Clin Oral Invest (2005) 9: 180–186
DOI 10.1007/s00784-004-0294-z

ORIGINAL ARTICLE

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Root surface conditioning with nicotine or cotinine reduces viability and density of fibroblasts in vitro

Received: 12 July 2004 / Accepted: 28 October 2004 / Published online: 19 May 2005

Abstract The purpose of study was to evaluate fibroblast attachment and cellular morphology on root surfaces chemically conditioned with nicotine or cotinine. A secondary objective was to determine if mechanical scaling and root planning of these chemically conditioned surfaces would alter cellular attachment. Root surface dentin specimens were prepared from uniradicular teeth of non-smoking patients. Specimens were randomly assigned to two experimental groups: no treatment (chemical conditioning only) and scaling and root planning after conditioning (SRPC). The concentrations of the tested substances were in the range of 0–1 mg/mL (nicotine) and 0–1 ?g/mL (cotinine). After a 24-h conditioning period, dentin slices were incubated with continuous lineage of fibroblastic cells from rat (McCoy cells) for another 24 h. Specimens were prepared for SEM analysis and microphotographs. The statistical analysis of the data indicated significant alteration of cellular morphology on fibroblasts that were grown on root surface exposed to nicotine concentrations greater than 1 ? g/mL. This effect of nicotine was not reduced by SRPC. On the other hand, in the SRPC group cellular density was greater. For cotinine-conditioned specimens, the greater concentrations also led to alteration on morphology, and these alterations were observed in the SRPC group as well. Cotinine did not induce significant changes on cellular density. The results indicated that fibroblasts are negatively influenced by nicotine present on the dentin substrate and also that scaling may reduce these effects. Cotinine treatment on root surfaces may alter cell morphology and density but these effects were less severe than that promoted by nicotine, and were not affected by scaling.

Keywords Adverse effects · Cotinine · Fibroblast · Nicotine

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Introduction

Cigarette smoking has been described as one of the most significant risk factors for the development and progression of periodontal diseases [2, 11]. Over 4,000 toxins are known to be present in cigarette smoke, including poisons such as carbon monoxide, toxic substances such as oxidation radicals, carcinogens such as nitrosamines, and addictive psychoactive substances such as nicotine [4, 23].

Nicotine enters the circulation through direct absorption by the soft tissues in the oral cavity or following previous adherence to tooth structures, and mainly through the lungs, where the inhaled smoke toxins gain direct access to the blood stream [20]. Serum nicotine and metabolites levels vary widely due to brand differences and smoking habits within individuals [2, 7, 13, 18]. Other factors influencing nicotine levels include individual metabolism, rates of absorption, time since last tobacco consumption, type of tobacco used, frequency and time length of the habit. In addition, the methods employed to measure nicotine play a role in the estimation of tobacco exposure. For tobacco and cigarette consumers, the levels of nicotine found are extremely variable, ranging from 96 ng to 1.6 mg/mL in saliva and from 15 ng to 1.8 ?g/mL in the plasma. Variable nicotine levels may, in part, be due to the short half-life (30 min) as nicotine is rapidly metabolized into cotinine. The levels of cotinine have been reported to a range in saliva and plasma between 106 ng to 1 ?g/mL and 48 ng to 1.1 ?g/mL,

respectively. This compound has a longer half-life in comparison with that of nicotine, lasting approximately 10–30 h in the plasma. The levels of cotinine remain relatively constant in active smokers. As a consequence of the long half-life of cotinine, serum levels of this drug have been used as a biochemical marker of regular tobacco use [8, 13, 15, 18].

In vitro investigations have demonstrated that nicotine can alter the cellular morphology and viability of cells derived from human periodontal tissue, as well as influence the ability of these cells to attach to a substrate and to synthesize extracellular matrix molecules [6, 12, 15, 17, 19, 20, 22]. However, little information is available in the literature regarding the direct effect of cotinine on fibroblasts [15]. In the present study, cellular morphology was also used as indirect measure of cell adhesion and cellular viability, since only viable fibroblasts can effectively attach to the substrate and exhibit a flattened bipolar or multipolar morphology. In addition, the density of cells was used to assess cells as a greater density indicates more intense cell proliferation.

