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The influence of cigarette smoke inhalation and its cessation on the tooth-supporting alveolar bone: a histometric study in rats

João Batista César-Neto, Bruno Braga Benatti, Enilson Antônio Sallum, Márcio Zaffalon Casati, Francisco Humberto Nociti Jr
Department of Prosthodontics and Periodontics, Division of Periodontics, School of Dentistry at Piracicaba, University of Campinas, São Paulo, Brazil

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Objective: It has been previously shown that smoking may enhance periodontal breakdown and impair bone healing around titanium implants. However, there is a lack of information concerning the effect of smoking on the tooth-supporting alveolar bone. Thus, the aim of this study was to histometrically evaluate the influence of cigarette smoke inhalation and its cessation on tooth-supporting alveolar bone.

Methods: Sixty male Wistar rats were randomly assigned to one of the following groups: group 1 – control ($n = 15$), group 2 – 2 months of cigarette smoke inhalation ($n = 13$), group 3 – 3 months of cigarette smoke inhalation and 2 months without exposure to cigarette smoke inhalation ($n = 16$) and group 4 – 5 months of cigarette smoke inhalation ($n = 16$). Five months after the beginning of cigarette smoke inhalation regime (2 months for group 2), the animals were killed and the mandible was removed and prepared for histological sections. The proportion of mineralized tissue in the furcation area (i.e. a 1000 μm zone under the furcation and between the roots) was obtained.

Results: Data analysis demonstrated that the animals continuously exposed to cigarette smoke inhalation presented a decreased proportion of mineralized tissue (groups 2 and 4), when compared to control and cessation groups (groups 1 and 3) ($p < 0.05$). Similar levels of proportion of mineralized tissue were observed in groups 1 and 3, showing a beneficial effect of cigarette smoke inhalation cessation on proportion of mineralized tissue.

Conclusion: Within the limits of the present study, it can be concluded that cigarette smoke inhalation may affect the tooth-supporting bone as early as 2 months after the initial exposure, and that smoke exposure cessation may revert its negative impact on the alveolar bone.

Professor Francisco H. Nociti Jr, DDS, MS, PhD, Department of Prosthodontics and Periodontics, Division of Periodontics, School of Dentistry at Piracicaba, University of Campinas, Av. Limeira 901, Caixa Postal 052, CEP 13414–903, Piracicaba, São Paulo, Brazil.
Fax: +55 19 3412 5218
e-mail: nociti@fop.unicamp.br

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The influence of systemic factors on bone has been widely investigated. Several factors such as cigarette smoking, low body weight, estrogen deficiency, alcoholism, caucasian race, inadequate physical activity, calcium intake and poor health have been reported to influence bone quality and osteoporosis incidence in both skeletal and oral bones (1–5).

Among the systemic factors that have been described to influence bone,

smoking is one of the most investigated and has been shown to affect bone in general (6–14). For example, postmenopausal women who smoke lose significantly more cortical bone and have more spinal osteoporosis than non-smoking counterparts (4), and are more likely to lose alveolar bone height and density than non-smokers with a similar periodontitis condition (5). Cigarette smoking may increase bone resorption at fracture ends (6), and interfere with osteoblastic function (7). A 15-patient clinical study revealed that 80% of the individuals with impaired osseous healing were smokers (8). Additionally, it is well recognized that cigarette smoking is associated with impaired wound healing after surgical treatment in the oral cavity (9), reduced bone height (10), increased bone loss rate and resorption of the alveolar ridge (11) and higher incidence of periodontitis (12) and type IV bone (13).

Evidence has now been provided to show that the impact of tobacco smoking on tissues may be reversible. A meta-analysis study reported that current smokers presented significantly reduced bone density when compared to former and never smokers (14). In oral tissues, Liede *et al.* (15) observed that periodontal status and mucosal health were better in individuals who had quit smoking when compared to current smokers. It has also been shown that gingival microcirculation recovers its normal function during the early stages of smoking cessation (16), and that the changes in the inflammatory response of the periodontium can also be reversible on quitting smoking (17). Bain (18) clinically examined the influence of smoking cessation on implant outcomes, and did not find any differences in the failure rate between non-smoking controls and the smokers who had quit.

