UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

JOÃO BATISTA CÉSAR NETO
Cirurgião-Dentista

Influência da Inalação da Fumaça de Cigarro e Sua Interrupção Sobre o Periodonto e o Tecido Ósseo ao Redor de Implantes de Titânio. Estudo em Ratos.

Tese apresentada à Faculdade de Odontologia de Piracicaba – Unicamp, como parte dos requisitos para obtenção do título de Doutor em Clínica Odontológica, Área de Periodontia

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A Comissão Julgadora dos trabalhos de Defesa de Tese de DOUTORADO, em sessão pública realizada em 31 de Maio de 2005, considerou o candidato JOÃO BATISTA CESAR NETO aprovado.

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PROF. DR. ENILSON ANTONIO SALLUM

PROF. DR. ANTONIO WILSON SALLUM

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SUMÁRIO

PREFÁCIO

Esta tese está baseada nos seguintes artigos científicos:

1- Bone density around titanium implants may be influenced by intermittent cigarette smoke inhalation: A histometric study in rats. **Int J Oral Maxillofac Implants. 2002 May-Jun;17(3):347-52**.

2- A comparative study on the effect of nicotine administration and cigarette smoke inhalation on bone healing around titanium implants. **J Periodontol. 2003 Oct;74(10):1454-9.**

3- Bone density around titanium implants may benefit from smoking cessation. A histologic study in rats. **Int J Oral Maxillofac Implants (accepted).**

4- Bone filling around titanium implants may benefit from smoking cessation. A histologic study in rats. **J Periodontol (accepted).**

5- The influence of cigarette smoke inhalation on bone density. A radiographic study in rats. **Pesq Odontol Bras (accepted).**

6- Matrix metalloproteinase-2 may be involved with increased bone loss associated with experimental periodontitis and smoking. A study in rats. **J Periodontol. 2004 Jul;75(7):995-1000.**

7- Smoking cessation may present a positive impact on mandibular bone quality and periodontitis-related bone loss. A study in rats. **J Periodontol. 2005 Apr;76(4):520-5.**

8- The influence of cigarette smoke inhalation and its cessation on the tooth-supporting alveolar bone. A histometric study in rats. **J Periodontal Res. (submitted)**.

RESUMO

O consumo de cigarros tem demonstrado um impacto negativo na taxa de sucesso de implantes osseointegráveis e é considerado um fator de risco verdadeiro para doença periodontal. Alguns estudos têm relatado que ex-fumantes apresentam taxas de sucesso de implantes osseointegráveis semelhantes a indivíduos que nunca fumaram e que o risco de perda de inserção periodontal também diminui após a interrupção do consumo de cigarros. Em vista disso, o objetivo do presente estudo é avaliar histologicamente, em modelo animal (ratos), a influência da inalação da fumaça de cigarro (IFC) sobre o tecido ósseo ao redor de implantes de titânio inseridos na tíbia dos animais, sobre o osso alveolar de suporte, sobre a evolução da periodontite induzida e a expressão de MMP-2 no tecido gengival. Além disso, numa segunda fase, investigou-se o efeito da interrupção da IFC nas situações descritas acima. Os resultados deste estudo mostram que a IFC influencia negativamente a densidade do osso preexistente e a qualidade do osso neoformado ao redor de implantes de titânio (menor contato osso-implante e preenchimento das roscas). Tanto a interrupção temporária quanto a definitiva promoveram um efeito positivo no osso ao redor dos implantes de titânio. Resultados semelhantes foram observados nas avaliações do osso alveolar de suporte. A IFC promoveu uma diminuição da densidade óssea alveolar e esse efeito foi revertido após a interrupção da IFC. Quanto à doença periodontal induzida, a IFC potencializou a perda óssea na região avaliada (furca) e maiores níveis de MMP-2 foram encontrados no tecido gengival adjacente a essa área. Além disso, os animais do grupo submetido à interrupção da IFC apresentaram níveis de perda óssea semelhantes ao grupo controle, demonstrando um impacto positivo da interrupção do consumo de cigarros sobre a progressão da periodontite induzida. Dentro dos limites do presente estudo conclui-se que: 1- a IFC exerce um efeito negativo tanto no osso preexistente (região medular) quanto no novo osso ao redor de implantes de titânio; 2- confirmou-se histologicamente que a IFC potencializa a perda óssea durante a periodontite e que a MMP-2 pode ser uma das moléculas envolvidas nesse processo; 3- os efeitos negativos da IFC, no tecido ósseo e periodontal, podem ser revertidos após a interrupção da IFC.

PALAVRAS-CHAVE: implantes de titânio, doença periodontal, osso alveolar, densidade óssea, tabagismo, interrupção do consumo de cigarros.

ABSTRACT

Smoking has been reported to negatively impact on titanium implants success rates and has been considered a true risk factor for periodontal disease. Some studies have shown that former-smokers present a implant success rate similar to the one of never-smokers, and that the risk of clinical attachment loss decreases after smoking cessation. Thus, the aim of the present investigation was to histologically evaluate, in an animal model (rats), the influence of cigarette smoke inhalation (CSI) on 1- bone tissue around titanium implants inserted in tibiae, 2- the tooth-supporting alveolar bone, and 3- bone loss resulting from ligature-induced periodontitis and MMP-2 expression in gingival tissue. Additionally, it was investigated the influence of CSI cessation on the conditions described above, e.g. bone healing around titanium implants and bone loss resulting from ligature-induced periodontitis. The results of the present study demonstrated that CSI exerted a negative influence on the preexisting and newly-formed bone around titanium implants and, both temporary and complete CSI cessation were able to revert its harmful effect. Similar findings were observed for the tooth-supporting alveolar bone, where CSI negatively affected bone density and such an effect was reverted after CSI cessation. With respect to the ligature-induced periodontitis, CSI enhanced bone loss in the furcation area and produced higher levels of MMP-2 in gingival tissue adjacent to periodontitis sites. In addition, CSI cessation exerted a positive impact on bone loss, with the cessation group showing a bone loss rate similar to the one of control group. Within the limits of the present study, it can be concluded that: 1- CSI exerted a negative effect on both preexisting and newly-formed bone around titanium implants; 2- CSI may enhance periodontal breakdown, and MMP-2 may take part of this process; 3- the negative effects of CSI, on bone around implants and periodontal tissues, may be reverted after smoking cessation.

KEYWORDS: titanium implants, periodontal disease, alveolar bone, bone density, smoking, smoking cessation

1 INTRODUÇÃO GERAL

Uma estimativa da Organização Mundial de Saúde afirma que há cerca de 1,3 bilhões de fumantes no mundo e que esta população está aumentando, principalmente nos países em desenvolvimento (WHO, 2005). Informações como essa tem atraído a atenção dos pesquisadores para o estudo das conseqüências do consumo de cigarros em eventos biológicos.

Em odontologia, o tabagismo tem sido relacionado a diversos eventos negativos tais como: fator de risco para câncer bucal (CRUZ *et al.*, 2002), maior severidade e incidência de doença periodontal (KERDVONGBUNDIT & WIKESJO, 2002), menor ganho de inserção após terapia periodontal (SCABBIA *et al.*, 2001), dificuldades na reparação de enxertos ósseos (JONES & TRIPLETT, 1992; KAN *et al.*, 1999), inadequado preenchimento sangüíneo dos alvéolos dentários pós-extração (MEECHAN *et al.*, 1988), menor taxa de sucesso de implantes de titânio (BAIN & MOY, 1993; De BRUYN & COLLAERT, 1994) e maior perda óssea ao redor de implantes já osseointegrados (HAAS *et al.*, 1996; LINDQUIST *et al.*, 1996). Dentre esses eventos, destaca-se a influência do tabagismo sobre a doença periodontal, uma das patologias mais freqüentes em indivíduos adultos, e sobre o prognóstico dos implantes de titânio, uma vez que essa opção terapêutica é relativamente nova e tem sido amplamente utilizada como solução protética nos dias de hoje. Embora alguns estudos clínicos já tenham relatado associações entre consumo de cigarros e uma maior severidade da doença periodontal e piores taxas de sucesso de implantes osseointegráveis, o número de estudos histológicos buscando evidências que suportem os achados clínicos ainda é limitado.

Alguns estudos avaliaram a influência da nicotina sobre o reparo ósseo ao redor de implantes de titânio e a evolução da periodontite induzida. Estudos histométricos em ratos observaram que injeções de nicotina aumentaram a perda óssea na região da furca de dentes submetidos à indução de doença periodontal (NOCITI *et al.*, 2000 e 2001). Quanto aos implantes de titânio, sabe-se que injeções de nicotina não influenciaram negativamente o reparo ósseo ao redor de implantes inseridos em tíbias de coelhos (NOCITI *et al.*, 2002; STEFANI *et al.*, 2002), o que diverge dos relatos clínicos em fumantes. Entretanto, a nicotina é apenas uma das mais de 4000 substâncias potencialmente tóxicas presentes na fumaça de cigarro. Na tentativa de melhor reproduzir o efeito do tabagismo, alguns estudos têm utilizado o modelo de fumo passivo, o qual permite a avaliação de todos os componentes da fumaça de cigarro atuando em conjunto. UENG et

al. (1997 e 1999) utilizaram o modelo de fumo passivo para avaliar o efeito da fumaça de cigarro sobre o reparo ósseo após distração osteogênica em tíbia de coelhos. Os resultados destes estudos mostraram que a fumaça de cigarro influenciou negativamente o reparo ósseo. Dentro dos limites de nosso conhecimento não existem relatos da utilização desse tipo de modelo em odontologia.

Apesar dos inúmeros relatos sobre os prejuízos que o tabagismo pode promover em diversas áreas da saúde, algumas investigações laboratoriais e clínicas têm demonstrado que os efeitos do consumo de cigarros e seus componentes podem ser reversíveis. Estudos em cultura de células observaram que os efeitos tóxicos da nicotina (PEACOCK *et al*., 1993), acetaldeído e acroleína (CATTANEO *et al*., 2000) (componentes da fumaça de cigarro) na proliferação e adesão de fibroblastos são revertidos quando essas substâncias são removidas do meio de cultura. Na tentativa de minimizar os efeitos negativos do tabagismo sobre a taxa de sucesso dos implantes de titânio, BAIN (1996) propôs um protocolo de suspensão temporária do consumo de cigarros. Tal protocolo consistia na interrupção do consumo de cigarros uma semana antes do procedimento cirúrgico e a manutenção dessa suspensão por mais 8 semanas, após a colocação dos implantes. Os resultados mostraram que o grupo de pacientes que seguiu o protocolo apresentou resultados semelhantes ao grupo de pacientes não fumantes. Estudos clínicos da área médica têm demonstrado que o tabagismo é um fator que pode influenciar a densidade óssea, mesmo em populações de baixo risco para perda de densidade óssea (ORTEGO-CENTENO *et al.,* 1997). Por outro lado, esses estudos também têm relacionado a interrupção do tabagismo com um aumento na densidade óssea, para níveis semelhantes aos de não-fumantes, (HOLLENBACH *et al*., 1993; WARD & KLESGES, 2001) e uma diminuição no risco de fraturas ósseas (KANIS *et al*., 2004). Em relação à doença periodontal, tem sido observado que os ex-fumantes respondem melhor ao tratamento cirúrgico e não-cirúrgico (GROSSI *et al.,* 1997) e que o risco de perda de inserção diminui após a interrupção do consumo e cigarros (TOMAR & ASMA, 2000). Embora existam diversos estudos clínicos avaliando o efeito do consumo de cigarros sobre a doença periodontal, a taxa de sucesso de implantes e a densidade óssea, o número de investigações no nível histológico é limitado. Assim, o presente estudo propôs-se a avaliar, histologicamente em ratos, o efeito da inalação da fumaça de cigarro (IFC) e sua interrupção sobre: 1-o tecido ósseo ao redor de implantes de titânio inseridos na tíbia dos animais, 2- o osso alveolar de suporte e 3- a evolução da periodontite induzida e expressão de MMP-2 no tecido gengival.

PROPOSIÇÕES GERAIS

Os objetivos do presente trabalho são:

1- Avaliar histometricamente a influência da IFC sobre o tecido ósseo pré-existente ao redor de implantes de titânio inseridos em tíbias de ratos;

2- Avaliar histometricamente a influência da inalação da fumaça de cigarro (IFC), comparada à nicotina, sobre o reparo ósseo ao redor de implantes de titânio inseridos em tíbias de ratos;

3- Avaliar histometricamente a influência da interrupção da IFC sobre o tecido ósseo pré-existente ao redor de implantes de titânio inseridos em tíbias de ratos;

4- Avaliar histometricamente o efeito da interrupção da IFC sobre o reparo ósseo ao redor de implantes de titânio inseridos em tíbias de ratos;

5- Avaliar radiograficamente o efeito direto da IFC e sua interrupção sobre o tecido ósseo da tíbia de ratos, sem a colocação de implantes;

6- Avaliar histometricamente o efeito da IFC sobre a progressão da periodontite induzida por ligaduras em ratos e investigar o papel da MMP-2 neste processo;

7- Avaliar histometricamente o efeito da interrupção da IFC na progressão da periodontite induzida por ligaduras em ratos e radiograficamente o efeito da IFC e sua interrupção na densidade óssea mandibular;

8- Avaliar histometricamente o efeito da IFC e sua interrupção sobre a densidade óssea da região da furca dos primeiros molares inferiores de ratos.

3.1 Capítulo 1

BONE DENSITY AROUND TITANIUM IMPLANTS MAY BE INFLUENCED BY INTERMITTENT CIGARETTE SMOKE INHALATION: A HISTOMETRIC STUDY IN RATS

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ABSTRACT

This study investigated the influence of cigarette smoke on bone healing around titanium implants placed in rats. After anesthesia, the tibiae surface was exposed and a screw-shaped titanium implant (4.0 mm in length - 2.2 mm in diameter) was placed bilaterally. The animals (n=32) were randomly assigned to one of the following groups: **Group 1** – control (n=18) and **Group 2** – intermittent cigarette smoke inhalation (n=14). After 60 days, the animals were sacrificed and undecalcified sections obtained. Bone density (the proportion of mineralized bone in a 500 µm-wide zone lateral to the implant) was measured in the cortical (Zone A) and cancellous bone (Zone B) areas. In Zone A, a slight difference in the bone density was noted between the groups (96.18% $\pm$ 1.08 / 95.38% $\pm$ 1.17 groups 1 and 2, respectively - $P>0.05$), but was not statistically significant. In contrast, bone density was significantly decreased in Zone B in the animals that were exposed to cigarette smoke (17.57% $\pm$ 6.45 / 11.30% $\pm$ 6.81, groups 1 and 2, respectively - $P<0.05$). In conclusion, although intermittent cigarette smoke exposure may not seriously affect cortical bone density, it may jeopardize bone quality around titanium implants in the cancellous bone area.

KEY WORDS: cigarette smoke, osseointegration, dental implants.

INTRODUCTION

For well over a decade titanium endosseous implants have been increasingly used in various edentulous situations[1-4]. However, there are local and systemic conditions which may impair bone

healing or may interfere with the maintenance of osseointegration[5]. It is well-recognized that cigarette smoking is associated with impaired wound healing after surgical treatment in the oral cavity[6], reduced bone height[7], increased bone loss rate[8], increased resorption of the alveolar ridge[7], higher incidence of periodontitis[9] and type IV bone[10]. In addition, smoking has been found to be an important factor in peri-implant soft tissue changes[11].

Smoking has also been one of the factors often discussed in relation to implant failure. Bain & Moy[12] assessed the various factors predisposing to implant failure in a group of 540 patients who had received 2194 implants. They found that smoking was by far the most significant factor: failure rates were 4.76% in non-smokers and 11.28% in smokers. In a later study, De Bruyn & Collaert[13] compared implant failures before loading in the maxillae of smokers and non-smokers. They found that at least one failure was detected in one in three smokers, compared with only one in 25 non-smokers (9% and 1%, respectively). Gorman et al.[16] evaluated the relationship between smoking and the failure rates of dental implants at second-stage surgery. They suggested that smoking is detrimental to implant success. Haas et al.[14] have also suggested that smokers suffer detrimental effects around successfully integrated maxillary implants. Lindquist et al.[15] investigated the influence of smoking and other possibly relevant factors on bone loss around mandibular implants. They demonstrated that smoking was the most important factor affecting the rate of peri-implant bone loss. Esposito et al.[5] reviewed the literature regarding factors associated with the loss of oral implants and concluded that smoking habit was one of the factors associated with biologic failures of the implants. Recently, Lambert et al.[17] reported long-term clinical outcomes of dental implants placed in smokers and non-smokers in a longitudinal clinical study. The authors concluded that smoking promoted an increased implant failure rate.

In addition to the clinical reports regarding the influence of smoking on bone healing around titanium implants, Stefani et al.[18] investigated the effect of nicotine administration on the osseointegration process around dental implants. A slight negative effect of nicotine on the bone-to-implant contact around implants with machined surfaces was observed, although this difference was not statistically significant. At that time, it was stated that nicotine, by itself, was not able to interfere with the bone healing around titanium implants.

To date, no information is available, at an experimental level, regarding the effect of cigarette smoke as a whole on the osseointegration process. Therefore, the present study was designed in order to

evaluate, by histologic analysis, the influence of cigarette smoke on bone healing around titanium implants placed in the tibiae of rats.

MATERIALS AND METHODS

ANIMALS

Thirty-two male Wistar rats (300-400g) were used in the entire study. The animals were kept in plastic cages with access to food and water *ad libitum*. Prior to the surgical procedures all animals were allowed to acclimatize to the laboratory environment for a period of 5 days. The protocol was approved by the University of Campinas Institutional Animal Care and Use Committee.

IMPLANTS SURGERY

General anesthesia was obtained by intramuscular administration of ketamine (0.5ml/kg). Skin was cleansed with iodine surgical soap. An incision of approximately 1 cm in length was made and the bone surface of the tibiae surgically exposed by blunt dissection. Under profuse saline irrigation, bicortical implant beds were drilled at a rotary speed not exceeding 1500 rpm and one screw-shaped commercially available pure titanium implant, of 4.0 mm in length and 2.2 mm in diameter, was placed bilaterally until the screw thread had been completely introduced into the bone cortex. Finally, soft tissues were replaced and sutured. Postoperatively, the animals received an antibiotic (1ml/Kg - Pentabiótico®, Wyeth-Whitehall Ltda, São Paulo, SP, Brazil) given as a single intramuscular injection.

EXPERIMENTAL DESIGN

Immediately after the implant surgery, the animals were randomly assigned to one of the following two treatment groups: **Group 1** – control (n=18) and **Group 2** – intermittent cigarette smoke inhalation (n=14). All animals of group 2 were intermittently housed in an animal cigarette smoke exposure chamber (Fig. 1) at a rate of 8 minutes three times daily until they were sacrificed (60 days). The animal cigarette device was specifically designed for this investigation. It was composed of a 45 X 25 X 20 cm^3 clear acrylic resin chamber, an air-pump and two inflow/outflow tubes. Five animals (group 2) were housed in the chamber at the same time, and the cigarette smoke of 10 cigarettes, containing 1.3 mg of nicotine each, was pumped into the chamber. Thus, the animals were forced to breathe the cigarette smoke that contaminated the air for 8 minutes. The animals of group 1 were not exposed to the cigarette smoke at anytime.

HISTOMETRIC PROCEDURE

After 60 days, the animals were sacrificed, the tibiae were removed and fixed in 4% neutral formalin for 48 hours. Undecalcified sections were prepared as previously described[19], i.e. the blocks were dehydrated by using an ascending series of ethanol (60-100%) and embedded in glycolmethacrylate resin (Technovit 7200® ; Heraeus Kulzer GmbH, Wehrheim, Germany). Subsequently, the sections (20-30 µm) were obtained and stained by using toluidine blue 1% staining. Bone density (i.e. the proportion of mineralized bone in a 500 µm-wide zone lateral to the implant) was obtained (Image-Pro®; Media Cybernetics, Silver Spring , MD, USA) bilaterally in the cortical (Zone A) and cancellous bone (Zone B) areas (Fig. 2).

