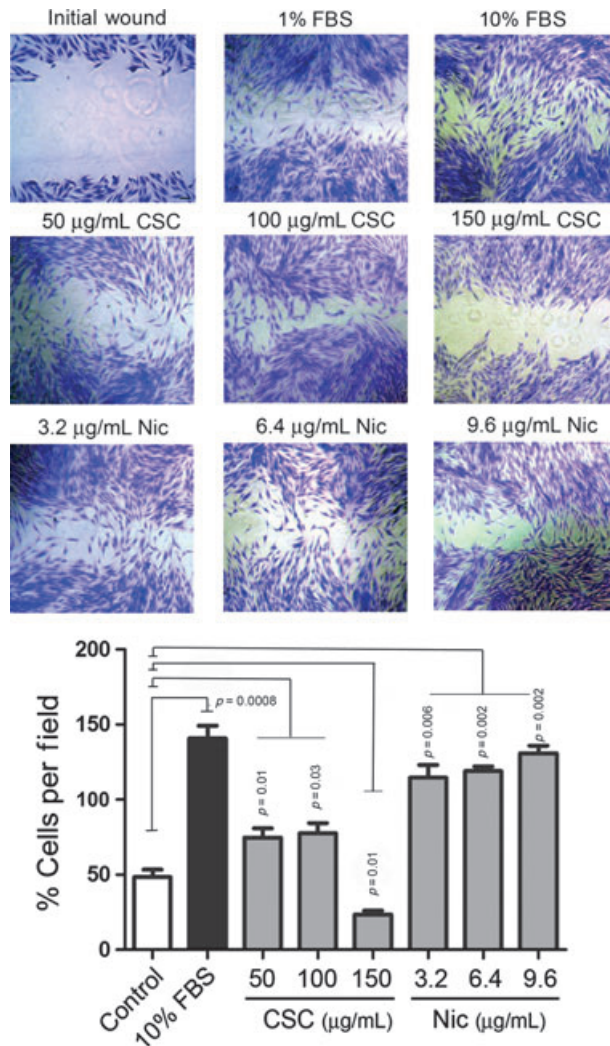


Fig. 2. Effect of CSC and nicotine on scratch wound closure. Scratch wounds were performed on a semi-confluent monolayer of human gingival fibroblasts. During wound closure, cells were exposed to different concentrations of CSC, or their nicotine equivalents, for 16 h. Fibroblasts were stained with crystal violet for visualization and quantification of migrating cells into the initial wound area. The resulting quantification is represented as the average number of cells per photographic field \pm SEM. Values of $p \leq 0.05$ are indicated above each bar of the graph (ANOVA followed by Dunnett's *post hoc* test).

the above-mentioned agents, cells were detached and seeded on the upper compartment of a transwell cell migration device. Cells were allowed to migrate for an additional 24 h in the presence of 1% FBS. We selected the highest and lowest concentrations of CSC and nicotine, considering our previous experiments using scratch wounds and collagen gel invasion assays. We observed that cell migration was stimulated by 10% FBS (positive control) and by 50 µg/mL CSC, 3.2 and 9.6 µg/mL nicotine (Fig. 5A and B). This stimulus represented an

increase in cell migration from an average of 3.6 cells per field (1% FBS) to 16.2 (10% FBS; $p = 0.0004$), 10.2 (50 µg/mL CSC; $p = 0.02$), 13.4 (3.2 µg/mL nicotine; $p = 0.003$) and 15.05 cells per field (9.6 µg/mL nicotine; $p = 0.004$). It was interesting to note that only 150 µg/mL CSC did not stimulate cell migration, exerting a similar effect to 1% FBS (control condition). Given that this experiment was performed in the presence of 1% FBS, we evaluated cell viability. Figure 3C shows that cell viability was not affected at the CSC or nicotine con-

centrations used in this assay. These results suggest that CSC, but not nicotine, negatively affected the ability of cells to migrate and invade a reconstituted ECM.