While significant progress has been made documenting the impact of smoking and its cessation on the skeletal bone (14, 19, 20) and periodontitis-related bone loss (10, 12), very limited information is available with respect to the influence of both smoking and its cessation on the tooth-supporting alveolar bone in general and local clinically healthy conditions. This probably occurs due to the identification and adjustment for risk and confounding factors, which are a significant challenge in clinical studies. Although a variety of statistical analytic techniques have been used to identify and overcome the influence of these factors, we often do not know what the potential risk and confounding factors may be. Therefore, based on the clinical relevance of this subject, the lack of information in the literature and the limitation of clinical studies, the present study aimed to investigate, in rats at the histological level, the influence of cigarette smoke inhalation and its cessation on the tooth-supporting alveolar bone around clinically healthy teeth.

Material and methods

Animals

Sixty male Wistar rats (300–400 g) were used. The animals were kept in plastic cages with food and water *ad libitum*. The protocol was approved by the University of Campinas Institutional Animal Care and Use Committee.

Experimental design

The animals were randomly assigned to one of the following groups.

Group 1: control: animals that were not exposed to cigarette smoke inhalation at any time during the experimental period ($n = 15$).

Group 2: 2 months of cigarette smoke inhalation ($n = 13$).

Group 3: 3 months of cigarette smoke inhalation and 2 months without exposure to cigarette smoke inhalation ($n = 16$).

Group 4: 5 months of cigarette smoke inhalation ($n = 16$).

The animals of groups 2, 3 and 4 were intermittently housed in a cigarette smoke exposure chamber as previously described. Briefly, the device consisted of a $45 \times 25 \times 20$ cm^3 clear acrylic chamber, an air-pump and two inflow/outflow tubes. Five animals were housed in the chamber at the same time, and the cigarette smoke of 10 cigarettes, each containing 1.3 mg of nicotine, 16.5 mg of tar, and 15.2 mg of carbon monoxide, was pumped into the chamber. Thus, the animals were exposed to cigarette smoke that contaminated the air for 8 min, three times daily during the cigarette smoke inhalation exposure period designated for each experimental group. It is important to emphasize that the animals of group 1 were not exposed to cigarette smoke at any time and the animals of group 3 stopped cigarette smoke inhalation 2 months before they were killed. The serum levels of nicotine and cotinine obtained by using this model has been previously reported (23).

Histometric procedure

After the experimental period (5 months for groups 1, 3 and 4, and 2 months for group 2), the animals were killed by decapitation. The jaws were removed and fixed in 4% neutral formalin for 48 h, and subsequently demineralized in a solution of equal parts of 50% formic acid and 20% sodium citrate for 45 days. Paraffin serial sections (6 μm) were obtained in a mesio-distal direction, and stained with hematoxylin and eosin. After excluding the first and the last section in which the furcation region was evident, five equally distant sections of each tooth were selected for histometric analysis. Using an image analysis system (Image-Pro®; Media Cybernetics, Silver Spring, MD, USA), the proportion of mineralized tissue in the furcation area (i.e. a 1000 μm-zone under the furcation and between the roots) was obtained by the measurement of a blinded examiner. Figure 1 represents the area of interest.

Statistical analysis

Mean values of alveolar bone density were determined for each group and compared statistically using the one-way ANOVA test ($\alpha = 0.05$). Pairwise multiple comparisons were carried out by the Tukey test ($\alpha = 0.05$) when the ANOVA test showed a significant difference.