Since fibroblasts are the main cell type of the periodontium, it is very important to evaluate fibroblastic response to noxious chemical stimuli. The aim of this in vitro study was to evaluate the effect of root exposure to nicotine or cotinine on the morphology and density of fibroblasts grown on dentin slices, and to determine the effect of scaling and root planning on fibroblast attachment and proliferation.

Material and methods

Sample selection

A total of 60 anterior teeth extracted due to loss of function because of severe periodontal disease were collected from patients who were diagnosed as having chronic periodontitis and free of any systemic diseases. The clinical parameters for the selection of teeth included: clinical attachment loss at least 8 mm, no masticatory function, with or without calculus and no history of periodontal treatment, including scaling and root planning on the previous 3 months. The study protocol was approved by the Research Ethics Committee of the School of Dentistry at Araraquara-UNESP.

Preparation of solutions of nicotine and cotinine

Nicotine (N-3876-Sigma Chemical Products, USA) and cotinine (C-5923-Sigma Chemical Products, USA) solutions were prepared by direct dilution in Minimum Essential Eagle medium (Instituto Adolfo Lutz, SP, Brazil) in concentrations ranging from 100 ng to 1 ?g/mL (nicotine) and 50 ng to 1 ?g/mL (cotinine). Ethanol vehicle control was used as a negative control. The concentrations used in the present study were based on the smallest and greatest values reported in saliva and plasma [2, 13, 15, 18].

Sample preparation and experimental groups

After extraction, teeth were rinsed and stored at 4°C in sterile saline (Fresenius Kabi, Campinas, SP, Brasil). Preparation of dentin samples started with creating a groove at the coronal height of the remaining periodontal ligament with a small round high-speed bur. This enabled us to use only the portion of root that was exposed to the disease process. Then, a single operator performed 20 scaling strokes from this groove up to the cementum-enamel junction using a 5/6 Gracey curette (Neumar, SP, Brazil).

The tooth crown was cut off 2 mm apically to the cementum-enamel junction, and the root was further sectioned longitudinally. After discarding both the sectioned crown and the root portion apical to the groove, dentin slices were obtained in the coronal-apical direction. These slices were further trimmed with rotary instruments to approximately 25 $mm^2$of area and 2 mm of thickness. A total of 90 specimens were prepared and autoclaved. These dentin specimens were randomly split into two experimental groups, as follows.

Group I: no treatment (chemical conditioning only):

- Dentin slices + Nicotine (20 specimens, five for each concentration)
- Dentin slices + Cotinine (20 specimens, five for each concentration)
- Dentin slices + Control (five specimens)

Group II: scaling and root planning after conditioning (SRPC):

- Dentin slices + Nicotine (20 specimens, five for each concentration)
- Dentin slices + Cotinine (20 specimens, five for each concentration)
- Dentin slices + Control (five specimens)

Treatment of the specimens

In the group “No treatment”, the specimens were immersed for 24 h in nicotine solution (100 ng-1 ?g-100 ?g-1 mg/mL) or cotinine solution (50 ng-100 ng-500 ng-1 ?g/mL). Control group specimens were immersed on culture medium containing 1 mL of the alcoholic vehicle for the same 24-h period. After conditioning, the specimens were rinsed two times with PBS (Sigma Chemical Products, USA). In the group “Scaling and Root Planning after Conditioning” the procedure with the drugs was exactly the same up to the PBS rinsing, when the specimens were scaled and root planned (SRP) as described.

Cell culture

McCoy cells, a continuous lineage of fibroblastic cells from rat (ATCC CRL 1696), were used in this study. The

cells were grown on Eagle Minimum Medium supplemented with 40 ?g/mL of gentamycin (Garamicina 60 mg, Schering-Plough, RJ, Brazil) and 7.5% fetal bovine serum (Cultilab, Campinas, SP, Brazil). These cells were maintained in an atmosphere of 98% humidity, 37°C and 5% CO_2.

After chemical conditioning, the specimens were placed in 24-well microplates (3.8 cm^2 cell growth area). To each well, 2 mL of culture medium containing 5×10^4 cells/mL were added. The specimens were incubated for 24 h.