STATISTICAL ANALYSIS

The data from Zones A and B (cortical and cancellous bone, respectively) were separately averaged. The hypothesis that there was no influence of intermittent cigarette smoke inhalation on the bone density around the implants was tested by using an intergroup analysis (Mann-Whitney test - alpha = 0.05), i.e., Zone A (Group 1) versus Zone A (Group 2) and Zone B (Group 1) versus Zone B (Group 2).

RESULTS

CLINICAL OBSERVATIONS

At the beginning of this investigation, a total of 36 animals were used. However, four animals from Group 2 died as a consequence of exposure to the cigarette smoke. Most of the deaths occurred during the first two days of exposure. After this period, the animals which survived, and were housed in the chamber for exposure to cigarette smoke, demonstrated some problems with respect to their breathing. In addition, a non-significant weight loss for animals of Group 2 was detected.

BONE DENSITY MEASUREMENTS

Statistical analysis did not reveal significant differences between Groups 1 and 2 with respect to the bone density at the cortical bone area - Zone A (96.18% $\pm$ 1.08 / 95.38% $\pm$ 1.17 for groups 1 and 2, respectively - $P>0.05$). In contrast, a significant difference was observed between Groups 1 and 2 regarding the bone density at the cancellous bone area - Zone B (17.57% $\pm$ 6.45 / 11.30% $\pm$ 6.81 for groups 1 and 2, respectively - $P>0.05$). Figures 3 and 4 illustrate the histologic results for the experimental groups.

DISCUSSION

The present investigation is part of a series of studies that has tried to document, at a histologic level, the influence of cigarette consumption and/or its compounds on periodontitis progression and bone healing around titanium implants.

Based on all epidemiologic and clinical studies that classified smoking as a risk factor for periodontitis progression, it was first reported, *in vivo*, the influence of nicotine administration on the progression rate of ligature-induced periodontitis in rats[20]. Later, the influence of nicotine administration on the bone healing around titanium implants placed in the tibiae of rabbits was histometrically evaluated [18]. A tendency for a lower percentage of bone-to-implant contact in the group that received nicotine daily was observed; however, this difference was not statistically significant.

In addition, it was also demonstrated that the implant surface may exert a positive role in the percentage of bone-to-implant contact in the animals which received the nicotine. Similarly, Lambert et al.[17] reported clinically higher success rates for HA-implants in smokers compared to machined surface implants. Nicotine is one of the 2000 potentially toxic substances in tobacco smoke and has been demonstrated, *in vivo* and *in vitro*, to influence many biologic events[20-24]. Despite this fact, within the limits of a previous study[18], it was hypothesized that nicotine would not be able to influence bone healing around titanium implants by itself and that the adverse effects of cigarette consumption on the success rates of the titanium implants would only be related to the cigarette smoke as a whole. Therefore, to investigate whether cigarette smoke inhalation would interfere with the bone healing around a titanium implant, the present study was proposed.

Ueng et al.[25-26], using a mechanism by which experimental animals (rabbits) could be exposed to cigarette smoke, reported that intermittent cigarette smoke exposure delayed mineralization during the bone healing process of distraction osteogenesis. In the present investigation, a similar device was used to expose the animals to cigarette smoke by changing the dimensions of the acrylic resin box to ones that would allow the inclusion of five animals (rats) each time (45 X 25 X 20 cm^3). In the present study, the amount of cigarettes used at the time of each exposure (i.e., 10 cigarettes/exposure) was determined by pilot studies which demonstrated that this was the highest volume of cigarette smoke that the animals could support for eight minutes - three times/day for 60 days. Nevertheless, some animals (4 rats) demonstrated more sensitivity to such volumes of smoke and died before completing the experimental period. CENDON-FILHA[27], using a

similar protocol (rats in exposure chamber and 10 cigarettes/exposure) reported lung emphysema in the animals after two years of daily exposure. Therefore, it was believed that the volume of smoke exposure to which each animal was submitted may have closely assimilated a heavy smoker, i.e., an individual who smokes more than 15 cigarettes daily.

Bain and Moy[12] first reported the negative effect of smoking on the success rate of osseointegrated implants. The smokers' failure rate was 11.28% (44/390), while the nonsmokers' failure rate was significantly lower, at 4.76% (86/1804). This observation was later confirmed in different populations using different implant systems. De Bruyn & Collaert[13] described the effect of smoking on initial implant failure before functional loading with fixed prosthetic restorations. The failure rate before loading was 9% in smokers versus 1% in nonsmokers and was statistically significant. They concluded that smoking is a significant factor in the failure of implants prior to functional loading.

Gorman et al.[16] analyzed more than 2000 implants regarding their survival at second stage surgery and concluded that smoking is detrimental to implant success. Lindquist et al.[15] showed that smoking was the most important factor of those correlated with increased peri-implant bone loss. Lambert et al.[17] reported that after three years, endosseous implant placement in smokers may be almost 1.5 times more likely to fail than in nonsmokers (2.9% difference), but both groups demonstrated a high success rate (94% versus 91.1% for nonsmokers and smokers, respectively). The difference between smokers and nonsmokers reported by Lambert et al.[17] (2.9%) is almost half of that reported by Bain and Moy[12] (6.52%). Possibly, the reason for this discrepancy lies in the fact that Bain and Moy studied 100% machined implants, while Lambert used mostly textured implants (HA-coated), signifying that the percentage of failures may be influenced by the implant design.

At a histologic level, the present study showed that intermittent cigarette smoke inhalation may influence bone density in the cancellous bone area around titanium implants, although no significant effect was observed in the cortical bone. The clinical relevance of such an observation requires further investigation, although it seems to support the high success rates observed for smokers in Lambert´s study. While in the present study, the animals were submitted to all of the compounds of the cigarette smoke as are humans, caution must be used to extrapolate the results. First, because the local effect of the cigarette consumption was not present in the present study. Second, the implants were not loaded and consequently, on a long-term basis, the real implications

of lower bone density in the cancellous bone region after loading the implants for a period of time could not be projected. Finally, despite the fact that rats have been used as a model to test some hypotheses regarding titanium implants[28-35], it may not entirely reproduce the events in humans. In addition, cigarette smoke is inhaled chronically by humans, i.e., the bone tissue is exposed to the compounds of the cigarette smoke for many years. Whether different results would be observed if animals were exposed for a longer period of time and/or before implant placement remains to be investigated.

CONCLUSION

In conclusion, within the limits of the present study, it was concluded that although cigarette smoke exposure may not seriously affect cortical bone, it may jeopardize bone quality around titanium implants in the cancellous bone area as seen in this exclusively histologic investigation.

Acknowledgment

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Figure 1: Schematic illustration of the cigarette smoke exposure device demonstrating that the acrylic chamber was composed of two sub-chambers: the cigarette (A) and the animal (B) compartments.

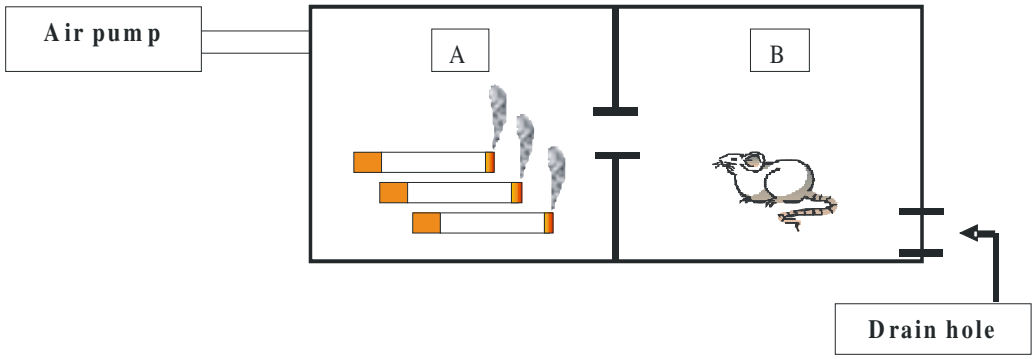


Figure 2: Schematic illustration of the histometric parameters evaluated.

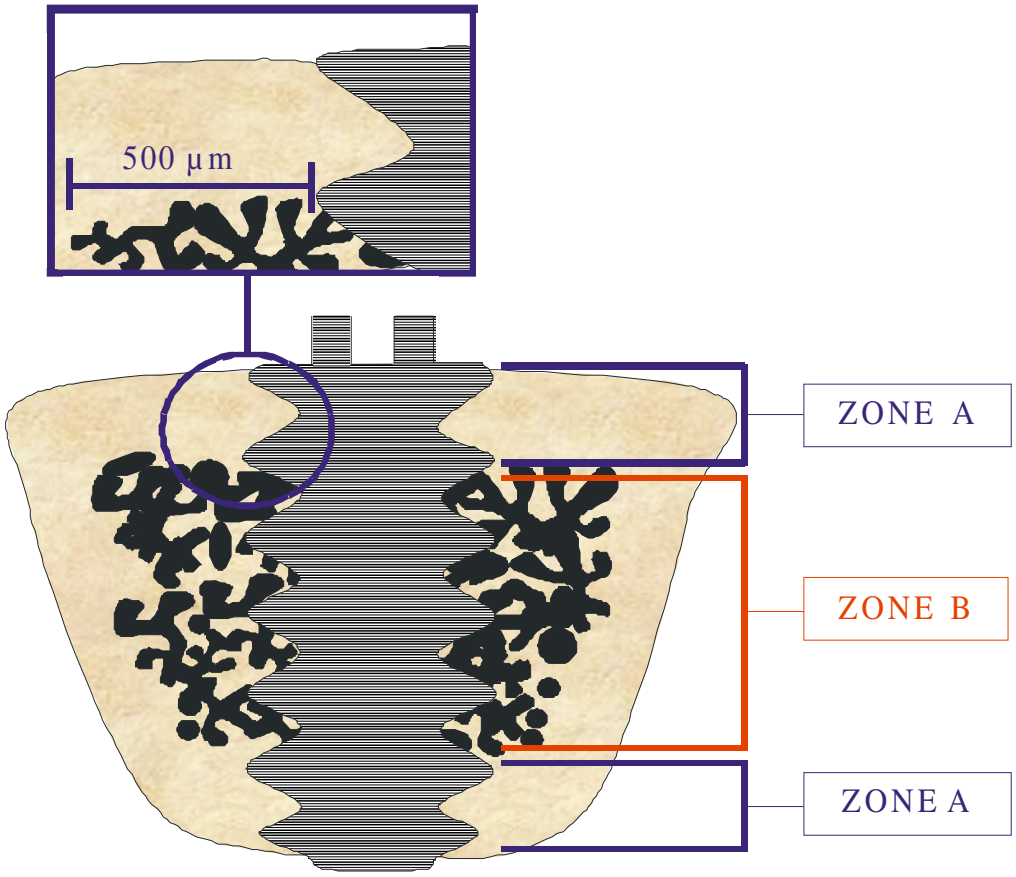
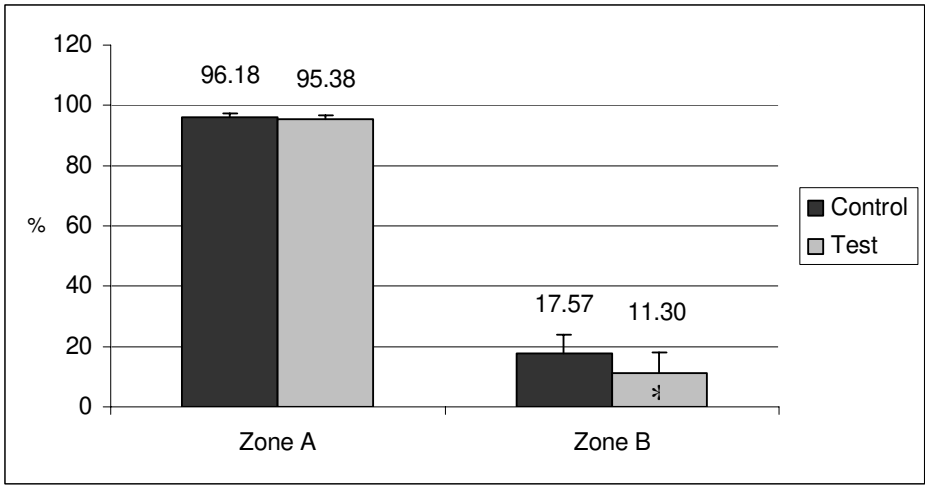
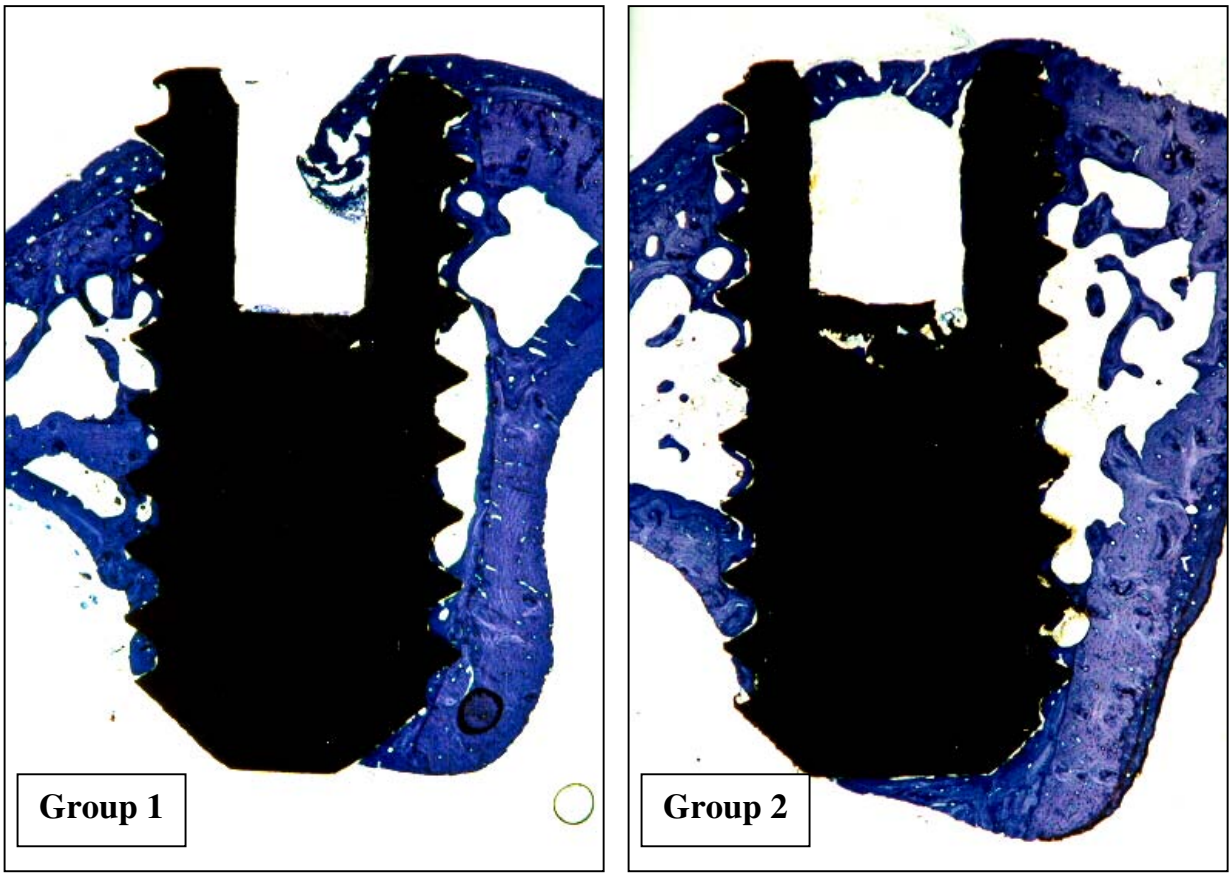


Figure 3: Mean and standard deviation (%) of the bone density around the implants for groups 1 and 2 at Zones A and B.



* Statistically significant difference (Mann-Whitney test - alpha = 0.05) - intergroup analysis for each zone

Figure 4: Photomicrographs illustrating the histological aspect observed around the implants inserted in the animals of groups 1 and 2. Toluidine blue / Original magnification = 6.25x.



3.2 Capítulo 2

A COMPARATIVE STUDY ON THE EFFECT OF NICOTINE ADMINISTRATION AND CIGARETTE SMOKE INHALATION ON BONE HEALING AROUND TITANIUM IMPLANTS.

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ABSTRACT

Background: A series of isolated studies has focused on the influence of smoking on bone around titanium implants. At this time, this study is proposed to, comparatively, investigate the impact of both conditions, e.g., nicotine administration and cigarette smoke inhalation, on the healing around implants.

Material and Methods: Forty-five Wistar rats were used. After anesthesia, the tibiae surface was exposed and one screw-shaped titanium implant was placed bilaterally. The animals were randomly assigned to one of the following groups: **Group 1** – control (n=19), **Group 2** – intermittent cigarette smoke inhalation (n=15) and **Group 3** – subcutaneous administration of nicotine (3mg/kg) twice daily (n=11). After sixty days, the animals were sacrificed. The degree of bone-to-implant contact (BIC) and the bone area (BA) within the limits of the threads of the implant were measured in the cortical (Zone A) and cancellous bone (Zone B) areas.

Results: In Zone A, cigarette smoke presented a significant negative influence on BIC and BA (Kruskal-Wallis – $P<0.05$). In contrast, the administration of nicotine did not influence either

parameter (P>0.05). In Zone B, cigarette smoke inhalation also resulted in a decreased percentage of BIC compared to the control group (P<0.05). In addition, the BA was significantly decreased in groups 2 and 3 when compared to control (P>0.05).

Conclusion: The negative impact of smoking on implant outcomes may be related to more than one molecule present in the cigarette smoke, and nicotine seems to, partially, contribute, especially in the cancellous bone.

KEY WORDS: cigarette smoke, nicotine, tobacco, osseointegration, dental implants.

INTRODUCTION

The long-term success of implant therapy has been reported by several authors[1-4], however, some systemic conditions have been correlated with higher rates of failure[5]. Smoking is one of the factors often discussed in relation to implant failure. It is well recognized that cigarette smoking is associated with impaired wound healing after surgical treatment in the oral cavity[6], reduced bone height[7], increased bone loss rate[8], increased resorption of the alveolar ridge[8], higher incidence of periodontitis[9] and type IV bone[10]. In the field of implantology, a greater incidence of implant failures before loading in the maxillae of smokers than in non-smokers (9% and 1%, respectively) [11] and higher rates of later failures (11.28% and 4.76%, for smokers and non-smokers respectively) [12] have been reported. Gorman et al.[13] evaluated the relationship between smoking and the failure rates of dental implants at second-stage surgery. They suggested that smoking is detrimental to implant success. Haas et al.[14] suggested that smokers suffer detrimental effects around successfully integrated maxillary implants and Lindquist et al.[15] related that smoking was the most important factor affecting the rate of peri-implant bone loss in mandible. Esposito et al.[5] reviewed the literature regarding factors associated with the loss of oral implants and concluded that smoking habit was one of the factors associated with biological failures of the implants. Recently, Lambert et al.[16] reported a longitudinal clinical study on the outcome of osseointegrated dental implants in smokers and non-smokers. The authors concluded that smoking promoted an increased implant failure rate.

As an attempt to understand and illustrate such impaired clinical outcomes, some studies were performed using animal models in order to provide a histological figure. Initially, Stefani et al.[17] and Nociti Jr. et al.[18] investigated the influence of nicotine administration on the osseointegration and bone density around dental implants. In both studies, the authors were not able to demonstrate a significant influence of nicotine on bone healing around titanium implants. It was then hypothesized

that nicotine, by itself, was not able to interfere with the bone healing around titanium implants. Therefore, a cigarette smoke exposure chamber was designed in order to investigate, in rats, the influence of the cigarette smoke as a whole on the bone around titanium implants[19,20]. It was showed that intermittent cigarette smoke inhalation altered the proportion of mineralized tissue around titanium implants in the cancellous area[19]. In addition, it was observed that the animals exposed to cigarette smoke presented a minor bone filling of the threads in both regions[20]. Although these studies are the only information available, in a histological level, regarding the influence of smoking and titanium implants, two relevant aspects had not been approached yet. First, the serum levels of nicotine and cotinine were not assessed by these studies, and a parallel with human levels of both parameters would be necessary to allow comparisons. Second, in all the studies, nicotine administration and cigarette smoke inhalation were separately evaluated; therefore, it did not allow comparisons between the treatments under similar conditions.