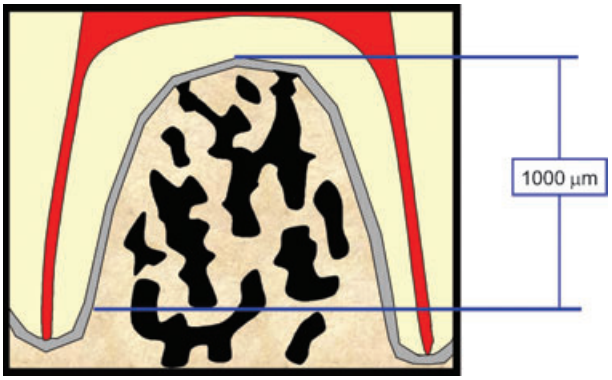

Fig. 1. Schematic illustration of the analyzed area (a 1000 μm-zone under the furcation limited by the roots).

Results

The results of the present investigation showed that, regardless of the duration of exposure (i.e. 2 and 5 months of cigarette smoke inhalation, for groups 2 and 4, respectively), the animals continuously exposed to the cigarette smoke presented a significant reduction in the proportion of mineralized tissue when compared to the animals from groups 1 and 3 (control and cessation, respectively) ($p < 0.05$). Furthermore, similar levels of proportion of mineralized tissue were observed for groups 1 and 3 ($p > 0.05$), showing a beneficial effect of cigarette smoke inhalation cessation on reverting the significant impact of cigarette smoke exposure on bone quality. Figure 2 graphically illustrates the results described above, and Fig. 3(A–D) illustrates the histological aspects.

Discussion

The present investigation evaluated, at the histological level, the influence of intermittent cigarette smoke inhalation (cigarette smoke inhalation) and its cessation on the tooth-supporting alveolar bone quality around the molar tooth in rats. The results of this investigation demonstrated that cigarette smoke inhalation exerted a negative effect on the tooth-supporting bone around clinically healthy teeth. In addition, cigarette smoke inhalation cessation was able to revert to the negative influence of smoke exposure on bone, resulting in bone quality levels similar to those observed for the control group. The present findings therefore suggest that, like skeletal bone, oral bone may also be affected by cigarette consumption, and these results are in line with clinical studies that suggest a negative influence of smoking on bone quality in areas also investigated for osteoporosis (14, 20, 21).

This investigation is part of a series of studies that initially intended to evaluate, in rats, the influence of cigarette smoke inhalation on periodontitis progression and on bone healing around titanium implants. It was observed that cigarette smoke inhalation decreased bone healing around the implants (21, 23), and promoted a higher rate of periodontal breakdown (22). A negative effect of cigarette smoke inhalation on the pre-existing bone in the tibiae was also observed (24). In addition, the impact of smoking on the skeletal and oral bone quality (tibiae and mandible, respectively) was radiographically investigated in rats submitted to cigarette smoke inhalation in comparison to non-exposed animals (25, 26). Data analysis demonstrated a negative impact of smoke exposure on both skeletal and oral bone. The results of the present investigation therefore seem to confirm previous findings (24–26), and additionally provide original information showing that the tooth-supporting bone might also be significantly affected by cigarette smoke inhalation.

Our group has previously shown that the smoke exposure regimen used in the present study may promote cotinine serum levels closely correlated to the ones obtained by smokers that consume between 10 and 20 cigarettes/day (27). However, because there are significant anatomic and metabolic differences between rats and humans, especially with respect to their biological and chronological ages, future comparisons of the present findings with results from studies performed in humans should be treated with caution.

The mechanisms by which smoking affects bone are not fully understood. The influence of hormones on bone density has been widely discussed; however, it seems that there are no differences between smokers and non-smokers with respect to hormonal profile (5, 14, 28–30). *In vitro* studies have shown that cigarette smoke