SEM preparation of specimens

After the 24-h incubation period, medium was removed from the wells by aspiration and the specimens fixed by immersion on 2.5% glutaraldehyde (Sigma Chemical Products, USA) for 15 min. After fixation, the specimens were dehydrated in increasing concentrations of ethanol (Sigma Chemical Products, USA) (10, 30, 50, 70, 90 and 100% for 15 min on each concentration) and were left at room temperature for 24 h. Subsequently, samples were fixed in metallic stubs and remained into a vacuum dessicator for 3 days. After sputter coating with gold (20 nm) the specimens were observed and photographed in a scanning electron microscope (T330A, JeoL JSM).

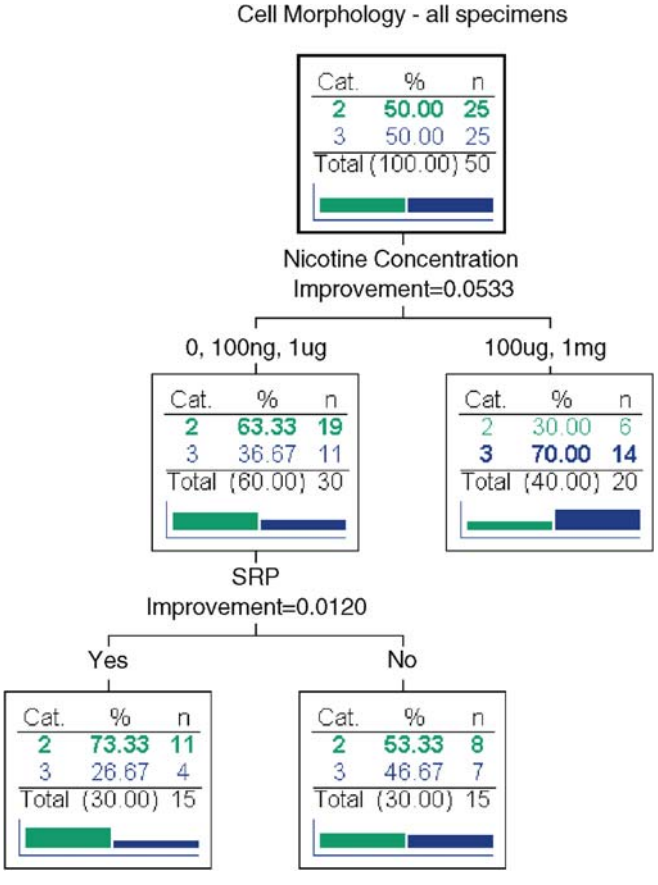

Fig. 1 Classification and regression tree for the effect of nicotine conditioning on cellular morphology. All samples are initially pooled in the uppermost box and if significant differences are found in the distribution of the scores, branches will be generated. The tree keep growing down until no further differences are found in the samples regarding the treatment groups

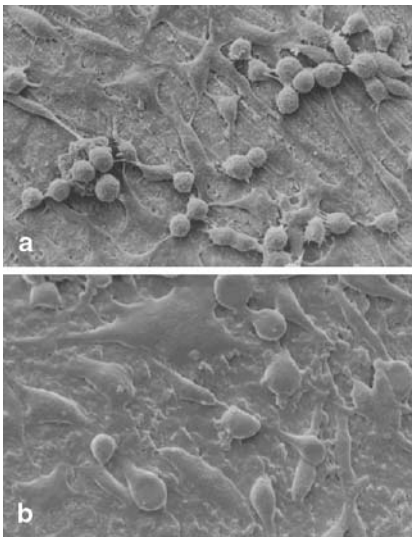

Fig. 2a Group II (SRPC)-Nicotine 100 ?g/mL. Presence of rounded and flattened cells. **b** Group II (SRPC)-Nicotine 1 ?g/mL. Presence of flattened cells. (Original magnification ×1,000)

All experiments were performed independently five times and three microphotographs of each sample were obtained at 500× magnification in random fields by a technician who was not aware of either the purpose of the study or the coding identifying the samples. The technician was only told to avoid the edges of the sample since the cells on these areas could have been disturbed when handling the samples with the tweezers. These microphotographs were used for cell density analysis. One of those fields was also photographed at 1,000× magnification for the morphology analysis.