Effects of CSC and nicotine on myofibroblastic differentiation

We analysed whether CSC or nicotine were able to interfere in the differentiation of myofibroblasts. Serum-starved human gingival fibroblasts were exposed to 100 µg/mL CSC or its nicotine equivalent (6.4 µg/mL) for 1 h. To induce myofibroblastic differentiation, human gingival fibroblasts were then treated with 5 ng/mL TGF- β 1 in α -MEM without serum for a period of 72 h. As shown in Fig. 6A and B, TGF- β 1 provided a potent stimulus to α -SMA production that represented a 3.3-fold increase in the normalized levels of this protein ($p = 0.004$). Moreover, α -SMA protein levels, normalized against β -actin, were reduced from 4.8 to 2.3 in the presence of 100 µg/mL CSC, reaching statistical significance ($p = 0.03$). Although the addition of nicotine in association with TGF- β 1 induced a reduction in α -SMA levels, this response did not reach statistical significance (Fig. 6A and B). Cell viability was confirmed in this experiment. To this end, cells were cultured for 72 h in α -MEM without serum, using a range of CSC concentrations. We confirmed that 100 µg/mL CSC was not affecting cell viability (Fig. 6C).

Discussion

Several studies have explored the role of nicotine on the altered wound healing response described in smokers (10–18). However, only recent studies have analysed the role of the whole mixture of components present in cigarette smoke that may affect cell responses involved in tissue repair (7,20,22). In the present study, using gingival fibroblasts as a model, we have evaluated the role of nicotine and CSC on cell viability, cell migration and myofibroblastic differentiation. Our results show that CSC exerts a cytotoxic effect on gingival fibroblasts, inhibits cell

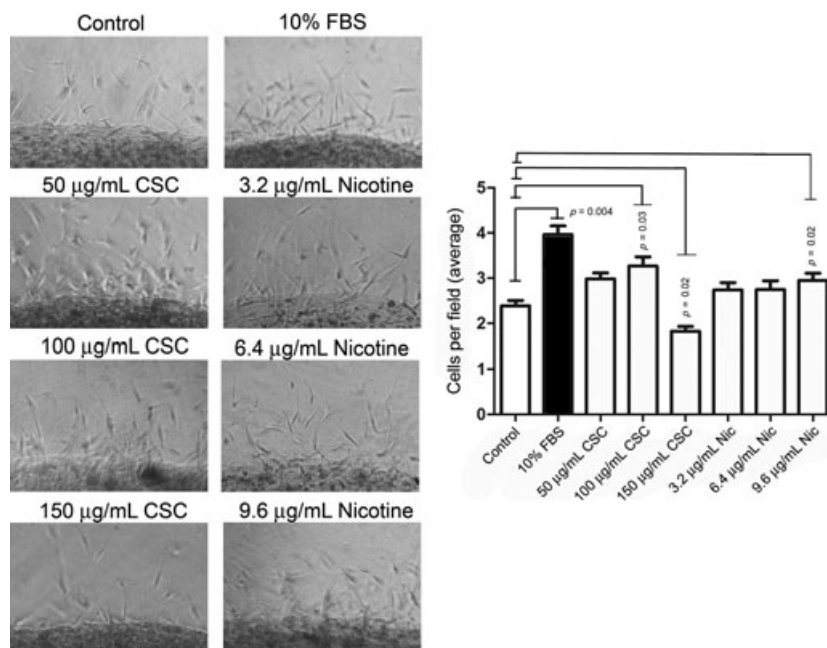


Fig. 3. Migration model using nested collagen matrices. Human gingival fibroblasts were allowed to contract within floating collagen matrices for 24 h, which were then embedded in cell-free collagen matrices. The figure shows the effect of increasing CSC concentrations, and their nicotine equivalents, on cell migration. 'Control' indicates vehicle-stimulated cells. Fetal bovine serum (FBS: 10%) was used as a positive control of cell migration. The graph shows the mean + SEM of migrating cells for each condition. Values of $p \leq 0.05$ are indicated above each bar of the graph (ANOVA followed by Dunnett's *post hoc* test).

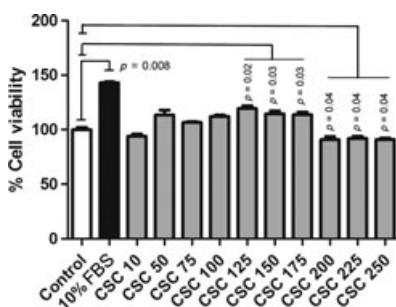


Fig. 4. Effect of CSC and 1% FBS on cell viability. Cells were exposed to CSC and 1% FBS for 24 h. Cell viability was determined through the MTS assay. The graph represents mean + SEM. Values of $p \leq 0.05$ are indicated above each bar of the graph (ANOVA followed by Dunnett's *post hoc* test).

migration and also interferes in the differentiation of myofibroblasts. Interestingly, cell viability was not affected by isolated nicotine over the range of concentrations tested in this study. In addition, nicotine stimulated cell migration and invasion. Finally,

nicotine marginally altered myofibroblastic differentiation when compared with the effect of CSC. These results contribute to clarify the differential role of cigarette smoke and nicotine on specific responses of mesenchymal cells during wound healing in gingival tissues.