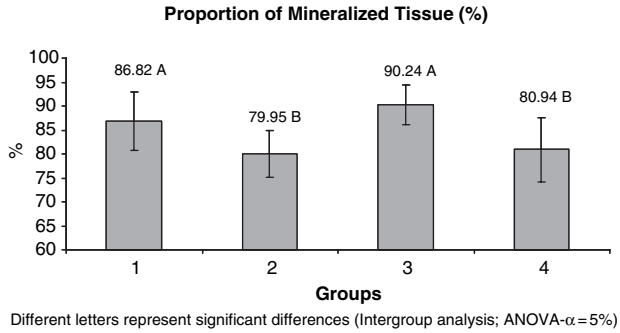

Fig. 2. Mean and standard deviation (%) of the proportion of mineralized tissue, in the area of interest, for groups 1 (control), 2 (2 months of cigarette smoke inhalation), 3 (3 months of cigarette smoke inhalation and 2 months without exposure) and 4 (5 months of cigarette smoke inhalation). Different letters represent significant differences (Intergroup analysis; ANOVA – $\alpha = 5\%$.

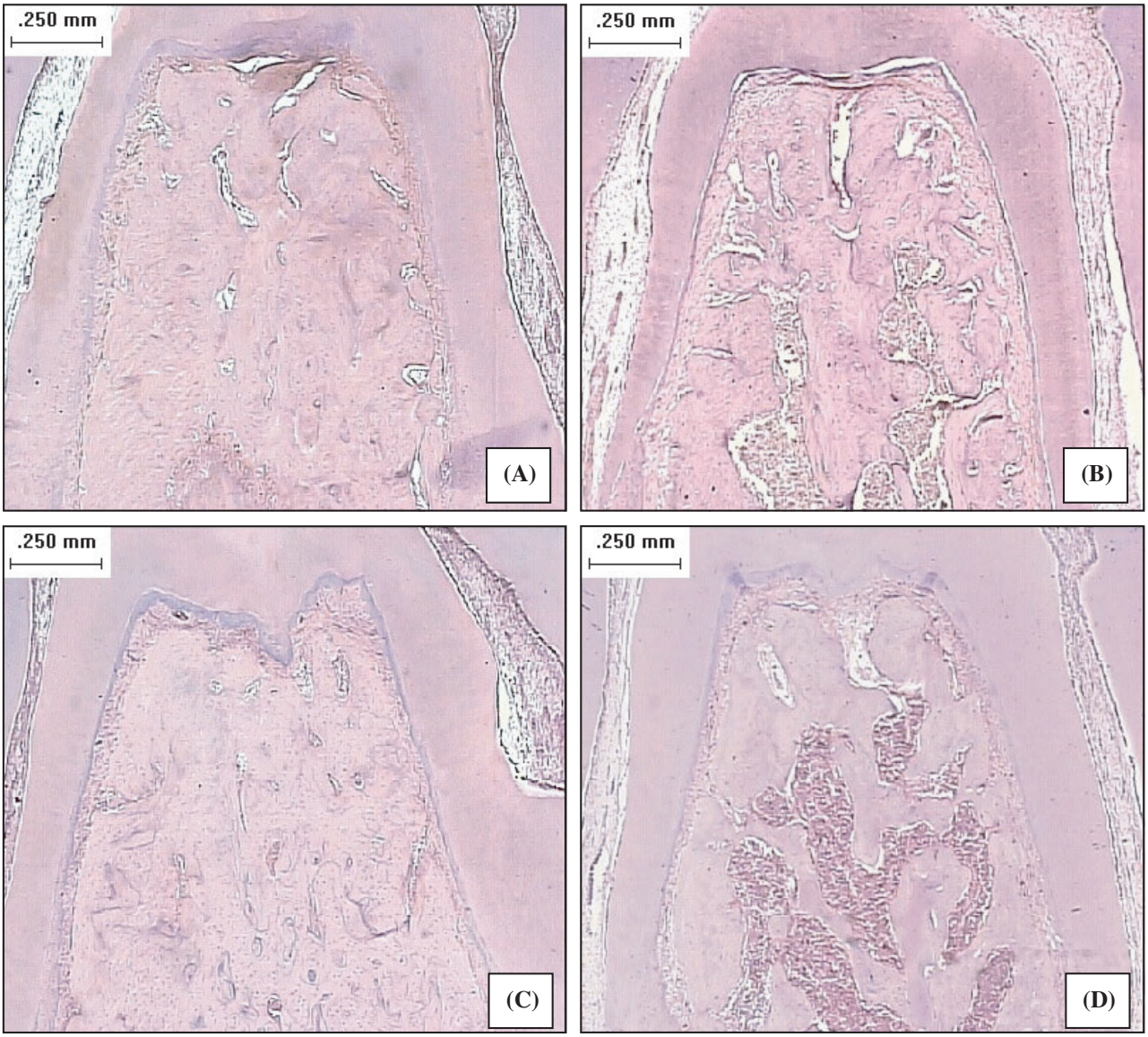

Fig. 3. Photomicrographs A to D illustrate the histological aspect of the furcation bone for groups 1 (control), 2 (2 months of cigarette smoke inhalation), 3 (3 months of cigarette smoke inhalation and 2 months without exposure) and 4 (5 months of cigarette smoke inhalation), respectively. Original magnification 5×; hematoxylin and eosin.

compounds have cytotoxic effects on the cells responsible for bone metabolism and remodeling. Nicotine has shown a biphasic effect on osteoblast cultures, with antiproliferative effects at high levels and stimulatory effects at very low levels (31). Henemyre *et al.* (32) observed that, at clinically relevant levels, nicotine is not toxic to osteoclasts. It appears to stimulate osteoclast differentiation and resorption of calcium phosphate, the major component of bone. The influence of a cigarette smoke extract was evaluated on human osteoprogenitor cells and osteoblast-like cells. Cigarette smoke extract inhibited the proliferation of osteoprogenitor cells and the differentiation of osteoprogenitor cells toward osteoblast-like cells (33). The chemotactic response of both cell types for fibronectin and platelet-derived growth factor-BB, important molecules for bone repair and remodeling, was inhibited by cigarette smoke extract (34). Cigarette smoke extract also inhibited the production of fibronectin by both cell types (34). In addition, nicotine has been reported to enhance the constriction of intact bone vasculature (35) and to impair angiogenesis (7, 36) and osteogenesis in ossification areas (36). Since the process of bone remodeling is the mechanism by which bone renews itself and remains structurally competent, alterations in vascular and cellular events involved in remodeling may be related to the smoking effects on bone density.