Data collection and analysis

Three examiners previously calibrated and blind to experimental groups evaluated independently the morphology of cells using the 1,000× magnification photomicrographs. This evaluation was performed according to the index system proposed by Gamal et al. [10] and modified by us: 0 = no cells, 1 = round cells, 2 = flat cells, and 3 = combination of round and flat cells. The same examiners assessed fibroblast density on all three 500× microphotographs using the index described by Jenkins et al. [16] and also modified by us: 0 = no cells or only occasional cells, 1 = presence of some cells with no confluence, 2 = partial cellular confluence, and 3 = a confluent layer of cells covering the entire surface of the dentin specimen.

Since data on this study were categorical in nature, nonparametric statistical methods were used for the analysis. Differences within and between groups were evaluated using Classification and Regression Trees [3]. This tech-

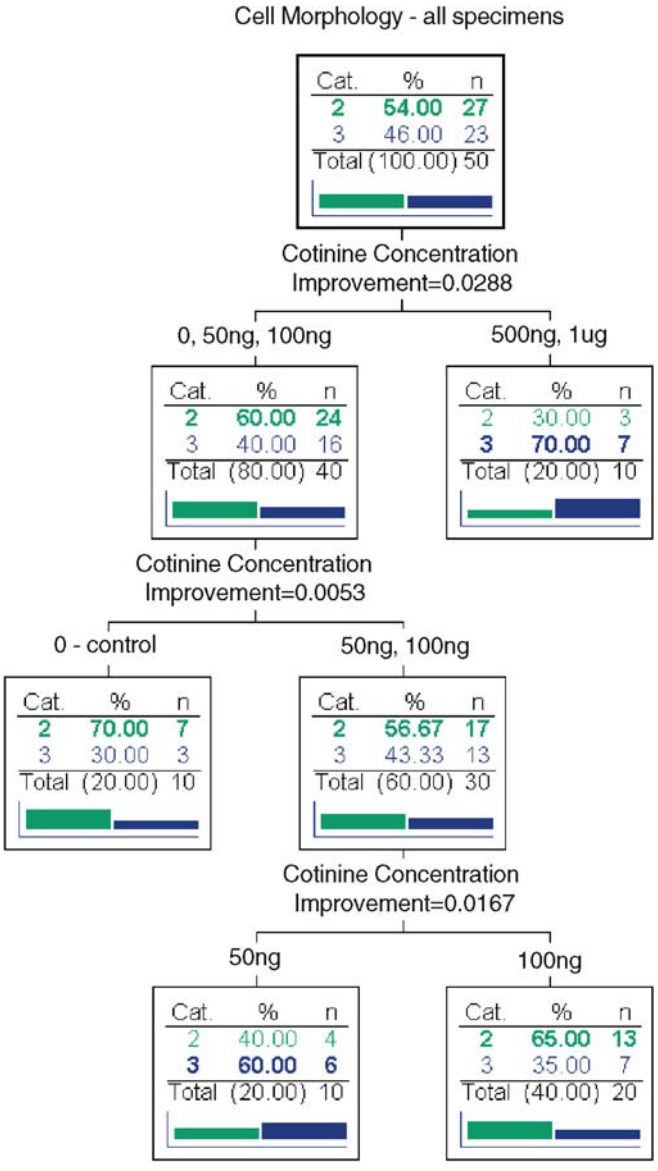

Fig. 3 Classification and regression tree for the effects of cotinine conditioning on cellular morphology

nique is intended to study associations between dependent and independent variables without establishing a mathematical function between variables, but rather by classifying each sampling unit from the values of the independent variables. This analysis was performed with S-Plus 2000 (Math Soft Inc.).

Results

The total sample included 100 microphotographs evaluated for cellular morphology and 300 for density assessments. One of the microphotographs was not considered because it was unintentionally flipped over during the incubation period.