Therefore, in the present study we hypothesized that nicotine alone may not be able to reproduce the negative impact of cigarette smoke inhalation around titanium implants inserted in rats with serum levels of nicotine and cotinine similar to that reported for smokers.

MATERIALS AND METHODS

ANIMALS

Forty-five male Wistar rats (300-400g) were included in the study. The animals were kept in plastic cages with access to food and water *ad libitum*. Prior to the surgical procedures all animals were allowed to acclimatize to the laboratory environment for a period of 5 days. The protocol was approved by the University of Campinas Institutional Animal Care and Use Committee.

IMPLANTS SURGERY

General anesthesia was obtained by intramuscular administration of ketamine (0.5ml/kg). Skin was cleansed with iodine surgical soap. An incision of approximately 1 cm in length was made and the bone surface of the tibiae surgically exposed by blunt dissection. Under profuse saline irrigation bicortical implant beds were drilled at a rotary speed not exceeding 1500 rpm. One screw-shaped commercially available pure titanium implant, of 4.0 mm in length and 2.2 mm in diameter, was placed bilaterally until the screw thread had been completely introduced into the bone cortex.

Finally, soft tissues were replaced and sutured. Postoperatively, the animals received antibiotic† given through a single intramuscular injection.

EXPERIMENTAL DESIGN

After the implant surgery, the animals were randomly assigned to one of the following treatment groups: **Group 1** – control (n=19), **Group 2** – intermittent cigarette smoke inhalation (n=15) and **Group 3** – subcutaneous injections of nicotine (3 mg/kg) twice daily (n=11). The group-two animals were intermittently housed in an animal cigarette smoke exposure chamber as previously described[19-20]. Briefly, the device consisted of a 45 X 25 X 20 cm^3 clear acrylic chamber, an air-pump and two inflow/outflow tubes. Five animals (group 2) were housed in the chamber at the same time, and the cigarette smoke of 10 cigarettes, containing 1.3 mg of nicotine each, was pumped into the chamber. Thus, the animals were forced to breath the cigarette smoke that contaminated the air for 8 minutes, three times daily until they were sacrificed (60 days). The group-three animals received subcutaneous injections of 3 mg/kg twice daily until they were sacrificed. The animals of group 1 were neither exposed to the cigarette smoke nor received subcutaneous injections at anytime.

NICOTINE AND COTININE SERUM LEVELS: ANALYTICAL METHODS

Blood samples were taken before the implant surgery, and after 30 and 60 days. The procedure was systematically performed 15 minutes after the treatments, e.g., cigarette smoke inhalation or nicotine adminstration. Serum samples were assayed for concentrations of nicotine and cotinine by high-pressure liquid chromatography, composed by two pumps#, programmed by a system controller¶, a UV-Vis detector** set at 260 nm and a reversed-phase column Luna†† (150mm X 4.6 mm I.D.X 5 μm). The mobile phase consisted of 20 mM dibasic potassium phosphate, 20 mM monobasic potassium phosphate containing 0.1% of triethylamine. The pH of the solution was adjusted to 6.3 with phosphoric acid and 10 % of acetonitrile was added to the final solution. The flow rate was 1.0 mL/min. 2-phenylimidazole was used as internal standards. All the reagents used to perform the method were HPLC grade. The extraction of the samples followed the methodology

† Pentabiótico®, Wyeth-Whitehall Ltda, São Paulo, SP, Brazil
# LC-10ADvp, Shimadzu Corporation, Tokyo, Japan
¶ SCL-10ADvp, Shimadzu Corporation, Tokyo, Japan
** SPD-10ADvp, Shimadzu Corporation, Tokyo, Japan
†† Column Luna, Phenomenex, USA

previously described by Nakajima et al.[21] but evaporated to dryness under nitrogen at ambient temperature. The injection volume was 20 μL and the limit of quantification was 10 ng/mL.

HISTOMETRIC PROCEDURE

After 60 days, the animals were sacrificed; the tibiae were removed and fixed in 4% neutral formalin for 48 hours. Undecalcified sections were prepared as previously described[22], i.e. the blocks were dehydrated by using an ascending series of ethanol (60-100%) and embedded in glycolmethacrylate‡. Subsequently, the sections (20-30 μm) were obtained and stained by using toluidine blue 1% staining. The percentage of bone-to-implant contact (BIC) and bone area (BA) within the threads of the implants was obtained bilaterally##, and the data from both sides were averaged to give a mean score for each animal. The data were arranged separately in cortical (Zone A) and cancellous bone (Zone B) areas, as previously described [19-20].

STATISTICAL ANALYSIS

The null hypothesis was tested by an intergroup analysis using the non-parametric Kruskal-Wallis test (alpha = 0.05), regarding Zones A and B separately (Group 1 vs Group 2 vs Group 3). Pairwise multiple comparisons were carried out by Tukey test (alpha = 0.05) in the case that Kruskal-Wallis test showed significant differences.

RESULTS

CLINICAL OBSERVATIONS

At the beginning of this investigation, a total of 55 animals were used. However, one animal from Group 1, five animals from Group 2 and four animals from Group 3 died before finishing the experimental period. Three deaths in Group 2 occurred during the first two days of exposure as a consequence of smoke inhalation, the other two during the collection of blood. After the initial period, the animals that survived, and were housed in the chamber for exposure to cigarette smoke, demonstrated some problems with respect to their breathing. All the deaths of Group 3 occurred during the collection of blood. In addition, a non-significant weight loss for animals of Group 2 and 3 was detected (data not shown).

‡ Technovit 7200® ; Heraeus Kulzer GmbH, Wehrheim, Germany

Image-Pro®; Media Cybernetics, Silver Spring , MD, USA

SERUM LEVELS OF NICOTINE AND COTININE

The serum levels of nicotine and cotinine were lower than the detectable limit for all groups before the implant placement. Group 1 (control) had also no detectable values in the second and third evaluations (30 and 60 days after implant insertion). On the other hand, Groups 2 and 3 presented detectable values of nicotine and cotinine in both evaluations. The mean values of nicotine were 346,1 ng/mL $\pm$ 114,3 and 376,03 ng/mL $\pm$ 53,85 at day 30 for groups 2 and 3, respectively; and 174,9 ng/mL $\pm$ 32,2 and 401,0 ng/mL $\pm$ 64,0 at day 60 for groups 2 and 3, respectively. The mean values of cotinine were 265,4 ng/mL $\pm$ 109,8 and 294,38 ng/mL $\pm$ 41,24 at day 30 for groups 2 and 3, respectively; and 149,9 ng/mL $\pm$ 27,4 and 181,2 ng/mL $\pm$ 17,8 at day 60 for groups 2 and 3, respectively.

HISTOMETRIC ANALYSIS

Statistical analysis revealed a significant difference regarding BIC between groups 1 and 2 either in cortical or cancellous bone (Zone A and Zone B, respectively). On the other hand, no differences were observed between groups 1 and 3, and groups 2 and 3 in both zones (Table 1).

Similar results were observed concerning BA in the cortical bone (zone A), e.g., a significant influence of smoke inhalation compared to control group and no differences between nicotine administration and the other groups (Table 1). Moreover, it was observed in the cancellous bone (zone B), that cigarette smoke inhalation and nicotine administration were able to decrease the percentage of mineralized bone within the limits of the implant threads (Table 1). Figures 1 to 3 illustrate the histological findings.

DISCUSSION

The present investigation evaluated the impact of subcutaneous injections of nicotine compared to intermittent cigarette smoke inhalation on bone healing around titanium implants inserted in the tibiae of rats using histological methods, e.g., degree of BIC and BA within the limits of the implant threads. The data for the cortical (Zone A) and cancellous (Zone B) regions were evaluated separately because of the anatomic and metabolic differences between these areas, and also because previous studies have reported that the bone in the medullar compartment may be more susceptible to systemic conditions[23-24]. The results of the present study demonstrated that cigarette smoke inhalation influenced negatively the bone healing for both histometric parameters in

cortical and cancellous bone. It seems that part of the effects produced by cigarette smoke is caused by nicotine and these effects are of great importance in cancellous bone.

Previous reports, using a similar protocol of cigarette smoke inhalation; have observed a negative influence of this treatment on the proportion of mineralized bone in cancellous zone lateral to the implant[19]. It has also been demonstrated that intermittent cigarette smoke inhalation produces a minor bone filling of titanium implant threads inserted in the tibiae of rats[20]. In addition, the present study demonstrated that intermittent cigarette smoke inhalation might affect BIC.

Ueng et al.[25-26], using a mechanism by which rabbits were exposed to cigarette smoke, reported that intermittent cigarette smoke exposure delayed the mineralization during the bone healing process of distraction osteogenesis. In the present investigation, the device used to expose the animals to the cigarette smoke was modified from previous reports[25-26] allowing the inclusion of five animals (rats) at a time (45 X 25 X 20 cm^3). The amount of cigarettes used at the time of each exposure (i.e., 10 cigarettes/exposure) was determined by former studies[19, 20] which demonstrated that it was the volume of cigarette smoke that the animals could support for eight minutes - three times/day for 60 days. Later, the assessment of serum level of cotinine demonstrated that either the protocol of intermittent cigarette smoke inhalation, used in the present investigation as well as in previous ones, or the regimen of nicotine administration produced serum levels correlated with human smokers that consume between 10 to 20 cigarettes/day[27]. The interval between the exposures/injections and the blood collections was based on the half-lives of nicotine and cotinine[28], the time necessary to metabolize nicotine into cotinine (half-life formation of the metabolite: 20-28 minutes)[29] and the time necessary for the animal to recover its normal functions of breathing and movement. A special care was taken with the animals, mainly in the intermediate collection, because pilot studies revealed a high incidence of deaths during this procedure.

In the present study, regarding nicotine and cotinine serum levels, a certain degree of variability, and changes between 30 and 60 days were observed. Possibly, the variability may be explained by two events. First, because a special care was taken during the intermediate collection of blood, it presented different duration according to the death risk of each animal. Second, it was not possible to standardize the blood flow of the animals what results in different time intervals to collect the amount of blood necessary for the analytical methods. Moreover, changes in nicotine absorption, as a

consequence of lung emphysema as previously reported, may have been the cause of different serum concentration during the time.

In contrast to a previous report[17], the present study showed that nicotine administration produced a negative influence on BA in the cancellous zone. Since the dosage (3mg/kg) and frequency of administration (twice daily) used in the present study are higher than the ones used in the study mentioned above (0,93 mg/kg, once daily, 8 animals), this data seems to confirm a previous report showing that nicotine may present a dose-dependent response in vivo[30].

In vitro studies may lead us to, mechanistically, approach the data presented by the present investigation. Several authors have reported that nicotine is a cytotoxic agent that produces negative effects on fibroblasts[31, 32, 33] and osteoblasts cell cultures[34, 35]. It has also been shown that acrolein and acetaldehyde, volatile components of cigarette smoke, have negative effects on fibroblasts cultures[36, 37, 38]. Besides, the carbon monoxide, another compound of cigarette smoke, has a great affinity for hemoglobin[39]. This link is 200 times stronger than the link to oxygen, consequently, decreasing the oxygen-carrying capacity of hemoglobin[39]. This information may be of great importance mainly for healing areas, where the cells are in high metabolism and need a great amount of oxygen. Another investigation related that hydrogen cyanide, a volatile component of cigarette smoke, inhibits enzyme systems necessary for oxidative metabolism and oxygen transport at the cellular level[40]. Taken together, the effects of these events may have the potential to jeopardize bone healing.

In contrast to most of clinical studies[11, 14-16] that reported early implant failure in smokers, in the present study, the observation of a lower bone-to-implant contact and poor bone quality around the implants guides us to correlate cigarette consumption with late implant failure. A similar finding can also be inferred from the study by Ekfeldt´s et al.[41]. They observed in a retrospective study that the number of lost implants in smokers was significantly higher after loading them (22) than before it (9).

Smokers, however, do not start smoking after implant placement, but may have been smoking for many years before, i.e., the bone tissue has been exposed to the compounds of the cigarette smoke for a long time. It remains to be investigated whether a different figure would be observed in case of animals that were exposed for a longer period of time before implant insertion. In

addition, despite the findings of the present investigation, the effect of nicotine-based products as adjunct to smoking cessation protocols on the implant outcomes remains unclear. Finally, a relevant aspect that is currently under investigation concerns the benefits that a cessation protocol can provide to the observations of the present study.

In conclusion, within the limits of the present study, intermittent cigarette smoke inhalation may result in lower bone-to-implant contact and a lower bone area within the limits of the threads, and part of these effects seems to be caused by nicotine, specially in cancellous bone.

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Figure 1: Photomicrograph illustrating the histological aspect observed around the implants placed in the animals of group 1. Toluidine blue / Original magnification = 12.5x .

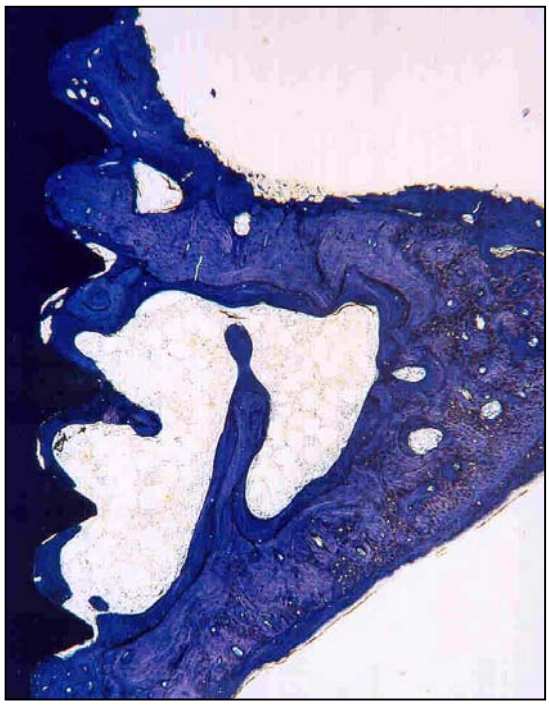

Figure 2: Photomicrograph illustrating the histological aspect observed around the implants placed in the animals of group 2. Toluidine blue / Original magnification = 12.5x .

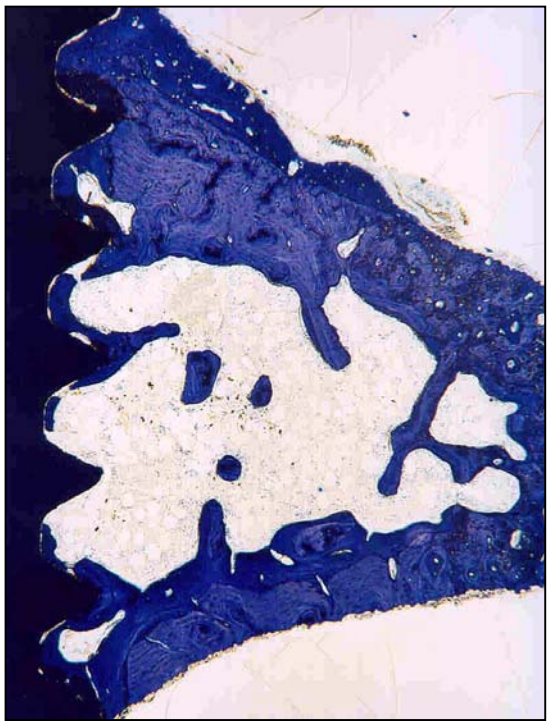

Figure 3: Photomicrograph illustrating the histological aspect observed around the implants placed in the animals of group 3. Toluidine blue / Original magnification = 12.5x .

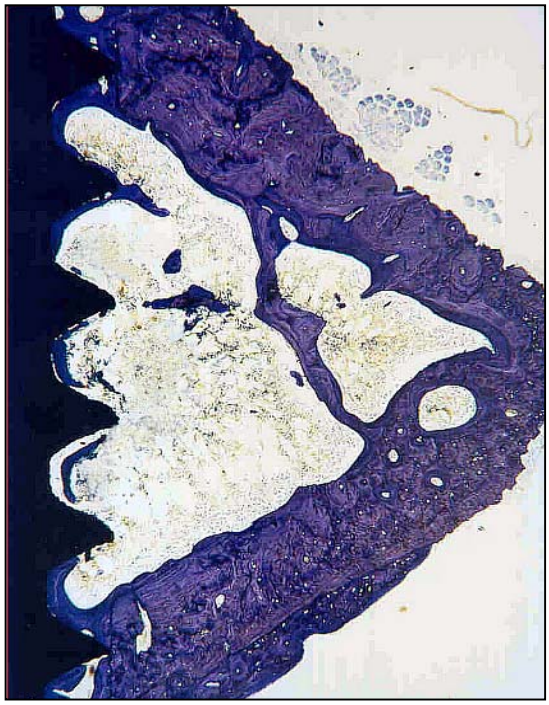

Table 1: Mean and standard deviation (%) representative of the histometric parameters evaluated.

GROUPS	ZONE 1		ZONE 2	
	BIC	BA	BIC	BA
1	55,60 ± 11,00a	86,47 ± 4,80a	36,67 ± 7,11a	32,01 ± 6,62ª
2	41,39 ± 15,64b	79,85 ± 6,17b	25,55 ± 13,34b	20,71 ± 8,57b
3	45,54 ± 3,06ab	81,54 ± 5,06ab	27,95 ± 11,93ab	21,91 ± 6,48b
	P=0,003	P=0,003	P=0,012	P<0,001

Letters should be considered by columns (Kruskal-Wallis test). If 2 different groups have the same letters, then they are not statistically significant, and vice versa.

3.3 Capítulo 3

BONE DENSITY AROUND TITANIUM IMPLANTS MAY BENEFIT FROM SMOKING CESSATION. A HISTOLOGIC STUDY IN RATS

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ABSTRACT:

Purpose: This study tested the hypothesis that interruption of cigarette smoke inhalation (CSI) would revert its impact on bone quality around implants.

Material and Methods: Sixty-nine rats were assigned to: Group 1 – control (n=16), Group 2 **–** CSI (n=17), Group 3 – CSI 83 days prior to implant placement (n=16), and Group 4 – CSI for 83 days, interruption 1 week before and 3 weeks after implant placement, and return to CSI for 39 days (n=20). Bone density (proportion of mineralized bone in a 500µm-wide zone lateral to the implant - BD) was obtained.

Results: In the cortical bone, a slight difference was noted (97.66% $\pm$ 3.69, 98.30% $\pm$ 0.95, 98.83% $\pm$ 0.73, 98.11% $\pm$ 1.14; groups 1, 2, 3 and 4 respectively - $P>0.05$). In contrast, continuous exposure to cigarette smoke (group 2) significantly decreased BD in the cancellous bone when compared to the other groups (25.69% $\pm$ 9.41, 18.08% $\pm$ 6.07, 25.46% $\pm$ 5.42, 26.20% $\pm$ 6.77; groups 1, 2, 3 and 4 respectively - $P<0.05$), with no significant difference between groups 1, 3 and 4 ($P>0.05$).

Discussion: This study suggests that smokers may present a satisfactory outcome if they were appropriately approached.

Conclusion: In conclusion, smoking may affect bone quality around titanium implants in cancellous bone, and cessation could result in a return toward to the levels of the control group.

KEY WORDS: smoking, cessation osseointegration, dental implants.

INTRODUCTION

Over the past 20 years endosseous titanium implants placed under various modifications of the original Bränemark protocol, have proven to be among the most predictable treatments in oral health care. Success rates in excess of 95% up to 15 years and beyond compare favorably with other methods of tooth replacement. Quality of life assessments comparing implant supported prostheses with removable partial and complete dentures have shown implant retained prosthesis to be a highly satisfactory method of tooth replacement[1-2]. However, some systemic conditions have been correlated with higher rates of failure[3]. Smoking is one of the factors often discussed in relation to implant failure[2,4-8]. 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[11-13], increased resorption of the alveolar ridge[11], higher incidence of periodontitis[14] and type IV bone[15].