A significant issue when studying the effects of tobacco smoke on cells is to identify a physiologically relevant range of concentrations of cigarette smoke. Cigarette smoke condensate is prepared from the research cigarette 1R3F, which is smoked into a chamber that collects the particulate content of cigarette smoke (32). In the present study, we used nicotine as a reference to define the concentration of CSC for our experiments. Nicotine has been found in saliva of smokers at concentrations ranging between 0.9 and 4.6 µg/mL (33). Serum nicotine concentrations in chronic smokers vary between 0.04 and 0.072 µg/mL, and in tissues it can achieve levels ranging from 0.6 to 1 µg/mL (34,35). Therefore, CSC concen-

trations between 50 and 150 µg/mL, as used in the present study, probably reflect a biologically feasible situation. Moreover, Zhang *et al.* (21) recently described that 100 µg/mL CSC contains 2.4 µg/mL nicotine. All our experiments were performed with consideration of the concentrations of nicotine (6.4%) present in 1R3F research cigarettes (19). Although this represents a limitation of the present study, all the cell responses to nicotine showed a similar trend within the concentrations tested (cell viability, 0.025–32 µg/mL; cell migration/invasion, 3.2–9.6 µg/mL; and myofibroblastic differentiation, 6.4 µg/mL).

In the present study, we propose that CSC may affect fibroblast functions. However, it is important to consider whether CSC components may reach gingival fibroblasts to modulate their cellular activities. Cigarette smoke condensate is composed of the particulate components solubilized by the organic solvent DMSO from filters that have been exposed to the combustion of research cigarettes (19). Considering the solubility properties of the agents present in CSC, it is probable that they can diffuse through biological membranes of superficial cells of the oral tissues, reaching deeper areas of the gingival connective tissue. Moreover, in gingival tissues exposed to inflammation, ulcerated gingival epithelium may be more prone to the effects of CSC components that might affect several cell types, including inflammatory cells and fibroblasts (36).

It has been proposed that tobacco-derived smoke may affect cell viability, modifying the normal physiology of periodontal tissues (37). However, the precise role and mechanism of action of specific components of cigarette smoke are still not well understood. Concerning the role of nicotine in cell viability and death, it has been observed that this agent may promote cell survival (38). Moreover, when gingival fibroblasts are stimulated with nicotine concentrations found in the serum of smokers, this agent protects these cells from apoptosis by the stimulation of nitric oxide production (39). These data are coincident with our observations, in which the viability of