The classification tree indicated significant changes on morphology of fibroblasts that were grown on roots exposed to nicotine concentrations greater than 1 ?g/mL (Fig. 1). Most specimens in these groups (70% of samples) presented a combination of round and flat cells. Furthermore, deleterious effects of nicotine were not reduced by scaling and root planning the specimens after conditioning (Fig. 2a). On the other hand, specimens conditioned with smaller concentrations of nicotine (100 ng or 1 ?g/mL) and subsequently submitted to scaling were associated with less severe effects on cell morphology. Most specimens from these groups submitted to SRPC presented flat cells (Fig. 2b), similarly to what was observed on control group specimens. However, specimens not scaled after conditioning with small concentrations of nicotine (100 ng and 1 ?g/mL) also presented a combination of rounded and flattened cells.

The results for the specimens treated with cotinine also indicated alterations on cell morphology with greater concentrations (500 ng, 1 ?g/mL), independent of root planning after the conditioning (Fig. 3). A total of 70% of the specimens conditioned with 500 ng and 1 ?g/mL presented a combination of round and flat cells (Fig. 4a), as opposed to 60% of the samples treated with smaller concentrations of cotinine as well as negative control specimens that showed only flat cells (Fig. 4b).

Figure 5 presents the classification tree for cell density in the specimens treated with nicotine. Differences on cell density were found between all concentrations of nicotine (100 ng, 1 ?g, 100 ?g, 1 mg/mL) and the negative control group. The majority of the microphotographs from negative control specimens presented cellular confluence covering the entire surface (Fig. 6a), while most of specimens conditioned with nicotine, regardless of the concentration, presented some cells but no confluence (Fig. 6b). Importantly, a gradual decrease of the cell density with increasing concentrations of nicotine was observed, suggesting a dose-response in cellular toxicity. Scaling the specimens

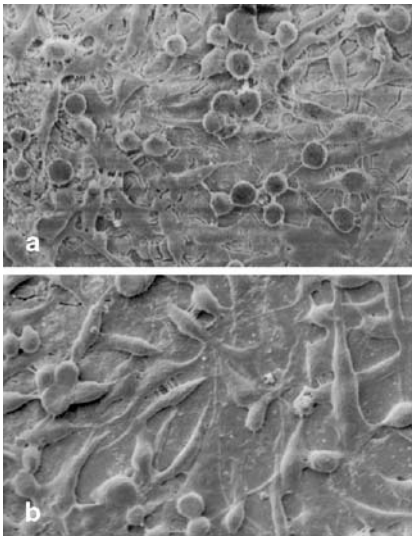

Fig. 4a Group I-Cotinine 1 ?g/mL, presence of combination of round and flat cells. **b** Group I-Cotinine 100 ng/mL, presence of flattened cells. (Original magnification ×1,000)