Several studies have provided evidence that the impact of tobacco smoking on oral structures may be reversible. In a 10-year study, Bolin et al.[10] showed that the progression of bone loss was significantly retarded in individuals who had given up smoking. Liede et al.[16] compared periodontal status, salivary proteolytic activity, and oral mucosal status in individuals who had quit smoking to regular smokers, and found that periodontal status and mucosal health were better in those who had quit smoking. Gingival microcirculation has also been shown to recover its normal blood flow in the early stages of smoking cessation[17], and that changes in the inflammatory response of the periodontium can also be reversible upon smoking cessation[18]. Additionally, former smokers have been reported to present periodontal bone height reduction rates similar to non-smokers[13], and therefore to lose significantly less marginal bone in a period over 20 years than individuals who declared to be smokers during the same period[12].

Unfortunately, there is much more evidence of the detrimental effect of smoking on implant outcomes than there is on the potential benefit of stopping smoking. In one study, Bain[19] clinically examined a cessation protocol in which potential implant patients who smoked were encouraged to stop for 1 week before and 8 weeks after implant placement. Based on a prospective study of 223 consecutive Bränemark system implants placed in 78 patients, the authors found no difference in failure rate between non-smoking controls and the smokers who quit, whereas a significant difference was noted between the continuing smokers and smokers who followed the cessation protocol.

Therefore, because only very limited studies are available in addition to the clinical relevance of this subject, the present study aimed to provide additional information on whether smoking cessation during the healing phase would impact on bone density around titanium implants, as well as whether complete rather than temporary cessation would be required to achieve bone quality similar to the non-smoking group.

MATERIALS AND METHODS

ANIMALS

Sixty-nine male Wistar rats (300-400g) were included in the study. The animals were kept in plastic cages with access to food and water *ad libitum*. Prior to the surgical procedures all animals were allowed to acclimatize to the laboratory environment for a period of 5 days. The protocol was approved by the University of Campinas Institutional Animal Care and Use Committee.

IMPLANT SURGERY

General anesthesia was obtained by intramuscular administration of ketamine (0.5ml/kg). Skin was cleansed with iodine surgical soap. An incision of approximately 1 cm in length was made and the bone surface of the tibiae surgically exposed by blunt dissection. Under profuse saline irrigation bicortical implant beds were drilled at a rotary speed not exceeding 1,500 rpm. One screw-type commercially available pure titanium implant, of 4.0mm in length and 2.2mm in diameter, was placed until the screw thread had been completely introduced into the bone cortex. Finally, soft tissues were replaced and sutured over the implant (cover screw was used). Postoperatively, the animals received an antibiotic (1ml/Kg - Pentabiótico®, Wyeth-Whitehall Ltda, São Paulo, SP, Brazil) given through a single intramuscular injection.

EXPERIMENTAL DESIGN (Figure 1)

Ninety days before implant surgery, the animals had been randomly assigned to one of the following groups: Group 1 – control (n=16), Group 2 **–** intermittent cigarette smoke inhalation CSI (n=17), Group 3 – CSI 83 days prior to implant placement (n=16), and Group 4 – CSI for 83 days, interruption 1 week before and 3 weeks after implant placement, and return to CSI for 39 more days (n=20). The animals of groups 2, 3, and 4 were intermittently housed in an animal cigarette smoke exposure chamber as previously described[20-21]. Briefly, the device consisted of a 45 X 25 X 20 cm^3 clear acrylic resin chamber, an air-pump and two inflow/outflow tubes (Figure 2). Five animals were housed in the chamber at the same time, and the cigarette smoke of 10 cigarettes, containing 1.3 mg

of nicotine, 16.5mg of tar, and 15.2mg of carbon monoxide each, was pumped into the chamber. Thus, the animals were forced to breathe the cigarette smoke that contaminated the air for 8 minutes, three times daily until they were sacrificed (60 days after implant placement). The animals of group 1 were not exposed to the cigarette smoke at any time. The serum levels of nicotine and cotinine obtained by using this model have been previously reported[22].

HISTOMETRIC PROCEDURE

Sixty days after implant placement, the animals were sacrificed; the tibiae were removed and fixed in 4% neutral formalin for 48 hours. Undecalcified sections were prepared as previously described[23], i.e. the blocks were dehydrated by using an ascending series of ethanol (60-100%) and embedded in glycolmethacrylate resin (Technovit 7200®; Heraeus Kulzer GmbH, Wehrheim, Germany). Subsequently, the sections (20-30 µm) were obtained and stained by using toluidine blue 1% staining. Bone density (i.e. the proportion of mineralized bone in a 500µm-wide zone lateral to the implant - BD) was obtained (Image-Pro®; Media Cybernetics, Silver Spring, MD, USA) bilaterally in the cortical (Zone A) and cancellous bone (Zone B) areas by a blinded examiner.

STATISTICAL ANALYSIS

The data from Zones A and B (cortical and cancellous bone, respectively) were separately averaged. The null hypothesis, i.e., BD was neither influenced by CSI nor by the cessation protocols, was tested by an intergroup analysis using the parametric one-way ANOVA test (alpha = 0.05), regarding Zones A and B separately (Group 1 vs Group 2 vs Group 3 vs Group 4). If statistical difference was detected by the one-way ANOVA test, a pair wise multiple comparison procedure was performed by the Tukey test (alpha = 0.05) to detect the differences between the groups.

RESULTS

HISTOMETRIC ANALYSIS

Although a slight difference was observed, the intergroup analysis (one-way ANOVA) did not reveal significant differences between the groups with respect to the bone density (BD) at the cortical bone area - Zone A (97.66% $\pm$ 3.69, 98.30% $\pm$ 0.95, 98.83% $\pm$ 0.73, 98.11% $\pm$ 1.14; for groups 1, 2, 3 and 4 respectively - P=0.38). In contrast, the intergroup analysis (one-way ANOVA) showed a significant difference among the groups (25.69% $\pm$ 9.41, 18.08% $\pm$ 6.07, 25.46% $\pm$ 5.42, 26.20% $\pm$ 6.77; groups 1, 2, 3 and 4 respectively - P=0.002). The pair wise comparison (Tukey test) indicated that continuous exposure to cigarette smoke (group 2) significantly decreased BD in the cancellous

bone ($P<0.05$) when compared to the other groups. Moreover, the pair wise comparison (Tukey test) showed no significant difference between groups 1, 3 and 4 ($P>0.05$) (Figure 3). Figure 4A-D illustrates the histologic results for the experimental groups.

DISCUSSION

The present investigation histologically evaluated the impact of intermittent cigarette smoke inhalation (CSI) on bone healing around titanium implants placed in the tibiae of rats (e.g. proportion of mineralized tissue in a 500µm-wide zone lateral to the implant surface - BD), and also sought to determine whether two different CSI cessation protocols would prevent the impact of CSI on bone. Data analysis demonstrated that the cortical bone (Zone A) was not significantly affected either by CSI or by any of the cessation protocols. On the other hand, CSI significantly reduced BD in the cancellous bone (Zone B). Additionally, definitive or temporary cessation protocols were both able to revert the CSI effect on BD.

During the early phase of implant procedure development, implant failure was generally attributed to poor surgical technique (infection, overheating of bone and over-instrumentation), poor prosthetic design or management, or patient-related factors (limited available bone, poor oral hygiene and occlusal overload). These findings were largely based on clinical observation, extrapolation from failures of tooth-supported prostheses and dogma. Jones and Triplett[24] evaluated the influence of smoking on wound healing in patients undergoing intraoral bone grafting and simultaneous implant placement, and may have been among the first to implicate smoking as a potentially risk factor. Smoking has been now one of the factors often discussed in relation to decreased success rates of dental implants. Bain and Moy[5] assessed various factors predisposing to implant failure in a group of 540 patients who had received 2194 implants. They found that smoking was by far the most significant factor: failure rates were 4.76% in non-smokers and 11.28% in smokers. In a later study, de Bruyn and Collaert[4] compared implant failures before loading in the maxillae of smokers and non-smokers. They found that at least one failure was detected in one in three smokers, compared with only one in 25 non-smokers (9% and 1%, respectively). Gorman et al.[6] evaluated the relationship between smoking and the failure rates of dental implants at second-stage surgery. They suggested that smoking is detrimental to implant success. Haas et al.[7] have also suggested that smokers suffer harmful effects around successfully integrated maxillary implants. Lindquist et al.[2] investigated the influence of smoking and other possibly relevant factors on bone loss around mandibular implants.

They demonstrated that smoking was the most important factor affecting the rate of peri-implant bone loss. Esposito et al.[3] reviewed the literature regarding factors associated with the loss of oral implants and concluded that smoking habit was one of the factors associated with biologic failure of the implants.

Recently, Lambert et al.[8] reported long-term clinical outcomes of dental implants placed in smokers and non-smokers in a longitudinal clinical study. The authors concluded that smoking promoted an increased implant failure rate. In addition to the clinical reports a series of studies has tried to document, at an histologic level, the influence of cigarette consumption and/or its compounds on bone healing around titanium implants. Stefani et al.[25] investigated the effect of nicotine administration on the osseointegration process around dental implants. A slight negative effect of nicotine on the bone-to-implant contact around implants with machined surfaces was observed, although this difference was not statistically significant. Nociti et al.[21] demonstrated that although cigarette smoke exposure may not seriously affect cortical bone, it may jeopardize bone quality around titanium implants in the cancellous bone area. César-neto et al.[22] investigated the impact of two conditions, i.e., nicotine administration and cigarette smoke inhalation, on the healing around implants, and concluded that the negative impact of smoking on implant outcomes may be related to more than one molecule present in the cigarette smoke and nicotine seems to partially contribute, especially in cancellous bone.

Reversibility of the effects of cigarette consumption has been studied both in medicine and dentistry. For lung disease, one of the most frequent pathologies associated with cigarette consumption, a former smoker is considered to run the same risk as a non-smoker 15 years after smoking cessation[26]. In addition, it has been shown that a current smoking habit had a stronger effect on mean total white blood cell counts (WBC) than cumulative exposure[27]. The effects of smoking on WBC demonstrated an almost immediate reduction after smoking cessation.

In dentistry, smoking cessation has also been shown to positively impact periodontal risk. In vitro studies[28-29] have suggested a reversible cytotoxic effect of cigarette compounds (i.e. Nicotine, acrolein and acetaldehyde) on periodontal cells. The relative risk was reported to be 3.97 for smokers and 1.68 for former smokers[30]. In addition, among former smokers, the risk decreased with the number of years since quitting (3.22 after 2 years and to 1.15 after 11 years). In a prospective study over 20 years[12], 507 individuals were radiographically evaluated, and the results showed that the

ones who stopped smoking during the experimental period lost significantly less marginal bone when compared to current smokers. Another longitudinal study[31] evaluated the changes on the periodontal status of 101 patients during 10 years. Clinically, an increased frequency of diseased sites in smokers was seen, while former and non-smokers presented decreased and similar frequencies. Radiographically, an increased bone loss for current smokers was noted when compared to former and non-smokers. No significant differences were observed between former and non-smokers. Moreover, smoking cessation has also been reported to be beneficial for periodontal treatment outcome.

Grossi et al.[32] demonstrated that former and non-smokers presented significantly more healing and reduction of *Bacteroides forsythus* and *Porphyromonas gingivalis* than current smokers. Therefore, the results of the present study appear to agree with previous reports showing a reversible condition promoted by cigarette consumption. In the implant field, very limited information is available with respect to the reversibility of the effects of smoking on implant outcomes. Bain[19] was the first to report that a smoking cessation protocol would improve success rates for osseointegration in smokers who follow it. Thus, the results of the present study support the concept that the effects of cigarette consumption on dental implants may be reversible, and therefore, suggest that smokers may realize a satisfactory outcome if they were appropriately approached.

Misclassification of smoking status has been a concern in the literature[33], and is considered a confounder in epidemiologic studies. Inaccurate reports may occur for many reasons such as individual metabolism, frequency of inhalation, depth of inhalation, capacity for dilution with room air, amount of cigarette stub left and cigarette brand[28]. Biochemical validation of smoking status seems to be useful in order to minimize the influence of confounders in clinical studies, mainly for the determination of light, regular and heavy smokers. In animal studies such confounders may be more accurately controlled. It has been previously reported[22] that the CSI regimen used in the present study promoted cotinine serum levels closely correlated with smokers that smoke between 10 to 20 cigarettes/day[34]. However, future comparisons with humans should be treated with caution, because of differences in the metabolism of nicotine between humans and rats, and the frequency of smoke administration used in this study.

CONCLUSION

Within the limits of the present investigation, it can be concluded that smoking effects on bone around titanium implants may be reversible, and that a temporary smoking cessation protocol may be as beneficial as a definitive one.

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Figure 1: Schematic illustration of the experimental design.

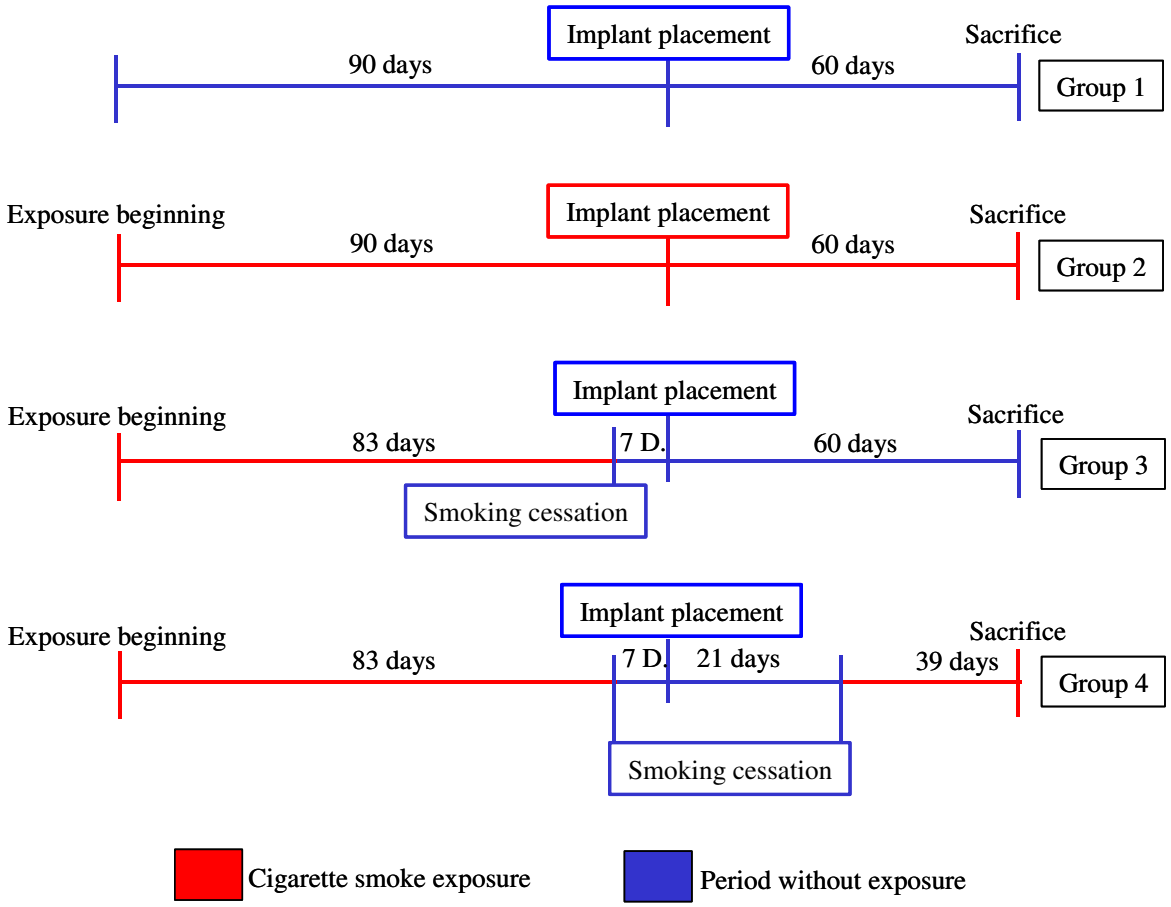


Figure 2: Schematic illustration of the cigarette smoke exposure device. The acrylic resin chamber was composed of two subchambers: the cigarette compartment (A) and the animal compartment (B).

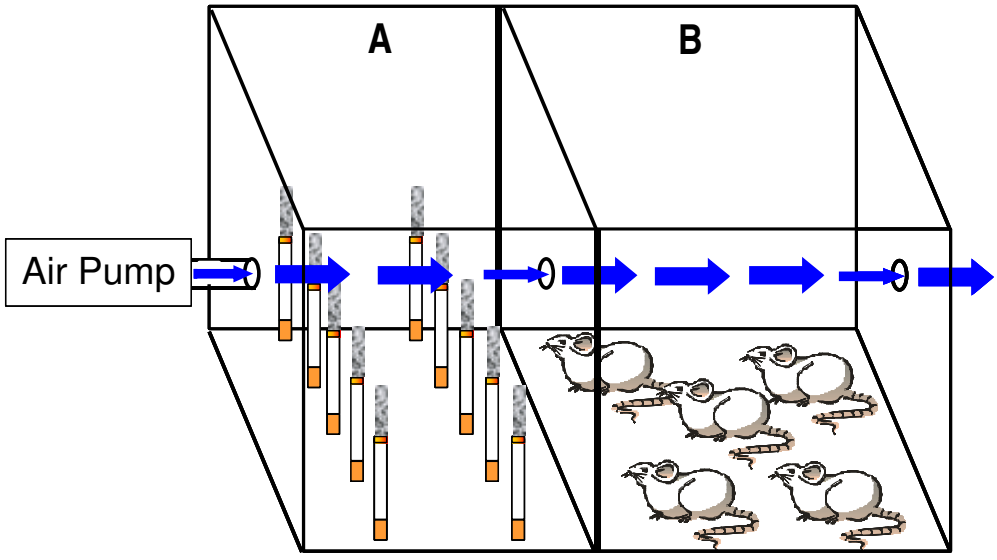
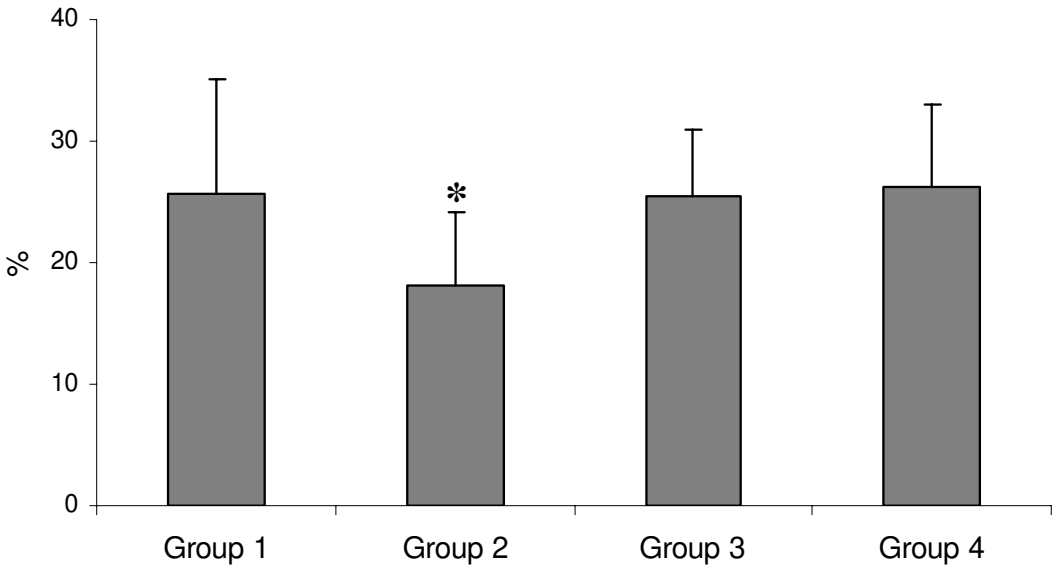
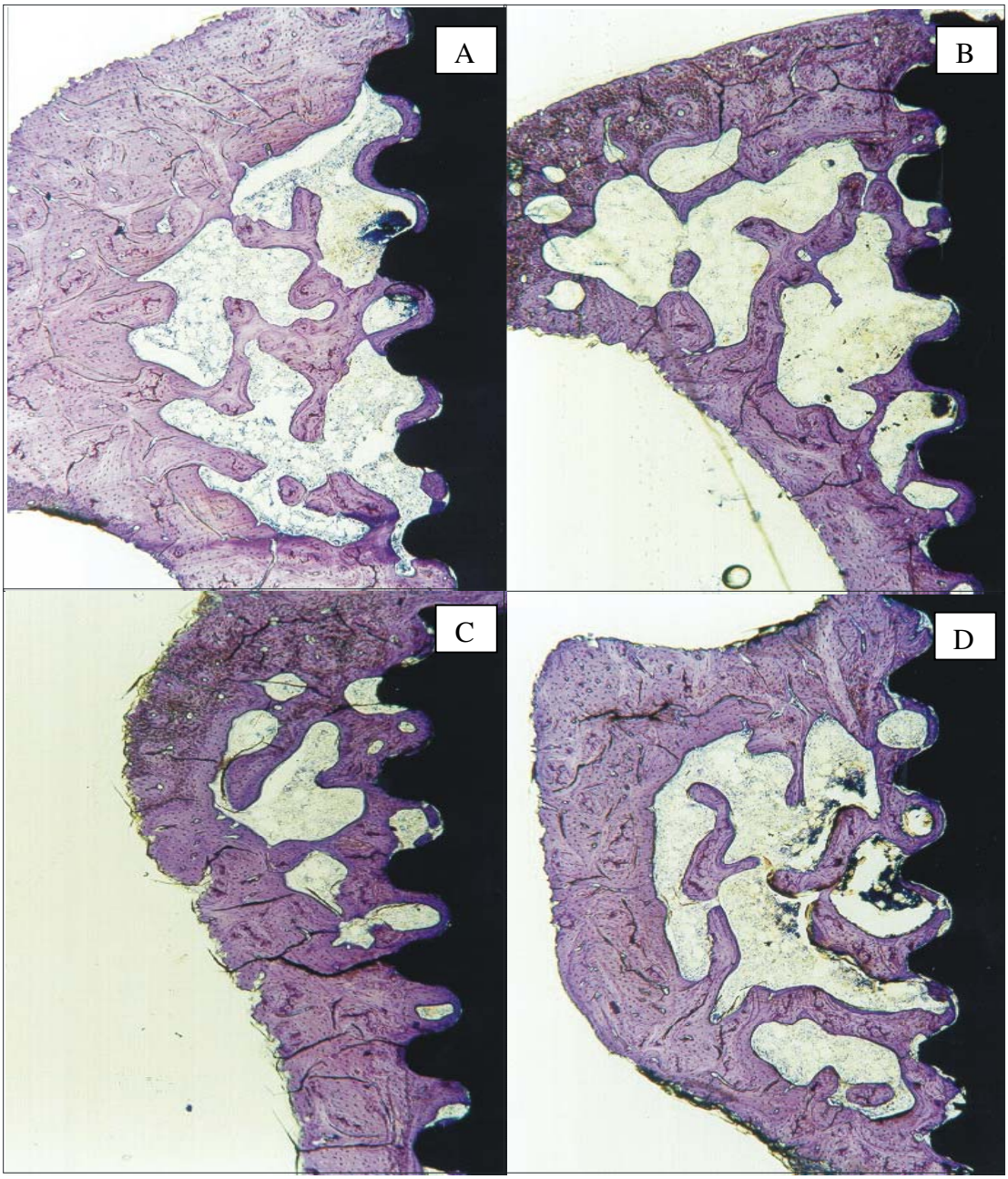