The present investigation also reported a beneficial effect of cigarette smoke inhalation cessation on bone. The reversible effect of smoking on bone has been previously documented in medicine with respect to its density

(14, 20, 30, 37) and fracture risk (37, 38). A meta-analysis study demonstrated that current smokers presented a significantly reduced bone density when compared to former and never smokers (14), and that former smokers presented bone densities intermediate or similar to those of never smokers (14, 20). The influence of smoking on fracture risk was investigated in 59,232 men and women from 10 prospective cohort studies (38). A smoking history was associated with a significantly increased risk of fracture, but the risk ratios were lower for former smokers than for current ones (38). The reversibility of smoking effects has also been investigated in dentistry. *In vitro* studies observed a reversible condition promoted by cigarette compounds (i.e. nicotine, acrolein and acetaldehyde) on fibroblast (39, 40). In addition to the positive effects of smoking cessation on the skeletal bone, a beneficial effect has also been reported for the alveolar bone decreasing periodontal risk (41), marginal bone loss (42), and bone loss rate (26). In the implant field, Bain (18) was the first to report that a smoking cessation protocol would improve success rates for osseointegration in smokers who follow it. Therefore, the results of the present investigation are in line with the studies that showed a reversible condition promoted by cigarette consumption, and may support the clinical concept that the effect of cigarette consumption on bone may be reversible.

In conclusion, within the limits of the present investigation, these findings provide important evidence on a reversible effect of cigarette smoke inhalation on the alveolar bone around clinically healthy teeth, and reinforce the hypothesis that, like skeletal bone, oral bone may also be influenced by smoking. Based on previous findings (43), nicotine may at least partially participate in this process, but further studies should be considered in order to better elucidate the mechanisms by which smoking affects alveolar bone.

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