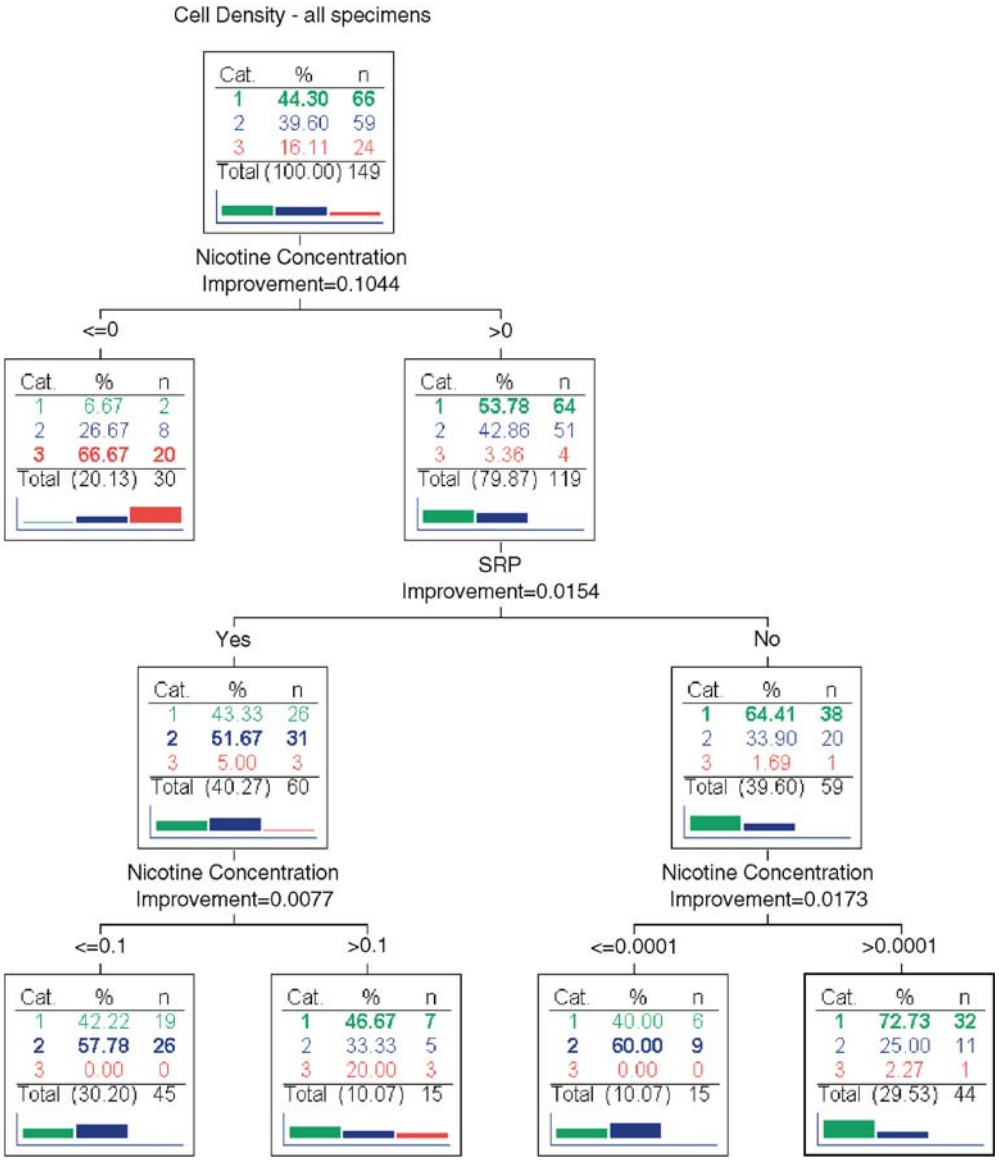

Fig. 5 Classification and regression tree for the effects of nicotine conditioning on cell density

after conditioning with nicotine resulted in greater cell density in comparison to the specimens that were not scaled.

For cotinine-treated specimens, the treatment (scaling or no treatment) rendered after chemical conditioning did not induce significant alterations on cell density (Fig. 7). Cellular confluence covering the entire surface was observed in 63.3% of the microphotographs from negative control and cotinine-treated specimens (up to 500 ng/mL). Furthermore, even in the specimens conditioned with 1 ?g/mL of cotinine and subsequently submitted to scaling, minimal differences on cell density were observed.

Discussion

The present study evaluated in vitro if conditioning a dentin surface with nicotine or cotinine would influence the density and morphology of fibroblasts grown on these dentin specimens. Secondarily, this study evaluated the effect of mechanical root planning after exposure to these substances to determine if this could alter experimental outcomes. This experimental approach was intended to simulate clinical setting where tobacco by-products may be adsorbed on root surfaces. This is a rather new approach, since in vitro studies on the deleterious effects of tobacco toxins do not use a dentin substrate. In fact besides the current study, only the study by Tanur et al. [21] also observed a negative influence of nicotine on attachment of fibroblasts to dentin surfaces, but in that study the cells were seeded onto the dentin substrate in the presence of varying concentrations of nicotine, instead of pre-conditioning dentin with nicotine.