Figure 3: Mean and standard deviation (%) of bone density around the implants for Group 1 – control, Group 2 **–** intermittent cigarette smoke inhalation (CSI), Group 3 – CSI 83 days prior to implant placement, and Group 4 – CSI for 83 days, interruption 1 week before and 3 weeks after implant placement, and return to CSI for 39 more days at Zones B.



* Statistically significant – Intergroup analysis showing lower bone density for group 2 compared to groups 1, 3, and 4 (one-way ANOVA – P=0.002).

Figure 4: Photomicrographs 4A to 4D illustrate the histological aspects observed in a 500μm-wide zone lateral to the implant surface in Groups 1 (control) **(A)**, 2 (intermittent cigarette smoke inhalation (CSI)) **(B)**, 3 (CSI 83 days prior to implant placement) **(C)**, and 4 (interruption 1 week before and 3 weeks after implant placement, and return to CSI for 39 more days) **(D)** (Toluidine blue / Original magnification = 6.25x).



3.4 Capítulo 4

BONE FILLING AROUND TITANIUM IMPLANTS MAY BENEFIT FROM SMOKING CESSATION. A HISTOLOGIC STUDY IN RATS

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ABSTRACT

Background: A harmful effect of smoking on titanium implants has been documented. However, only a limited number of studies investigated the influence of smoking cessation on implants outcome. Thus, the aim of this study was to investigate, at the histological level, whether i) smoking cessation influences bone healing around titanium implants, and ii) comparatively evaluate temporary versus complete cessation protocols.

Material and Methods: Sixty-six male Wistar rats were assigned to: Group 1 – control (n=16), Group 2 **–** intermittent cigarette smoke inhalation (CSI) 90 days prior and 60 days after implant placement (n=17), Group 3 – CSI 83 days prior to implant placement (n=17), and Group 4 – CSI for 83 days, cessation 1 week before and 3 weeks after implant placement, and return to CSI for 39 days (n=16). The animals were sacrificed 60 days after implant placement, and the degree of bone-to-implant contact (BIC) and the bone area (BA) within the limits of the threads of the implants were obtained in the cortical (Zone A) and cancellous bone (Zone B) areas.

Results: Data analysis demonstrated a significant effect of CSI on BA, and that either temporary or complete CSI cessation protocols resulted in values similar to the control group **(**88.9% $\pm$ 4.29, 80.66% $\pm$ 6.55, 84.27% $\pm$ 6.96, 85.71% $\pm$ 4.7 in zone A, and 51.28% $\pm$ 6.49, 38.69% $\pm$ 10.78, 48.87% $\pm$ 8.47, 49.47% $\pm$ 8.04; in zone B for groups 1, 2, 3 and 4 respectively). Additionally, a slight, but not statistically significant, effect of both protocols was noted on BIC (55.34% $\pm$ 14.57, 47.83% $\pm$

11.36, 51.09% ± 11.97, 47.76% ± 12.43 in zone A, and 42.69% ± 10.78, 33.21% ± 12.51, 39.67% ± 11.96, 37.46% ± 10.28; in zone B for groups 1, 2, 3 and 4 respectively).

Conclusion: Within the limits of the present investigation, both temporary and complete CSI cessation protocols positively impacted on the bone healing around titanium implants in both cortical and cancellous bone.

KEY WORDS: cigarette smoke, smoking cessation, osseointegration, dental implants.

INTRODUCTION

Titanium implants have been considered a predictable method of oral rehabilitation with success rates higher than 95% up to 15 years[1]. However, some conditions have been correlated with higher failure rates[2]. Among the systemic factors related to implant failure, smoking is one of the most discussed[2-8], and clinical studies have reported that smokers present not only higher rates of implant failure than non-smokers [4-6, 8], but also a greater detrimental effect around successfully integrated implants[3,7]. In addition, a series of histological studies has suggested a negative influence of smoking on bone healing around titanium implants inserted in rats[9-12].

In the medical field, evidences were provided on the beneficial effect that smoking cessation presents on bone density[13-16]. In dentistry, several studies have suggested that the impact of tobacco smoking on the oral structures may also be reversible. In a 10-year study, Bolin et al.[17] showed that the progression of bone loss was significantly retarded in individuals who had given up smoking. Liede et al.[18] compared periodontal status, salivary proteolytic activity, and oral mucosal status in individuals who had quit smoking to regular smokers; and found that periodontal status and mucosal health were better in those who quit smoking. Gingival microcirculation has also been shown to recover its normal functions on the early stages of smoking cessation[19], and that changes in the inflammatory response of the periodontium can be reversible on quitting smoking[20].

Although highly recommended in the dental implant field, the most obvious remedy to the adverse effects of cigarette consumption, smoking cessation, has not been extensively explored. In a prospective study of 223 consecutive Bränemark system implants placed in 78 patients, Bain et al.[21] found no difference in the failure rate between non-smoking controls and the smokers who quit, whereas a significant difference was noted between the continuing smokers and smokers who followed the cessation protocol. Thus, based on the clinical relevance of this subject, and the limited number of available studies, the present study aimed to histologically investigate in rats i), whether

smoking cessation during the healing phase would impact on bone formation around titanium implants, and ii) whether a complete rather than a temporary cessation protocol would be required in order to achieve bone profiles similar to the non-smoking group.

MATERIALS AND METHODS

ANIMALS

Sixty-six male Wistar rats (300-400g) were included in the study. The animals were kept in plastic cages with access to food and water *ad libitum*. Prior to the surgical procedures all animals were allowed to acclimatize to the laboratory environment for a period of 5 days. The protocol was approved by the University of Campinas Institutional Animal Care and Use Committee.

IMPLANTS SURGERY

General anesthesia was obtained by intramuscular administration of ketamine (0.5ml/kg). Skin was cleansed with iodine surgical soap. An incision of approximately 1 cm in length was made and the bone surface of the tibiae surgically exposed by blunt dissection. Under profuse saline irrigation bicortical implant beds were drilled at a rotary speed not exceeding 1,500rpm. One screw-shaped commercially available pure titanium implant, of 4.0mm in length and 2.2mm in diameter, was placed until the screw thread had been completely introduced into the bone cortex. Finally, soft tissues were replaced and sutured. Postoperatively, the animals received antibiotic[ϕ] (1ml/Kg), given through a single intramuscular injection.

EXPERIMENTAL DESIGN (Figure 1)

Ninety days before implant surgeries, the animals were randomly assigned to one of the following groups: Group 1 – control (n=16), Group 2 **–** 90 days of intermittent cigarette smoke inhalation (CSI) prior and 60 days after implant placement (n=17), Group 3 – CSI 83 days prior to implant placement (n=17), and Group 4 – CSI for 83 days, interruption 1 week before and 3 weeks after implant placement, and return to CSI for 39 more days (n=16). The animals of groups 2, 3, and 4 were intermittently housed in an animal cigarette smoke exposure chamber as previously described[10, 11]. Briefly, the device consisted of a 45 X 25 X 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, containing 1.3 mg of nicotine, 16.5mg of tar, and 15.2mg of carbon monoxide each, was pumped into the chamber. Thus, the animals were forced to breathe the

[ϕ] Pentabiótico®, Wyeth-Whitehall Ltda, São Paulo, Brazil)

cigarette smoke that contaminated the air for 8 minutes, three times daily until they were sacrificed (60 days after implant placement). The animals of group 1 were not exposed to the cigarette smoke at any time. The serum levels of nicotine and cotinine obtained by using this model has been previously reported[12].

HISTOMETRIC PROCEDURE

After the sacrifice (60 days after implant placement), the tibiae were removed and fixed in 4% neutral formalin for 48 hours. Undecalcified sections were prepared as previously described[22], i.e. the blocks were dehydrated by using an ascending series of ethanol (60-100%) and embedded in glycolmethacrylate‡. Subsequently, the sections (20-30 μm) were obtained and stained by using toluidine blue 1% staining. The percentage of bone-to-implant contact (BIC) and bone area (BA) within the threads of the implants were obtained by a blinded examiner##. The data were arranged separately in cortical (Zone A) and cancellous bone (Zone B) areas, as previously described [10-11].

STATISTICAL ANALYSIS

Data from Zones A and B (cortical and cancellous bone, respectively) were separately averaged. The null hypothesis, i.e., BIC and BA were neither influenced by CSI nor by the cessation protocols in zones A and B separately, was tested by an intergroup analysis using the non-parametric Kruskal-Wallis test (alpha = 0.05) (Group 1 vs Group 2 vs Group 3 vs Group 4). Pairwise multiple comparisons were carried out by Dunn`s test (alpha = 0.05) in case that Kruskal-Wallis test showed significant differences.

RESULTS

HISTOMETRIC RESULTS

Although a slight difference was observed, statistical analysis did not reveal significant differences among the groups with respect to bone-to-implant contact (BIC) either in cortical or cancellous bone (P>0.05) (55.34% $\pm$ 14.57, 47.83% $\pm$ 11.36, 51.09% $\pm$ 11.97, 47.76% $\pm$ 12.43 in zone A, and 42.69% $\pm$ 10.78, 33.21% $\pm$ 12.51, 39.67% $\pm$ 11.96, 37.46% $\pm$ 10.28 in zone B; for groups 1, 2, 3 and 4 respectively - P>0.05). In contrast, continuous exposure to cigarette smoke (group 2) significantly decreased BA when compared to the control group (group 1) in both cortical and cancellous bone. In addition, no differences were observed, with respect to BA, between groups

‡ Technovit 7200® ; Heraeus Kulzer GmbH, Wehrheim, Germany

Image-Pro®; Media Cybernetics, Silver Spring , MD, USA

3 and 4 (cessation groups) and the control group (P>0.05) (88.9% ± 4.29, 80.66% ± 6.55, 84.27% ± 6.96, 85.71% ± 4.7 in zone A, and 51.28% ± 6.49, 38.69% ± 10.78, 48.87% ± 8.47, 49.47% ± 8.04 in zone B, for groups 1, 2, 3 and 4 respectively). Figures 2 to 4 histologically and graphically illustrate the results for each experimental group.

DISCUSSION

The present investigation histologically evaluated whether smoking cessation would affect bone healing around titanium implants placed in animals intermittently exposed to cigarette smoke, and compared the possible benefits that temporary versus complete cessation protocols would exert on the new-formed bone. The results of the present study confirmed that CSI might significantly affect bone volume in the cortical and cancellous bone around the implant. Additionally, data analysis demonstrated that both temporary and complete cessation protocols positively impacted on the new-formed bone, resulting in BA values similar to the non-smoking control group.

During the early time of implant procedure development, implant failure was generally attributed to poor surgical technique (infection, overheating of bone and over-instrumentation), poor prosthetic design or management, or patient-related factors (limited available bone, poor oral hygiene and occlusal overload). These findings were largely based on clinical observation, extrapolation from failures in tooth-supported prostheses and dogma. However, evidences began to be available correlating systemic conditions with higher failure rates. Esposito et al.[2] reviewed the literature regarding factors associated with the loss of oral implants, and concluded that smoking habit was one of the strongest factors associated with biologic failures of implants. Several other clinical studies have provided additional evidence that smokers present higher rates of implant failure [4-6, 8], as well as suffer detrimental effects around successfully integrated implants[3, 7]. More recently, a series of studies have suggested, at the histologic level, that cigarette smoke and its compounds may affect bone volume around titanium implants, and therefore take part in the process by which smoking negatively affect implant outcome. Stefani et al.[9] observed a slight negative effect of nicotine on the bone-to-implant contact around implants with machined surfaces, although this difference was not statistically significant. Nociti et al.[10] demonstrated that cigarette smoke exposure may jeopardize bone quality around titanium implants in the cancellous bone area. Finally, César-Neto et al.[12] comparatively investigated the impact of nicotine administration or cigarette smoke inhalation, on the healing around implants; and found that the negative impact of smoking on implant outcomes may be

related to more than one molecule present in the cigarette smoke and nicotine seems to partially contribute. Therefore, the findings by the present study that CSI affects bone around titanium implants reproduce and confirm previous reports.

In the implant field, very limited information is available with respect to the reversibility of the effects of smoking on implants outcome. Bain[21] was the first to report that a smoking cessation protocol would improve implant success rates in smokers who follow it. A similar figure is observed in medicine with respect to several tissues. A meta-analysis study demonstrated that current smokers presented a significantly reduced bone mass when compared to former and never smokers[14] and, that former smokers presented bone mass that is intermediate or similar to never smokers[13,14]. They additionally reported that smoking has an independent, dose-dependent effect on bone loss, which increases fracture risk, and that smoking cessation may present a beneficial effect[14]. Regarding bone healing, it was observed that patients who quit smoking, for periods longer than 6 months, after instrumented spinal fusion presented nonunion rates similar to nonsmokers[23]. For lung disease, one of the most often cigarette-related pathologies, a former smoker is considered to run the same risk as a non-smoker 15 years after smoking cessation[24]. In addition, it was reported that cigarette consumption negatively affects white blood cell counts, and such effect is promptly reverted after smoking cessation[25]. 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 periodontal cells[26,27]. Smoking cessation also exerted a beneficial effect on periodontal risk, which decreased with number of years since quitting[28]. Longitudinal studies showed that patients who stopped smoking lost significantly less marginal bone than current smokers[18,29]. Additionally, smoking cessation has also been considered beneficial to periodontal therapy, presenting more healing and reduction of *Bacterioides forsythus* and *Phorphyromonas gingivalis*[30]. The results of the present investigation are in agreement with the studies that showed a reversible condition promoted by cigarette consumption, and support the concept that the effect of cigarette consumption on dental implants may be reversible.

Misclassification of smoking status has been a concern in the literature[31], and is considered a confounder in clinical studies. Therefore, biochemical validation of smoking status seems to be useful in order to minimize the influence of such confounders. In this sense, a previous study[12] has reported that a CSI regimen similar to the one used by the present study resulted in cotinine serum levels

closely correlated with smokers that smoke between 10 to 20 cigarettes/day[32]. However, future comparisons with humans should be treated with caution, because of differences in the metabolism of nicotine between humans and rats, and the frequency of smoke administration used in this study. Moreover, additional studies should be considered in order to investigate whether the decreased bone volume around implants, promoted by cigarette consumption, would be clinically relevant. If such relationship was confirmed, advisement about smoking cessation should be considered as part of the implant patient approach. Within the limits of the present investigation, both temporary and complete CSI cessation protocols positively impacted on bone healing around titanium implants, in both cortical and cancellous bone. Therefore, a short-term cessation protocol during the healing phase may result in bone filling of the threads similar to non-exposed animals. However, further studies should be considered in order to determine whether the return to a smoking condition would impact on the bone around the implant in a long-term basis.

Acknowledgment

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Figure 1 – Schematic illustration of the experimental design.

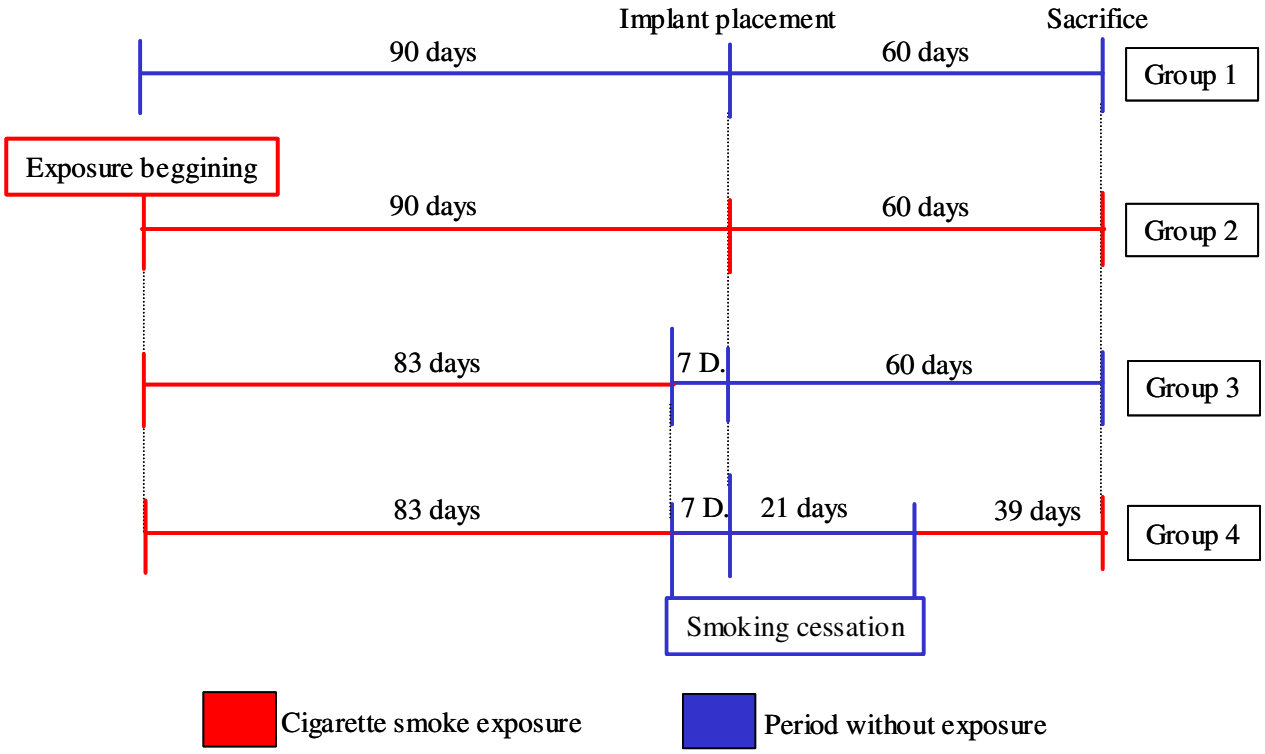


Figure 2: Photomicrographs 2A to 2D illustrate the histological aspect observed around the implants for groups 1, 2, 3 and 4, respectively. Toluidine blue / Original magnification = 6.25x - Bar=0.5mm.