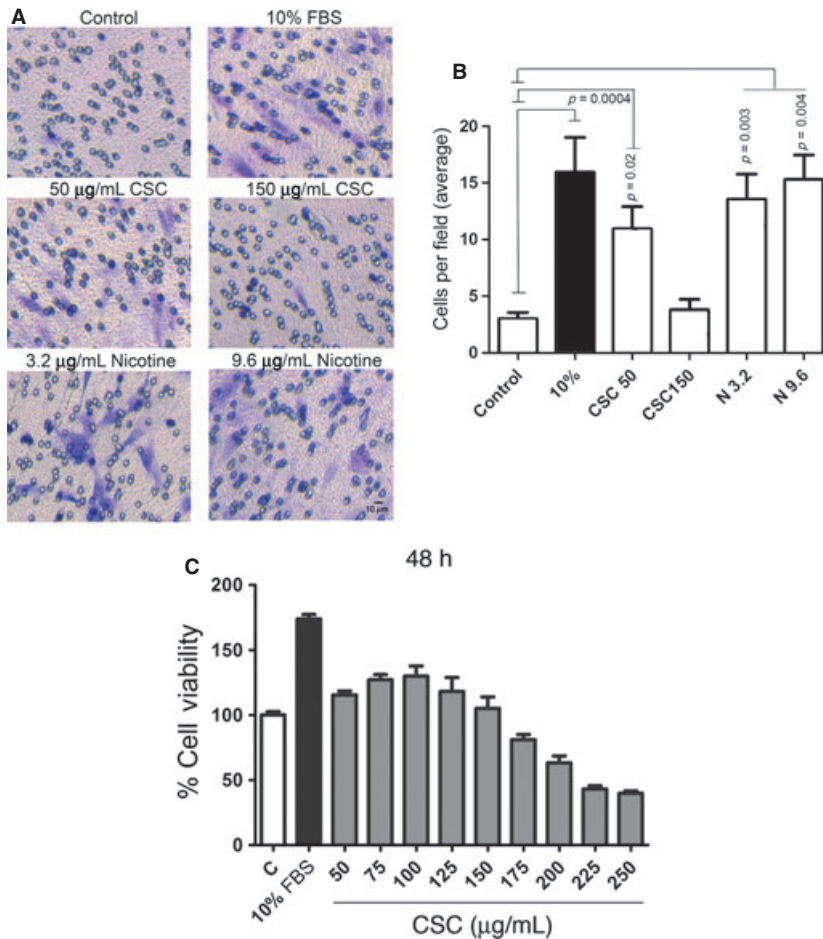


Fig. 5. Transwell invasion assay. (A) Human gingival fibroblasts were pretreated with different CSC concentrations, or their nicotine (N) equivalents, for 24 h. Then, cells were seeded on the top of a transwell cell culture device. After 24 h of cell migration, fibroblasts were stained with crystal violet for visualization and quantification. (B) The graph shows the resulting quantification, represented as mean number of cells per photographic field + SEM. Values of $p \leq 0.05$ are indicated in each bar of the graph (ANOVA followed by Dunett's *post hoc* test). (C) Cell viability was determined by the MTS assay using human gingival fibroblasts during a 48 h period in the presence of 1% FBS.

gingival fibroblast was not affected by nicotine. In contrast, CSC may have a dual effect on cell survival/viability. We observed that 50 µg/mL CSC induced a modest but statistically significant increase in cell viability at 48 and 72 h. In connection with this finding, low concentrations of CSC (4 µg/mL) may stimulate the ERK pathway, which has been linked with cell survival and proliferation (7,40). Moreover, stimulation of chicken embryo fibroblasts with whole cigarette smoke induces the activation of cell survival signaling pathways, including interleukin-8, Protein Kinase B/Akt, p53 and p21 (41).

Gingival mesenchymal cell migration occurs after injury to repopulate the tissues that have been affected by either chronic inflammatory lesions or acute damage. In order to migrate, cells must modify their actin cytoskeleton, extending long lamellipodia at their migration front (42). During migration, cells must also recognize, through integrin receptors, specific molecules in the extracellular environment to permit the attachment to extracellular matrix molecules. Another important mechanism involved in cell migration is the proteolytic degradation of extracellular matrix components necessary to permit the

advance of cells through this environment (42). It is possible to propose that CSC or nicotine may affect cell migration at different levels. To assess these effects, we used three different models of cell migration (nested cell migration, transwell invasion assays and a scratch wound closure experiment). Although these assays clearly differ in the migration traits observed in each case (42), all of them demonstrated similar results, showing that at low CSC concentrations (50 and 100 µg/mL) cell migration was stimulated, whereas a higher dose (150 µg/mL CSC) induced a clear decrease in this response. In the case of nicotine, all the concentrations tested induced an increase in cell migration. In a previous study, it was observed that nicotine inhibits cell migration at concentrations ranging between 16.223 and 162.23 µg/mL (16). Considering that cells and tissues are exposed to nicotine in the presence of CSC, it is highly probable that nicotine is not the main agent affecting mesenchymal cell migration in this environment. Recent studies have also shown that whole cigarette smoke has a detrimental effect on cell migration (22). On the contrary, a striking observation of the present study was that CSC may stimulate cell migration at low concentrations. Cell migration is promoted by the proteolytic degradation of ECM components (42). Previous studies from our group showed that low concentrations of CSC (4 µg/mL) stimulate the production of the serine protease urokinase in human gingival fibroblasts (7). In addition, it has been observed that stimulation of gingival fibroblasts with 100 µg/mL CSC promotes collagen degradation (21). However, the corresponding concentration of nicotine (2.4 µg/mL) did not have this effect. Therefore, it is tempting to propose that CSC stimulated cell migration through a primary effect on proteolytic activity.