Nevertheless, evaluating these substances would be valid only if they were found in vivo. Many studies [2, 8, 13] have determined the levels of both nicotine and cotinine in chronic smokers. The concentrations used here were based on the smallest and greatest values reported in

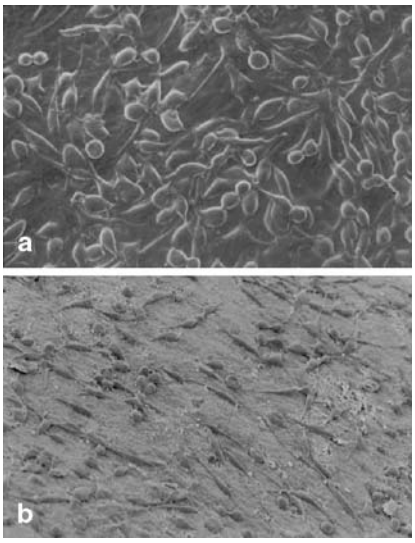

Fig. 6a Control group, a confluent layer of cells covering the entire surface. **b** Group I-Nicotine 1 mg/mL, presence of some cells with no confluence. (Original magnification ×500)

saliva and plasma, and also included the minimum and maximum values found by Cuff et al. [7] on root surfaces.

Fibroblasts in culture need to attach to a solid or semi-solid substrate to grow, and their morphology can be used as an indication of cellular viability. Thus, flattened cells are attached to the substrate, while rounded cells are not attached. This lack of attachment can be due to cellular necrosis or apoptosis [9]. To differentiate the cause of rounded cell morphology we used cell density as an indirect indication of proliferation, as well as the presence of mitotic figures.

The results demonstrated that conditioning specimens with nicotine in concentrations greater than 1 ?g/mL altered the morphology of cells, independent of SRPC. The treatment with concentrations of nicotine of 100 ?g and 1 ?g/mL resulted in predominance of the combination of round and flat cells, in contrast with the control that presented a greater number of flattened cells. Lahmouzi et al. [17] reported similar alterations in the morphology of the gingival fibroblasts of rats with nicotine dosages greater than 1 mM (150 ?g/mL) and Giannoupoulou et al. [12] found vacuolization of the cytoplasm of human PDLF after exposing these cells to concentrations greater that 1 ?g/mL of nicotine. On the other hand, Tipton and Dabbous [22] showed that human gingival fibroblasts exhibited severe alterations as highly vacuolated cytoplasm soon after exposure to 0.075% and 0.05% of nicotine, but this effect was reversible and the cells appeared morphologically normal 3 days after exposure.

In the present study, the specimens that were mechanically scaled after conditioning with 100 ng/mL or 1 ?g/mL of nicotine presented flat cells, similarly to what was observed in the negative control. This suggests that SRP may reduce the negative effect of nicotine and render the dentin surface more biocompatible to fibroblasts only when the concentration of nicotine was equal or smaller than 1 ?g/mL. It is tempting to suggest that clinically scaling and root planning may reduce the amount of nicotine adsorbed onto the root of smokers, thus allowing better adhesion, migration and proliferation of fibroblasts.

In support to this conclusion, Cuff et al. [7] observed that the concentrations of nicotine adsorbed onto the root surfaces of smokers could be significantly diminished by root planning. However, Ito et al. [14] showed that conditioning of root surfaces with high concentrations of nicotine led to adsorption of the drug into dentinal tubules. This implies that adsorbed nicotine can be very difficult to remove by root planning alone and that some form of chemical treatment could be necessary. The latter findings [14] may explain the present observations in which greater concentrations of adsorbed nicotine after scaling were probably still high enough to influence fibroblast attachment and viability, as suggested by the rounded cell morphology and also could justify the influence of the highest concentration of cotinine (1 ?g/mL) on cell morphology regardless of scaling after conditioning. This hypothesis is reinforced by the fact that specimens conditioned with smaller concentrations of cotinine presented cell morphology similar to that observed in the negative control group.