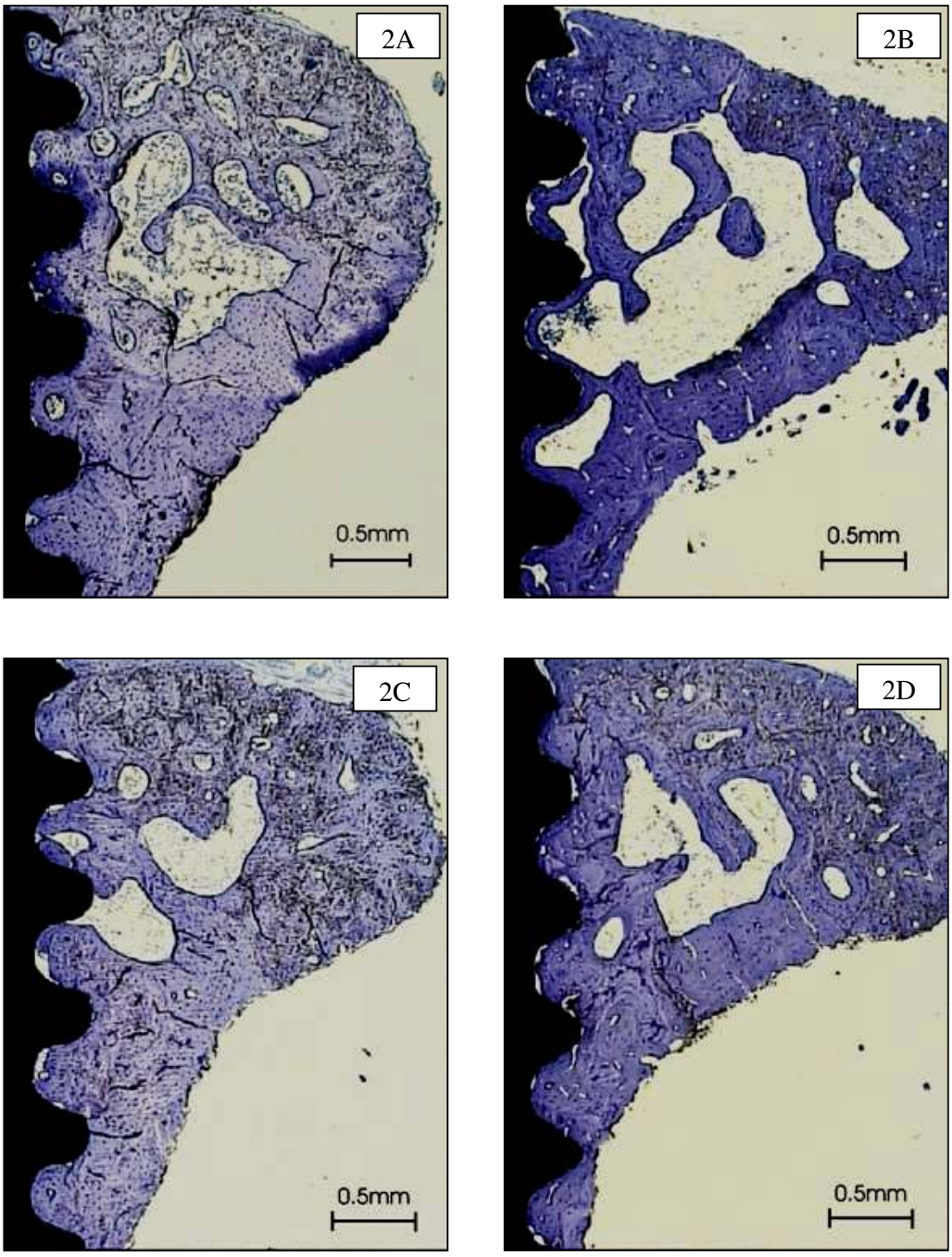


Figure 3: Mean and standard deviation (%) representative of bone-to-implant contact, in the cancellous and cortical bone, for groups 1, 2, 3 and 4.

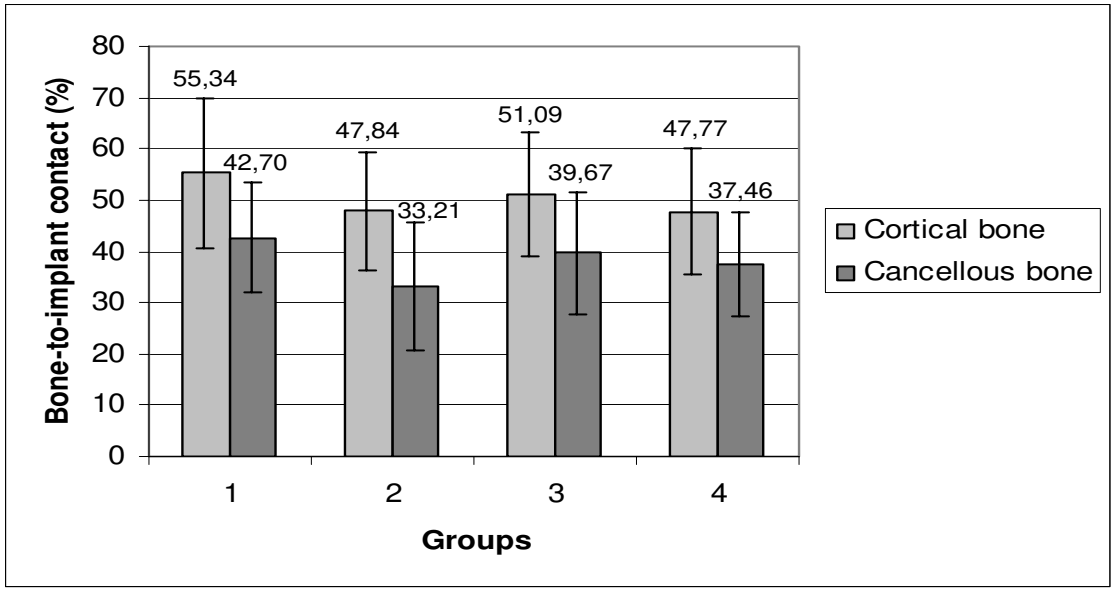
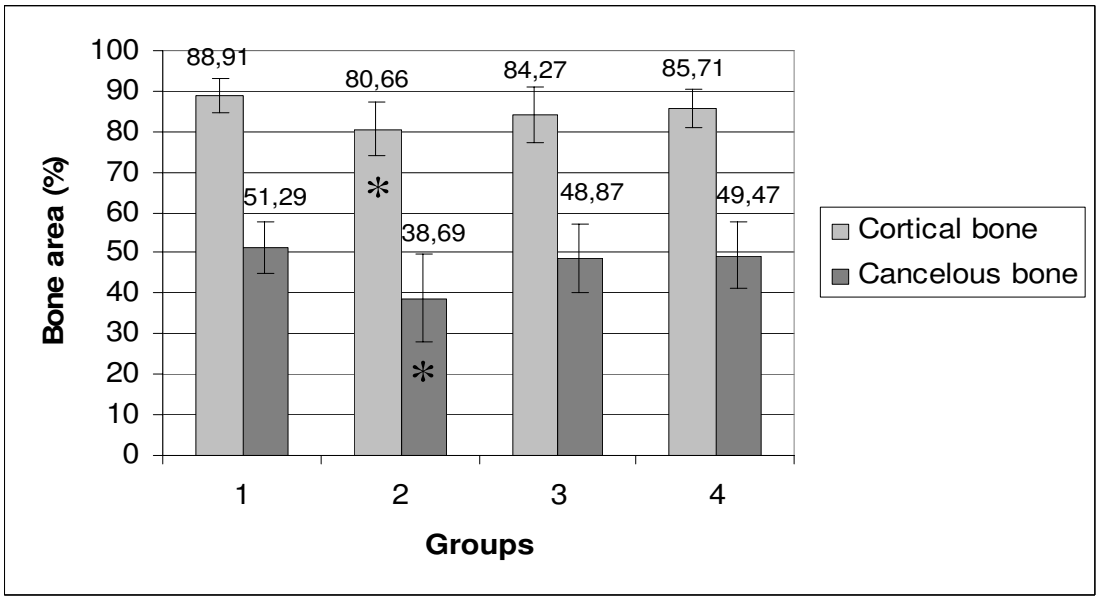


Figure 4: Mean and standard deviation (%) representative of bone area within the limits of the threads of the implant, in the cancellous and cortical bone, for groups 1, 2, 3 and 4.



*** Statistically significant – Intergroup analysis by Kruskal-Wallis test - $\alpha = 0.05$**

3.5 Capítulo 5

THE INFLUENCE OF CIGARETTE SMOKE INHALATION ON BONE DENSITY. A RADIOGRAPHIC STUDY IN RATS.

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ABSTRACT

The aim of this study was to evaluate the influence of cigarette smoke inhalation (CSI) and its cessation on tibiae bone quality. Forty-one male Wistar rats were randomly assigned to one of the following groups: Group 1 – control (n=14), Group 2 **–** 3 months of CSI and 2 months without exposure to CSI (n=12), and Group 3 – 5 months of CSI (n=15). At the end of the experimental period the animals were sacrificed, the tibiae removed and immediately radiographed for photodensitometric analysis. The results showed that continuous exposure to cigarette smoke promoted a significantly reduced bone density ($P<0.05$) (3.22 Al eq $\pm$ 0.58; 2.93 Al eq $\pm$ 0.45; 1.86 Al eq $\pm$ 0.35**;** for groups 1, 2 and 3, respectively). Similar levels of bone density were observed for the control and cessation groups (groups 1 and 2 - $p>0.05$). Thus, within the limits of the present study, it can be concluded that smoking may affect tibiae bone quality, and CSI cessation results in a return towards the level of the control group.

DESCRIPTORS: smoking, bone density, rats.

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RESUMO

O objetivo deste estudo foi avaliar a influência da inalação da fumaça de cigarro (IFC) e o efeito de sua interrupção na qualidade óssea da tíbia. Quarenta e um ratos Wistar foram aleatoriamente designados a um dos seguintes grupos: Grupo 1 – controle (n=14), Grupo 2 **–** 3 meses de IFC e 2 meses sem exposição a fumaça (n=12) e Grupo 3 – 5 meses de IFC (n=15). Ao final do período experimental os animais foram sacrificados, as tíbias removidas e imediatamente radiografadas para a análise fotodensitométrica. Os resultados mostraram que a exposição contínua a fumaça de cigarro promoveu uma significante redução na densidade óssea (P<0,05) (3,22 Al eq $\pm$ 0,58; 2,93 Al eq $\pm$ 0,45; 1,86 Al eq $\pm$ 0,35**;** para os grupos 1, 2 e 3, respectivamente). Níveis semelhantes de densidade óssea foram observados nos grupos controle e interrupção (grupos 1 e 2 - p>0.05). Portanto, dentro dos limites do presente estudo, pode-se concluir que a IFC pode influenciar a qualidade óssea, e que a interrupção da inalação parece reverter esse efeito negativo resultando numa densidade óssea semelhante a do grupo controle.

Descritores: tabagismo, densidade óssea, ratos.

INTRODUCTION

Over the past 20 years, endosseous titanium implants have proven to be amongst the most predictable treatments in oral health care. Quality of life assessments show implant-retained prosthesis to be a highly satisfactory method of tooth replacement[1,18]. However, some systemic conditions have been correlated with higher rates of failure[8]. Among these conditions, smoking is one of the factors most discussed in relation to implant failure[3,4,6,8,18].

Studies have provided evidences that the impact of tobacco smoking on tissues may be reversible. Liede et al.[17] observed that periodontal status and mucosal health were better in individuals who had quit smoking when compared to current smokers. Gingival microcirculation has also been shown to recover its normal function on the early stages of smoking cessation[19], and the changes in the inflammatory response of the periodontium can also be reversible on quitting smoking[20]. Bain et al.[4] clinically examined the influence of smoking cessation on implant outcome, and did not find differences in failure rate between non-smoking controls and the smokers who had quit.

The reason of a higher rate of implant failure in smokers is not fully elucidated. One possibility may be a harmful effect of smoking on bone density, since poor bone quality is a factor

associated with implant failure[12]. A clinical study has already suggested that a higher incidence of poor bone quality is observed in smokers[3]. Although most of the animal studies did not report a correlation between nicotine administration and lower bone density[2, 9, 24, 25], nicotine is just one of the potentially toxic compounds of cigarette smoke. Still, the number of studies evaluating the effect of cigarette smoke as a whole and smoking cessation on bone is limited. Thus, based on the clinical relevance of this subject, and the limited number of studies available, this study is proposed to investigate the influence of cigarette smoke inhalation and its cessation on bone density.

MATERIALS AND METHODS

ANIMALS

Forty-one male Wistar rats (300-400g) 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 (CSI) at any time during the experimental period (n=14), Group 2 – 3 months of CSI followed by 2 months without exposure (n=12) and Group 3 – CSI during 5 months (n=15) (Figure 1).

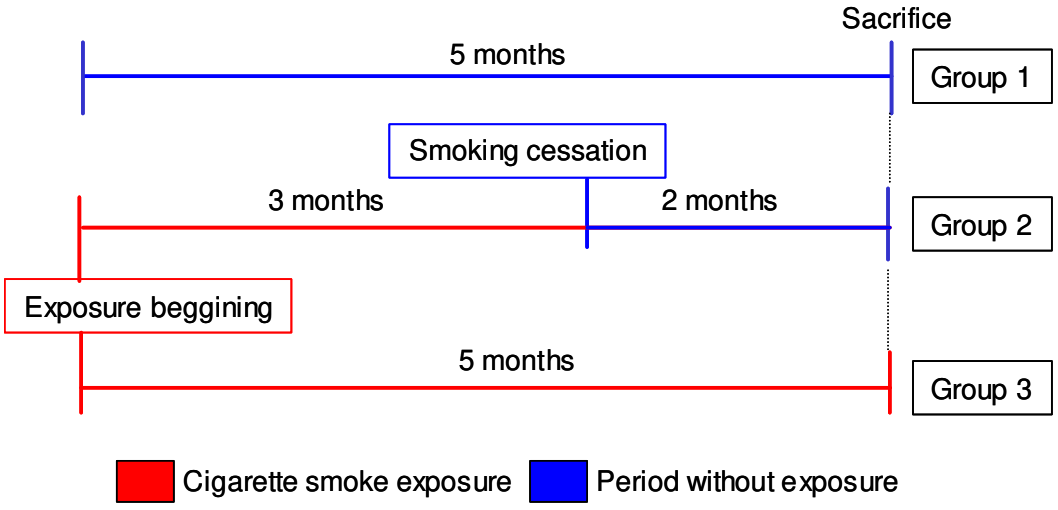


Figure 1 – Schematic illustration of the experimental design.

The animals of groups 2 and 3 were intermittently housed in an animal cigarette smoke exposure chamber as previously described[6,21,22]. Briefly, the device consisted of a 45 X 25 X 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, containing 1.3 mg of nicotine, 16.5mg of tar, and 15.2mg of carbon monoxide each, was pumped into the chamber. Thus, the animals were exposed to cigarette smoke that contaminated the air for 8 minutes, 3 times daily until they were sacrificed. The animals of group 1 were not exposed to cigarette smoke at any time. The serum levels of nicotine and cotinine obtained by using this model has been previously reported[6].

RADIOGRAPHIC ANALYSIS:

Immediately after the sacrifice, the tibiae were removed and radiographed using an X-ray unit (GE 1000, General Eletric Company, Milwalkee, Wisconsin, USA) with an exposure time of 0.3 seconds (60Kvp, 10mA) and 31 X 41mm radiographic film (Insight Film, Eastman Kodak, Rochester, NY, USA). A reference radiograph of an aluminum step-wedge was also taken using the same apparatus and exposure time. The films were developed in an automatic processing machine (Gendex GXP Dental X-Ray Processor- Des Plaines, IL, USA). The total length of the tibiae was measured and the mean point was determined as a reference for photodensitometric analysis (MRA Equipamentos Eletrônicos, Ribeirão Preto, SP, BRAZIL). A blind examiner performed five measurements, in order to scan the bone area between the 2 corticals at the level of the previously determined reference point. Figure 2 illustrates the analyzed area. The measurement of bone density was compared with the step-wedge standard to compensate for processing variations and to provide a unit for quantifying bone density, that was expressed in millimeters of aluminum equivalence (Al eq).

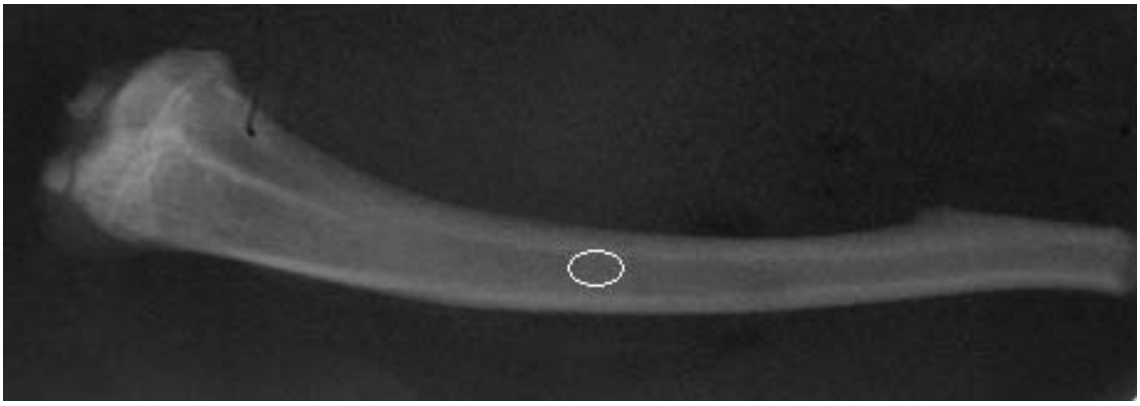


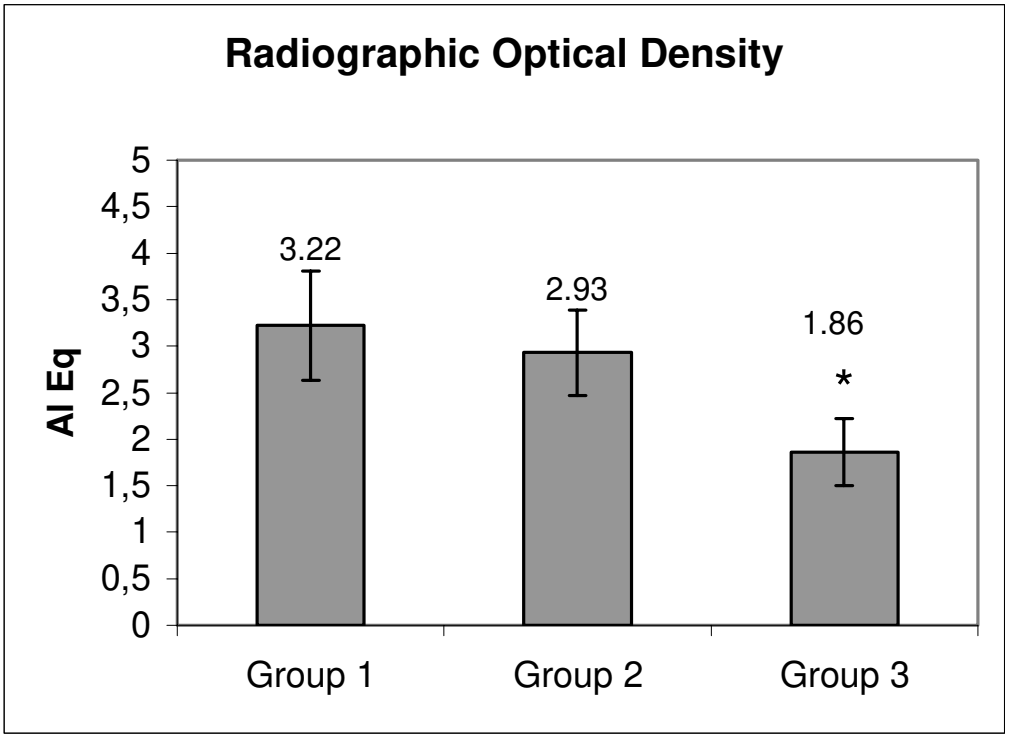
Figure 2 - Radiographic image illustrating the tibiae area where photodensitometric measurements were performed.