Myofibroblasts are specialized cells that are differentiated during wound repair and fibrotic conditions (43). Induction of the myofibroblastic phenotype is controlled by growth factors, such as TGF-β1, the expression of a spliced form of fibronectin called

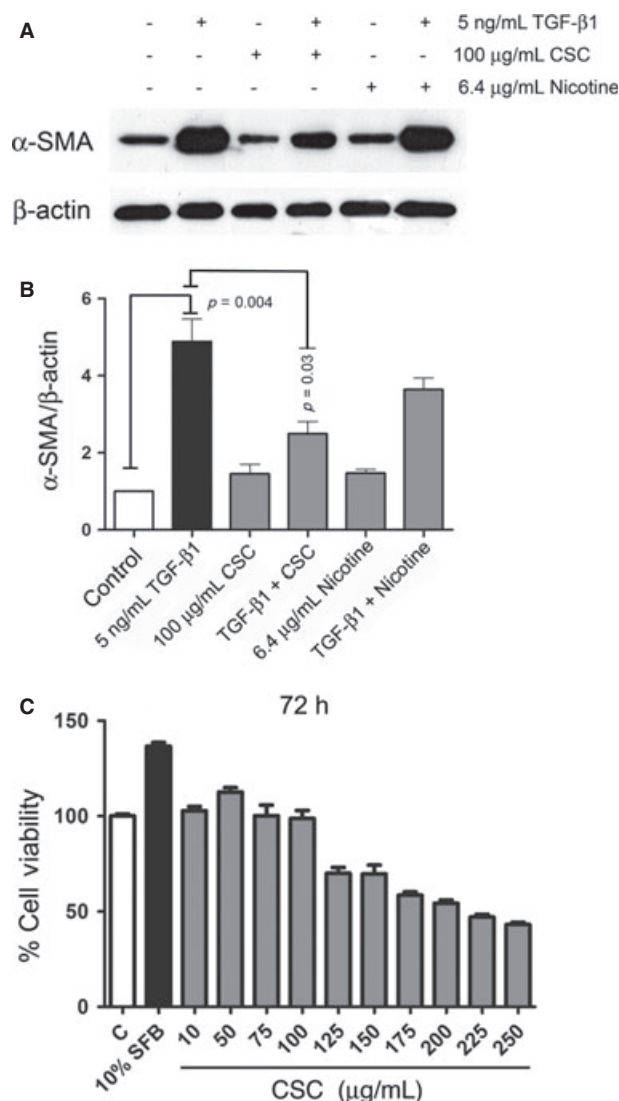


Fig. 6. Effect of CSC and nicotine on myofibroblastic differentiation. (A) Human gingival fibroblasts were exposed to CSC (100 μg/mL), or its nicotine equivalent (6.4 μg/mL), for 1 h and then treated with transforming growth factor β1 (TGF-β1; 5 ng/mL). α-Smooth muscle actin (α-SMA) expression was evaluated by western blotting. (B) The graph shows α-SMA quantification, normalized to β-actin. The graph represents mean + SEM. Values of $p \leq 0.05$ are indicated above each bar of the graph (ANOVA followed by Dunnett's *post hoc* test). (C) Effect of CSC on cell viability at 72 h in the presence of 1% FBS. Cell viability was determined by the MTS assay.

EDA-fibronectin and the mechanical tension present in the tissue environment (43). During tissue repair, these cells are actively involved in tissue remodeling and contraction. Therefore, factors that negatively affect the differentiation of myofibroblasts may have an impact on wound repair. Previous studies have analysed the role of nicotine in myofibroblastic differentiation, with the observation that 162.23 μg/mL nicotine may inhibit the

expression of the myofibroblast marker α-SMA (18). Although our study demonstrated that nicotine was able to inhibit α-SMA when cells were stimulated with 6.4 μg/mL nicotine, we were able to observe a more powerful effect when cells were exposed to 100 μg/mL CSC. It is difficult to define with the present cell culture model the precise *in vivo* role for nicotine and CSC on myofibroblastic differentiation. However, in accordance with previous

studies (18), we propose that this specific response may be negatively modulated by both nicotine and other undetermined components present in CSC.

Within the limits of the present study, these results show that CSC components other than nicotine may affect key cell functions involved in wound repair.

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