Even though cotinine is the main metabolite of nicotine, and considering the structural similarity between cotinine and nicotine molecules, these substances may have different pharmacological activities. James et al. [15], testing

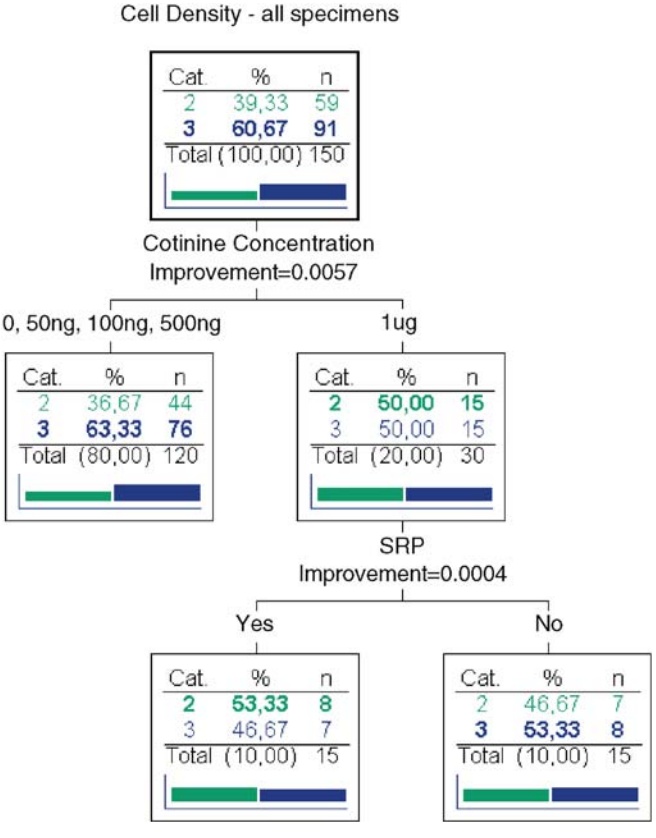

Fig. 7 Classification and regression tree for the effects of cotinine conditioning on cell density

the effects of cotinine on adhesion and proliferation of PDLF, showed that cellular proliferation and adhesion were inhibited only by concentrations greater than 10 ?g/mL, which is far greater than the highest concentration found in vivo (approximately 1 ?g/mL) and used here.

The reduction of cell density promoted by nicotine conditioning was directly proportional to the concentration of the drug. Similar dose-dependent results were found by Lahmouzi et al. [17], who reported a 50% reduction on cell density with concentrations of 4 mM. As occurred with cell morphology, except for the specimens conditioned with the smallest concentration of nicotine (100 ng/mL), scaling resulted in an increased cell density in comparison with the specimens that were not scaled. This result suggests that reduced concentrations of nicotine do not reduce cell proliferation. This is in agreement with Chang et al. [5] and Alpar et al. [1], who could not find a significant decrease on cell proliferation with concentrations of nicotine up to 10 and 5 ?M, respectively, but these authors also reported dose-dependent inhibition on cell proliferation with greater concentrations. Importantly, Alpar et al. [1] also found that the deleterious effect of nicotine were reversible with removal of the drug, which supports our findings in terms of greater cell density after mechanical scaling of the samples. Nevertheless, we could not confirm the findings of Peacock et al. [19], who showed in HGF stimulation of cell adherence and proliferation with low concentrations (0.025 ?M) of nicotine.

For the cotinine-treated specimens, cell density was not altered by concentrations up to 500 ng/mL, regardless of scaling. However, a small reduction on cell density was observed after conditioning with 1 ?g/mL, but only on specimens submitted to scaling. Surprisingly, the non-scaled specimens presented greater cell density than the scaled ones, and this difference was minimal only on specimens treated with 1 ?g/mL of cotinine. This fact may be attributed to differences on the pharmacological activities between nicotine and cotinine. Alternatively, this may be due to lower rate of adsorption of this molecule to the dentin substrate. Ongoing studies are being conducted to evaluate the rate of adsorption of both nicotine and cotinine on diseased root surfaces.

In summary, the results described here indicate that fibroblasts are negatively influenced by nicotine adsorbed on the dentin substrate and also that scaling may reduce these deleterious effects. Although cotinine, the main metabolite product of nicotine in vivo, presents molecular similarity with nicotine and can be found in high concentrations in saliva and gingival crevicular fluid, it does not affect fibroblast attachment and morphology as much as nicotine. This study provides additional information regarding cellular behavior in smokers, which may serve as the basis for future studies on new treatment strategies for smokers.

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