STATISTICAL ANALYSIS

Mean values of radiographic optical density were determined for each group and compared statistically using one-way analysis of variance (ANOVA) (α=0.05). Pairwise multiple comparisons were carried out by the Tukey test (α=0.05) in case the ANOVA test showed significant differences.

RESULTS

The results showed that continuous exposure to cigarette smoke (group 3) promoted a significantly reduced bone density (P<0.05) when compared to control and cessation groups (1 and 2, respectively) (3.22 Al eq $\pm$ 0.58; 2.93 Al eq $\pm$ 0.45; 1.86 Al eq $\pm$ 0.35**;** for groups 1, 2 and 3, respectively). Similar levels of bone density were observed for cessation and control groups (p>0.05). Figure 3 graphically illustrates the results described above.



* Statistically significant - Intergroup analysis ANOVA (α=0.05).

Figure 3: Mean and standard deviation of tibiae bone density, expressed in millimeters of aluminum equivalence (Al eq), according to each group.

DISCUSSION

The mechanism by which smoking influences the success rate of titanium implants is still unclear. The hypothesis that cigarette smoke inhalation would have an influence on the degree of bone-to-implant contact and bone filling of the implant threads (parameters evaluated in newly formed bone) was tested in a series of studies of our laboratory[6,21,22]. In general, these studies concluded that CSI has a negative impact on both parameters related to the newly formed bone around implants and nicotine seems to partially contribute to such an effect. Interestingly, it was also observed a decreased proportion of mineralized tissue in the pre-existing bone adjacent to the implant in the animals submitted to CSI[6]. This result emerged the question of a possible effect of CSI on pre-existing bone. Thus, the present investigation was designed to better elucidate this hypothesis, since it was not possible to exclude an influence of the healing process adjacent to the pre-existing bone. The result of this study showed that CSI significantly reduced bone density and is in agreement with our previous results[6,21,22] confirming the hypothesis that CSI affects bone metabolism not only in healing areas but also in areas of pre-existing bone.

Within the limits of our knowledge, this is the first study that investigated in an animal model the influence of CSI on bone quality of an area not adjacent to titanium implants. Despite smoking being considered one of the main risk factors of osteoporosis, most of the animal studies did not report a correlation between nicotine (one of the main compounds of cigarette smoke) administration and lower bone density[2, 9, 24, 25]. A previous study of ours also reported that nicotine did not present a negative impact on bone healing around implants inserted in tibiae of rabbits[24]. On the other hand, the result of the present study showed that cigarette smoke, as a whole, presented a negative impact on bone quality and is in line with the hypothesis suggested by Akhter et al.[2], that tobacco agents other than nicotine are responsible for the decreased bone density and increased fracture risk observed in smokers.

One of the most widely employed techniques to assess bone density in humans is dual energy X-ray absormetry (DEXA). However, DEXA technique in mandible is difficult and expensive, and can only be used in edentullous areas[14]. Photodensitometry of periapical and panoramic radiographs has been utilized to estimate mandibular bone mass[14,15,16]. A significant correlation between skeletal bone mineral density and alveolar bone mass, determined by a photodensitometer, has been reported[11,14,15]. Photodensitometric measurements were also used to compare mandibular

bone differences between normal and osteoporotic women[16]. A recent study reported that the sensitivity of dexa measurements is unsuitable when assessing small bone samples in mice[7]. Therefore, based on this body of evidence, the use of a photodensitometer to assess bone mass may be considered a suitable method to evaluate bone changes in areas where DEXA is not indicated, like bone samples of small animals.

The reversibility of the effects of cigarette consumption has been studied both in medicine and dentistry. A meta-analysis study demonstrated that current smokers presented a significantly reduced bone mass when compared to former and never smokers[27] and, that former smokers presented bone mass that is intermediate or similar to never smokers[10,27]. They additionally reported that smoking has a dose-dependent effect on bone loss, which increases fracture risk, and that smoking cessation may present a beneficial effect[27]. 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 periodontal cells[5,23]. Smoking cessation also exerted a beneficial effect on periodontal risk, which decreased with the number of years since quitting[26]. A prospective study over 20 years showed that patients who stopped smoking lost significantly less marginal bone than current smokers[13]. In the implant field, very limited information is available with respect to the reversibility of the effects of smoking on implants outcome. Bain[4] was the first to report that a smoking cessation protocol would improve success rates for osseointegration in smokers who follow it. The results of the present investigation are in agreement with the studies that showed a reversible condition promoted by cigarette consumption, and support the clinical concept that the effect of cigarette consumption on bone may be reversible.

During the early time of implant procedure development, implant failure was generally attributed to poor surgical technique (infection, overheating of bone and over-instrumentation), poor prosthetic design or management, or patient-related factors (limited available bone, poor oral hygiene and occlusal overload). These findings were largely based on clinical observation, extrapolation from failures in tooth-supported prostheses and dogma. But some studies began to correlate systemic conditions with higher rates of failure[3,4,6,8,18]. Now, smoking has been one of the factors often discussed in relation to decreased success rates of dental implants. Our findings suggest that smoking may affect bone quality, and that smoking cessation may revert the tobacco harmful effect on bone. However, it is not possible to establish a direct correlation between animal

findings and a clinical situation, due to the differences between humans and rats and the frequency of smoke administration used in this study. Accordingly, the hypothesis generated by the present study should be clinically evaluated since bone quality is critical for implant success[12]. Once the clinical relevance of the present hypothesis is confirmed, recommendations regarding smoking cessation should be considered to form part of the implant patient approach. Further studies should still be performed in order to determine the time necessary to bone density recover its normal level after smoking cessation.

Conclusion

Within the limits of the present study, it can be concluded that smoking may affect tibiae bone density, and CSI cessation exerts a beneficial effect on bone quality promoting a return towards the level of the control group.

Acknowledgment

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3.6 Capítulo 6

MATRIX METALLOPROTEINASE-2 MAY BE INVOLVED WITH INCREASED BONE LOSS ASSOCIATED WITH EXPERIMENTAL PERIODONTITIS AND SMOKING. A STUDY IN RATS

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ABSTRACT

Background: Smoking has been associated with periodontitis severity, and considered a risk factor for its development. It has been reported that matrix metalloproteinase (MMP) produced by host cells plays a major role in periodontal tissue destruction. Thus, the present study tested, in rats, the hypothesis that local increased levels of MMP-2 would be associated with the enhanced periodontitis-related bone loss after intermittent cigarette smoke inhalation (CSI).

Material and Methods: Twenty-seven adult male Wistar rats were used. A ligature was placed around one of the mandibular first molars of each animal and they were randomly assigned to one of the following groups: **1** – control (n=13) or **2** – CSI (n=14). Sixty days later, the animals were sacrificed, gingival tissues harvested, and the specimens processed for decalcified sections. Extracts from the gingival tissues were prepared and assayed for MMP-2 expression.

Results: Intergroup comparisons (unligated sites) showed that CSI might directly affect alveolar bone (0.16 $\pm$ 0.03mm^2 versus 0.24 $\pm$ 0.09mm^2 for non-smokers and smokers, respectively – P=0.001). Moreover, CSI significantly enhanced bone loss resulting from experimental periodontitis (0.64 $\pm$ 0.36mm^2 versus 1.50 $\pm$ 0.50mm^2 for non-smokers and smokers, respectively - P<0.05). In addition, zymography demonstrated that CSI also enhanced MMP-2 levels and activity in the gingival tissues around ligated teeth.

Conclusion: Within the limits of the present investigation, it can be assumed that CSI effect on MMP-2 levels and activity may account for the increased periodontitis progression rate observed in smokers.

KEY WORDS: cigarette smoke, experimental periodontitis, MMP-2

INTRODUCTION

Periodontal disease is a leading cause of tooth loss in humans, and epidemiological studies have demonstrated that it affects a great part of the world population[1]. The hallmark of periodontitis is a chronic destruction of periodontal attachment apparatus following an intense inflammatory response to bacteria. Matrix metalloproteinase (MMP) represents a family of peptidases that plays an important role in the turnover of extracellular matrix components in physiological and pathological conditions. Studies have reported that members of MMP family are implicated in periodontal tissue destruction, including MMP-1, MMP-8, MMP-9 and MMP-2[2-5]. MMP-2 can degrade type IV collagen, the major protein found in the basement membrane, and also denatured type I collagen that is the major protein in periodontal tissue[7-8]. The increase of transcription levels or active MMP-2 in the periodontal tissue can potentate collagen breakdown during periodontitis, conforming it was shown in chronic adult periodontitis[9].

Clinical observations of differences between smokers and non-smokers regarding their periodontal status have stimulated extensive research activity in this field. Cigarette smoking has been shown to represent a strong risk marker, and possibly, a true risk factor for periodontal disease[10-11]. Experimental evidence has demonstrated that cigarette smoking may negatively influence the healing outcome following various periodontal therapeutic procedures[12-17]. Moreover, it has been shown that smokers not only run an increased risk of developing periodontal disease, but smoking also seems to enhance the severity of existing periodontal disease[10,18]. The mechanisms by which tobacco smoke interferes with periodontitis progression are not completely understood.

The effect of smoking on increasing elastase activity levels and a correlation with MMPs activity has recently been suggested as one possible reason for the increased risk and/or progression in periodontitis[4,19] reported for smokers. Furthermore, higher levels of TNF-α, which plays a role on the transcription levels of MMPs genes, has also been reported for smokers and compared with non-smokers[20-21].

Thus, the goal of the present study was to test the hypothesis that local increased levels of MMP-2 would be associated with the enhanced periodontitis-related bone loss observed after intermittent cigarette smoke inhalation.

MATERIALS AND METHODS

ANIMALS

Twenty-seven adult male Wistar rats (300-400g) were included in the study. The animals were kept in plastic cages with access to food and water *ad libitum*. Prior to the surgical procedures all animals were allowed to acclimatize to the laboratory environment for a period of 5 days. The protocol was approved by the University of Campinas Institutional Animal Care and Use Committee.

EXPERIMENTAL DESIGN

General anesthesia was obtained by intramuscular administration of ketamine (0.5ml/kg). One of the mandibular first molars of each animal received a cotton ligature to induce experimental periodontitis. One day later, the animals were randomly assigned to one of the following treatment groups: **Group 1** – control (n=13) or **Group 2** – intermittent cigarette smoke inhalation (CSI) (n=14). Animals of group 2 were intermittently housed in an exposure chamber (45x25x20cm^3) as previously described[22-23]. Briefly, 5 animals were housed in the chamber at the same time for 8 minutes/3x daily, and the cigarette smoke of 10 cigarettes, containing 1.3 mg of nicotine, 16.5mg of tar and 15.2mg of carbon monoxide each, was pumped into the chamber.

NICOTINE AND COTININE SERUM LEVELS: ANALYTICAL METHODS

In order to assess serum levels of nicotine and cotinine, blood samples were taken before the placement of the ligatures (baseline), 30 and 60 days later. The procedure was systematically performed 15 minutes after cigarette smoke inhalation. Serum samples were assayed for concentrations of nicotine and cotinine by high-pressure liquid chromatography. The device was

composed by two pumps[#], programmed by a system controller[¶], a UV-Vis detector[**] set at 260nm and a reversed-phase column Luna[††] (150mm X 4.6mm I.D.X 5μm). The mobile phase consisted of 20mM dibasic potassium phosphate, 20mM monobasic potassium phosphate containing 0.1% of triethylamine. The pH of the solution was adjusted to 6.3 with phosphoric acid and 10 % of acetonitrile was added to the final solution. The flow rate was 1.0ml/min. 2-phenylimidazole was used as internal standards. All the reagents used to perform the method were HPLC grade. The extraction of the samples followed a previously described methodology by Nakajima *et al.*[24], but evaporated to dryness under nitrogen at ambient temperature. The injection volume was 20μL and the limit of quantification was 10ng/mL.

HISTOMETRIC ANALYSIS

Sixty days after ligature placement, the jaws were removed and gingival samples harvested from the first molars region. The jaws were fixed in 4% neutral formalin for 48h, 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. Using an image analysis system[Ψ], the area of bone loss in the furcation region was histometrically determined. Measurements were averaged to allow an intergroup and intragroup analysis (Mann-Whitney test - α=0.05).

PROTEASE ASSAYS

Gingival samples were obtained (buccal aspect of first molar), placed on ice and subsequently frozen (-80ºC). Tissue extracts were prepared as previously described by Robinson et al. (1992). Gingival samples were homogenized in a solution containing 0.25%Triton X-100, 10mM $CaCl_2$, 2mM PMSF and 2mM NEM and centrifuged (6,000g X 20 minutes at 4ºC). MMPs were extracted with a 50mM Tris-HCl buffer (pH 7.4) containing 100mM $CaCl_2$, 2mM PMSF e 2mM NEM for 30 minutes at 40ºC. Extract aliquots were stored at -20ºC.

In order to assess proteolytic activity, equal amounts of protein were mixed with an equal volume of non-reducing sample buffer (2%SDS; 125mM Tris-HCl, pH 6.8, 10% glycerol and 0.001%

[#] LC-10ADvp, Shimadzu Corporation, Tokyo, Japan
[¶] SCL-10ADvp, Shimadzu Corporation, Tokyo, Japan
[**] SPD-10ADvp, Shimadzu Corporation, Tokyo, Japan
[††] Column Luna, Phenomenex, USA
[Ψ] Image-Pro®; Media Cybernetics, Silver Spring, MD, USA

bromophenol blue) and then electrophoresed. Electrophoresis gels were then washed twice in 2% Triton X-100 for 20 min at room temperature, and incubated at 37°C for 16h in 50mM Tris-HCl buffer (pH 7.4), containing 5mM $CaCl_2$ (Tris-$CaCl_2$). Following incubation, the gels were stained with 0.05% Coomassie Brilliant Blue G-250. Gelatinolytic activity was detected as unstained bands. The relative molecular masses of proteases were determined by the relation of log Mr to the relative mobility of Sigma SDS-PAGE LMW marker proteins. In order to evaluate MMPs activity, electrophoretic bands were scanned and the absorbance was analyzed[Π].

RESULTS

CLINICAL OBSERVATIONS

Clinically, at the time of the sacrifice, signs of gingival inflammation were observed around the ligated teeth for both groups, while unligated teeth kept a healthy appearance. Furthermore, at the end of the experimental period, autopsy revealed that CSI resulted in a significant pigmentation of the lungs (dark color) (figure 1A and 1B).

SERUM LEVELS OF NICOTINE AND COTININE

Baseline results showed nicotine/cotinine values lower than the detectable limit for both groups. Animals from the control group kept non-detectable levels during the second and third evaluations (30 and 60 days). On the other hand, animals exposed to cigarette smoke presented the following mean levels of nicotine and cotinine, respectively, 346.1ng/ml $\pm$ 114.3 and 265.4ng/ml $\pm$ 109.8 at day 30; and 174.9ng/ml $\pm$ 32.2 and 149.9ng/ml $\pm$ 27.4 at day 60.

HISTOMETRIC RESULTS

Intragroup analysis demonstrated that ligature placement promoted a significant bone loss when compared to unligated sites ($P<0.05$). In addition, intergroup comparisons (group 1 vs group 2 - unligated sites) showed that CSI resulted in a wider periodontal ligament area in the furcation region (0.16 $\pm$ 0.03mm^2 / 0.24 $\pm$ 0.09mm^2 for groups 1 and 2, respectively – $P=0.001$), suggesting that alveolar bone may be affected regardless of the presence of the dental biofilm. Moreover, with respect to the ligated teeth, data analysis showed that CSI significantly enhanced bone loss resulting from experimental periodontitis (0.64 $\pm$ 0.36mm^2 / 1.50 $\pm$ 0.50mm^2 for groups 1 and 2, respectively - $P<0.05$). Figures 2A to 2D histologically illustrate the bone loss in the furcation region for all the experimental groups.

[Π] ImageMaster System /Total Lab®, Amersham Biosciences Corp., Piscataway, NJ, USA

PROTEINASE ASSAYS

Two major bands, with an approximate molecular mass of 72kDa and 66kDa, were detected in the zymographic assays (figure 3). The bands corresponded to zimogens and active forms of MMP-2 respectively. Zymogram analysis demonstrated that periodontitis sites presented higher levels of active and latent MMP-2 when compared to healthy sites. Furthermore, CSI resulted in higher levels of both forms of MMP-2 when compared ligated sites. On the other hand, CSI did not affect MMP-2 levels in areas where gingival tissues were clinically healthy.

DISCUSSION

Smokers have been reported to present 2.7 times greater probability of developing periodontal disease than non-smokers, independent of age, sex and plaque index[25]. Jansson and Lavstedt[26] reported that smoking is correlated to a greater marginal bone loss and Bergstrom *et al*.[27] demonstrated that chronic smoking compromises periodontal health. In spite of the great importance of these studies, the biological events by which cigarette consumption influences the pathogenesis of periodontal disease remain still not fully understood. The results of the present studies reaffirmed cigarette consumption as a challenge in periodontics since an increased rate of bone loss was observed in the animals that were submitted to CSI after inducing experimental periodontitis. In addition, it was also demonstrated that CSI can significantly, at least locally, affect MMP-2 activity, and therefore, may partially explain our histological findings.

MMP-2 is one of the major enzymes involved in the degradation process of soft tissues[28]. Although the role of MMP-2 in periodontal disease is still unclear, recent evidences have shown that this enzyme is increased in the gingival tissue of periodontitis patients[9], suggesting that it may be involved in the pathogenesis of periodontitis, being activated by periodontopathogenic bacteria[3,29]. MMPs are expressed at low levels in the absence of inflammation[30], which is in agreement with our results where low MMP-2 levels were observed at unligated sites. As reported by others[9,31], the present study found higher levels of MMP-2 in periodontitis when compared to healthy sites. In addition, as also previously reported for MMP-8 and elastase activities[4,19], here we demonstrated increased MMP-2 activity around ligated teeth as a consequence of cigarette smoke exposure. Because MMP-2 is the most widely distributed MMP and may be produced by several periodontal cells e.g. fibroblasts, keratinocytes, endothelial cells, monocytes/macrophages and osteoblasts[32], we

then assume that, besides MMP-8 and elastase, MMP-2 may also play a significant role during periodontitis development in smokers.

The mechanism of MMP-2 activation is different from other MMPs since MMP-2 cannot be activated by serine proteinase. According to a model proposed by Strongin et al.[33], latent MMP-2 binds to MT1-MMP (a membrane type MMP) by using TIMP-2 (tissue inhibitor of MMP-2) as a bridging molecule to form a trimolecular complex. Subsequently, cleaving its propeptide domain activates MMP-2. Pattamapun et al.[9] demonstrated that MMP-2 regulation by *Porphiromonas gingivalis* in human periodontal ligament cells might involve MT1-MMP as suggested. Interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α) have also been reported to regulate MMP-2 expression in different cell types[34-35]. In the present investigation, within the limits of its experimental design, it is not possible to determine the mechanism by which cigarette consumption affected MMP-2 expression and activity. However, there are evidences demonstrating that smoking can regulate IL-1 and TNF-α[20-21,36], and therefore, these cytokines may be involved. Nevertheless, further studies should be considered in order to determine, mechanistically, how smoking regulates MMP-2 expression and how this regulation is involved in the control of tissue degradation in smokers.

In the present study, nicotine and cotinine serum levels were assessed, and the cotinine serum levels closely correlated with smokers that smoke between 10 to 20 cigarettes/day[37]. However, a certain degree of variability was observed and perhaps changes in nicotine absorption, as a consequence of lung emphysema, as previously reported[38], may have been the cause of such variation over time. Importantly, because differences in the metabolism of nicotine between human and rats, and the frequency of smoke administration used in this study, caution should be considered during future comparisons with human subjects.

In conclusion, within the limits of the present investigation, cigarette smoke effect on MMP-2 expression and activity may account for the increased periodontitis progression rate observed in smokers. On the clinical level, if such relation between smoking and enzyme activity in periodontal tissues were confirmed by further studies, it might be useful in order to design specific therapeutic approaches for periodontal disease in smokers.

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Figure 1: Clinical appearance of the lungs for (A) group 1 (control) and (B) group 2 (CSI) after sacrificing the animals illustrating the effects of smoke exposure. Note that in A the lungs present a normal aspect with a light pink color, while in B autopsy revealed that CSI resulted in a significant pigmentation of the lungs (dark color) as a consequence of cigarette smoke inhalation.

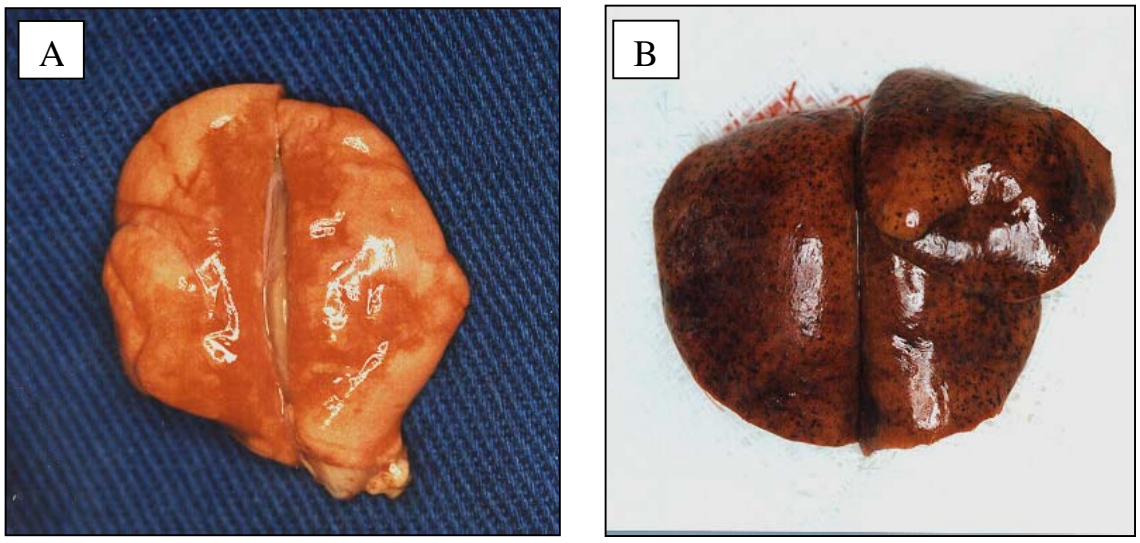


Figure 2: Photomicrographs illustrating bone loss in the furcation region of mandibular molars, which were quantitatively analyzed. Figure A and B are representatives of group 1 (control) ligated and group 2 (CSI) ligated teeth, respectively (Original Magnification 12.5x - H & E).

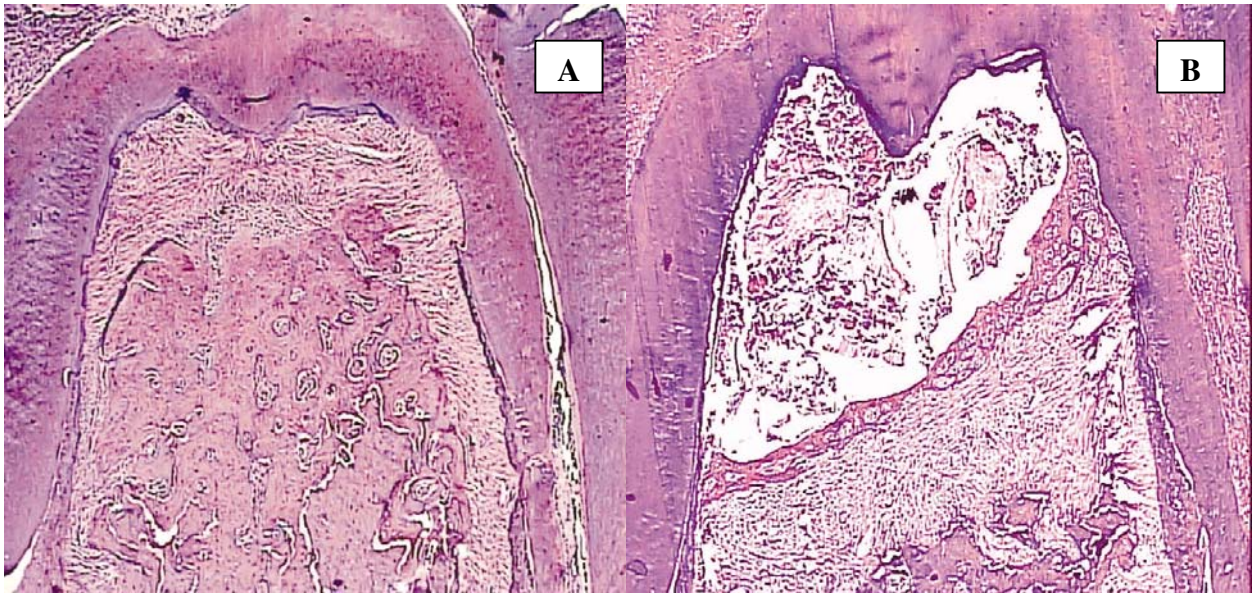
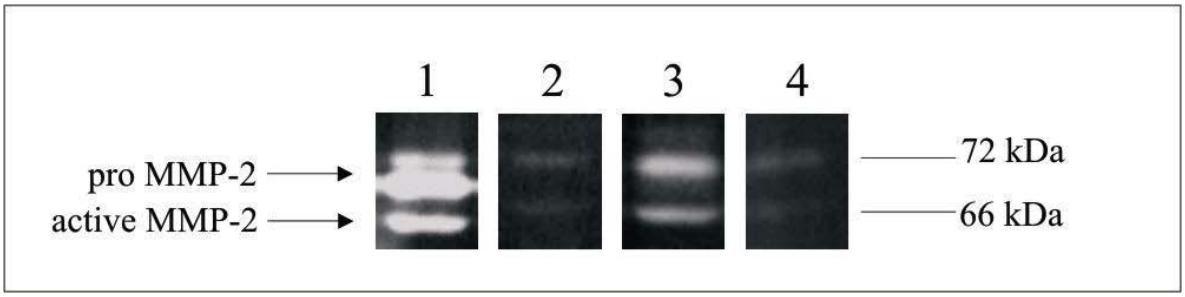


Figure 3: Gelatin zymogram of gingival extracts. Columns 1 to 4 represent animals exposed and not exposed to cigarette smoke (ligated and unligated), respectively. Note that ligated teeth (columns 1 and 3) presented higher MMP-2 levels for both groups, while unligated teeth (columns 2 and 4) presented almost non detectable levels. In addition, ligated teeth in animals exposed to cigarette smoke (column 1) showed higher levels of MMP-2 than ligated teeth in animals not exposed (column 3).



3.7 Capítulo 7

SMOKING CESSATION MAY PRESENT A POSITIVE IMPACT ON MANDIBULAR BONE QUALITY AND PERIODONTITIS-RELATED BONE LOSS. A STUDY IN RATS.

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ABSTRACT

Background: It has been previously shown that cigarette smoke inhalation (CSI) enhances bone loss in ligature-induced periodontitis. In this study, the hypothesis that the interruption of smoke exposure would revert the impact of CSI on mandibular bone quality and periodontitis-related bone loss was tested.

Material and Methods: Fifty-three Wistar rats were randomly assigned to one of the following groups: Group 1 – control (n=16), Group 2 **–** 83 days of CSI prior to ligature placement (n=17), and Group 3 – 90 days of CSI before and 60 days after ligature placement (n=20). Animals were sacrificed 60 days after ligature placement, the jaws removed and immediately radiographed for photodensitometry analysis. Bone loss was histometrically evaluated.

Results: CSI did not affect unligated sites in either condition ($P>0.05$), however, smoke inhalation maintained during the whole experimental period significantly enhanced bone loss in ligated teeth ($P<0.05$). Moreover, similar levels of bone loss were observed for ligated teeth between the control

and cessation groups (0.90mm^2 $\pm$ 0.33; 0.96mm^2 $\pm$ 0.32; 1.64mm^2 $\pm$ 0.65**;** groups 1, 2 and 3, respectively). Radiographically, continuous exposure to cigarette smoke promoted a significantly reduced bone density ($P<0.05$) (1.74Al eq $\pm$ 0.38; 1.74Al eq $\pm$ 0.14; 0.68Al eq $\pm$ 0.10**;** groups 1, 2 and 3, respectively).

Conclusion: Within the limits of the present investigation, it can be assumed that CSI may enhance bone loss in ligature-induced periodontitis, and negatively impact mandibular bone quality. Additionally, smoke exposure cessation seems to revert its impact on mandibular bone, and, therefore, may be of clinical relevance.

KEY WORDS: cigarette smoke, ligature-induced periodontitis, rats, cessation.

INTRODUCTION

It has been demonstrated that there is a strong positive correlation between cigarette consumption and increased incidence and severity of periodontal disease[1-3]. Clinical observations of differences between smokers and non-smokers regarding their periodontal status have stimulated extensive research activity. Cigarette smoking has been shown to represent a strong risk marker, and possibly, a true risk factor for periodontal disease[4-5]. Moreover, it has been shown that smokers not only run an increased risk of developing periodontal disease, but also enhance the severity of existing periodontal disease[4, 6].

The hallmark of periodontitis is a chronic destruction of periodontal attachment apparatus following an intense inflammatory response to bacteria. The influence of smoking on the periodontal biofilm is still controversial. In general, there is some agreement that cigarette consumption has a negative impact on the host response. Animal studies reported that nicotine[7,8] and cigarette smoke inhalation (CSI)[9], when administered after periodontitis induction, enhanced ligature-induced periodontal breakdown, and could be associated with a higher level of matrix metalloproteinase-2 (MMP-2) in the gingival tissues[9]. Furthermore, higher levels of tumor necrosis factor-alpha (TNF-α) were observed in the gingival crevicular fluid of smokers with moderate to severe periodontal disease[10]. It has also been reported that tobacco components may alter morphology and metabolism of periodontal cells without the presence of bacterial contamination[11-18]. In vitro studies have shown that nicotine negatively affects osteoblasts[11,12], gingival[13] and periodontal ligament fibroblasts[14], and stimulates osteoclast activity[12,15]. Recent reports showed that acrolein and acetaldehyde, volatile components of cigarette smoke, also have a negative effect on fibroblast cultures[16-18]. Likewise, it has

been reported that peripheral mononuclear cells of smokers release more IL-1β when treated with cigarette smoke[19]. Clinical studies have shown that abstinence from smoking reduces incisional wound infection[20], reduces complications of postmastectomy breast reconstruction[21], reduces the risk of senile cataract[22] and helps to reverse the negative impact of cigarette smoking on outcome after spinal fusion[23]. Regarding periodontal disease, a recent prospective study[24] reported that individuals who stopped smoking lost significantly less marginal bone than those who did not. Therefore, the aim of the present study was to test, in a rat model, the hypothesis that CSI cessation would revert its impact on mandibular bone quality and on periodontitis-related bone loss.

MATERIALS AND METHODS

ANIMALS

Fifty-three male Wistar rats (300-400g) were included in the study. The animals were kept in plastic cages with access to food and water *ad libitum*. Prior to the surgical procedures all animals were allowed to acclimatize to the laboratory environment for a period of 5 days. The protocol was approved by the University of Campinas Institutional Animal Care and Use Committee.

EXPERIMENTAL DESIGN (Figure 1)

At the beginning of the study, the animals were randomly assigned to one of the following groups: group **1** – control (n=16), group **2 –** 83 days of intermittent cigarette smoke inhalation (CSI) previous to ligature placement (n=17), and group **3** – 90 days of CSI before and 60 days after ligature placement (n=20). Animals of group 2 and 3 were intermittently housed in an exposure chamber (45x25x20cm^3) as previously described[25-27]. Briefly, 5 animals were housed at the same time in the chamber for 8 minutes/3x daily, and the cigarette smoke of 10 cigarettes containing 1.3 mg of nicotine, 16.5mg of tar and 15.2mg of carbon monoxide each, was pumped into the chamber. A ligature was placed, under general anesthesia obtained by intramuscular administration of ketamine (0.5ml/kg), around one of the mandibular first molars of each animal. The ligature was left in position for 60 days before the sacrifice of the animals. The serum levels of nicotine and cotinine obtained using this model have been previously reported[27].

RADIOGRAPHIC ANALYSIS:

Immediately after the sacrifice, the jaws were removed and radiographed using an X-ray unit* with an exposure time of 0.3 seconds (60Kvp, 10mA) and 31 X 41mm radiographic film$^{\Upsilon}$. A reference radiograph of an aluminum step-wedge was then taken using the same apparatus and exposure time. The films were developed in an automatic processing machine$^{\phi}$ and the optical density was measured with a densitometer$^{\Psi}$ with an aperture of approximately 2mm. Five measurements were performed in the mandible, within the limits of the area indicated in figure 2. The optical density of the step-wedge was also evaluated in order to express the results in aluminum equivalence (Al eq).

HISTOMETRIC ANALISIS:

The jaws were fixed in 4% neutral formalin for 48h, 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. Using an image analysis system$^{\partial}$, the area of bone loss in the furcation region was histometrically determined.

STATISTICAL ANALISIS:

An intergroup analysis tested the hypothesis that there were no differences in bone loss rate in the furcation region of ligated teeth among groups 1, 2 and 3 (non-parametric Kruskal-Wallis test - α=0.05). Using the same procedure, the periodontal ligament area in the furcation region of the unligated teeth was compared among the groups. In addition, the paired t test (α=0.05) was used for intragroup comparisons of interradicular bone loss between ligated and unligated teeth. The mean values of radiographic optical density were averaged and compared statistically using one-way analysis of variance (ANOVA) (α=0.05). Pairwise multiple comparisons were carried out by the Tukey test (α=0.05) when the ANOVA test showed significant differences.

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* GE 1000, General Eletric Company, Milwalkee, Wisconsin, USA
$^{\Upsilon}$ Insight Film, Eastman Kodak, Rochester, NY, USA
$^{\phi}$ Gendex GXP Dental X-Ray Processor- Des Plaines, IL, USA
$^{\Psi}$MRA Equipamentos Eletrônicos, Ribeirão Preto, SP, BRAZIL
$^{\partial}$ Image-Pro®; Media Cybernetics, Silver Spring, MD, USA

RESULTS

RADIOGRAPHIC ANALISIS

Data analysis demonstrated that group 3 presented a significantly lower bone density in the body of the mandible when compared to groups 1 and 2 ($p<0.05$). In addition, there were no statistical differences between groups 1 and 2 ($p>0.05$) with respect to mandibular basal bone density (Figure 3).

HISTOMETRIC ANALYSIS

Intragroup analysis revealed a greater bone loss ($P<0.05$) for the ligated teeth when compared to unligated ones. Intergroup analysis showed that CSI did not affect unligated sites ($P>0.05$) ($0.15 \pm 0.06mm^2$; $0.15 \pm 0.04mm^2$; $0.18 \pm 0.06mm^2$**;** for groups 1, 2 and 3, respectively). However, ligated teeth from group **3** ($1.64 \pm 0.65mm^2$) presented a significantly higher area of bone loss when compared to groups **1** ($0.90 \pm 0.33mm^2$) and **2** ($0.96 \pm 0.32mm^2$) ($P<0.05$). No statistically significant differences were observed between groups **1** and **2** ($P>0.05$). Figures 4 to 5 illustrate the bone loss and periodontal ligament area in the furcation region for the experimental groups.

DISCUSSION

The influence of smoking on periodontal disease has long been debated. Although the mechanism by which smoking influences periodontal status is still unclear, it is generally accepted that tobacco smoke exerts a harmful effect on periodontal health. Several compounds of cigarette smoke, such as nicotine[11-15], acrolein and acethaldeide[16-18], have been reported to directly affect periodontal cells. In vitro studies have demonstrated that nicotine may be cytotoxic, and may contribute to the development of periodontal disease by decreasing cell viability and altering leukocyte[28] and monocyte[28,29] functions. Higher levels of TNF-α have also been demonstrated in the gingival crevicular fluid of smokers with periodontitis[10] and, a higher expression of MMP-2 in gingival tissue adjacent to sites with periodontitis in rats submitted to CSI[9]. Additionally, animal studies demonstrated that nicotine and cigarette smoke might enhance bone loss when administered in the presence of dental biofilm[7-9].

Although longitudinal studies demonstrate powerful evidence of the relationship between smoking and periodontal disease, intervention trials have provided the strongest evidence for the identification of a causal relationship. In intervention trials, the hypothesized risk factor is eliminated from the test group while the control group is followed without any intervention. The results of the

present intervention study are in agreement with previous reports showing that cigarette smoke inhalation (CSI) may significantly enhance bone loss resulting from ligature-induced periodontitis in rats[9]. Furthermore, having first demonstrated that CSI significantly affected bone loss resulting from ligature-induced periodontitis[9], data analysis demonstrated that CSI cessation might positively affect the rate of bone loss resulting from periodontitis. The reversibility of the effects of cigarette consumption has been studied both in medicine and dentistry. For lung disease, one of the most frequent pathologies associated with cigarette consumption, a former smoker is considered to run the same risk as a non-smoker 15 years after smoking cessation[30]. In addition, it has been shown that a current smoking habit has a stronger effect on mean total white blood cell counts (WBC) than cumulative exposure[31]. The effects of smoking on WBC presented an almost immediate reduction after smoking cessation. In dentistry, smoking cessation has also been shown to positively affect periodontal risk. In vitro studies[16,32] have suggested a reversible cytotoxic effect of cigarette compounds (i.e. nicotine, acrolein and acetaldehyde) on periodontal cells. The relative risk for developing periodontal disease was reported to be 3.97 for smokers and 1.68 for former smokers[33]. In addition, among former smokers, the risk decreased with the number of years since quitting (3.22 after 2 years and 1.15 after 11 years). In a prospective study over 20 years[24], 507 individuals were radiographically evaluated, and the results showed that the ones who stopped smoking during the experimental period lost significantly less marginal bone when compared to current smokers. Another longitudinal study[34] evaluated the changes in periodontal status of 101 patients during 10 years. Clinically, an increased frequency in diseased sites was observed in smokers, while former and non-smokers presented decreased and similar frequencies. Radiographically, an increased bone loss was noted for current smokers when compared to former and non-smokers. No significant differences were observed between former and non-smokers. Moreover, smoking cessation has also been reported to be beneficial for periodontal treatment outcome. Grossi et al.[35] demonstrated that former and non-smokers presented significantly more healing and reduction of *Bacteroides forsythus* and *Porphyromonas gingivalis* than current smokers. Therefore, the results of the present study are in agreement with previous reports showing a reversible condition promoted by cigarette consumption.

Misclassification of smoking status has been a concern in the literature[36], and is considered a confounder in epidemiological studies. Inaccurate reports may occur for many reasons such as individual metabolism, frequency of inhalation, depth of inhalation, capacity for dilution with room air,

amount of cigarette stub left and cigarette brand[37]. Biochemical validation of smoking status seems to be useful in order to minimize the influence of confounders in clinical studies, mainly for the determination of light, regular and heavy smokers. In animal studies, such confounders may be more accurately controlled. It has been previously reported[27] that the CSI regimen used by the present study promoted cotinine serum levels closely correlated with smokers that smoke between 10 to 20 cigarettes/day[3]. However, future comparisons with humans should be treated with caution, because of differences in the metabolism of nicotine between humans and rats, and the frequency of smoke administration used in this study.

In the present investigation, radiographic analysis additionally demonstrated that CSI significantly affected mandibular bone density, leading to a lower optical density of the mandibular bone. Furthermore, radiographic analysis suggests that the adverse effect of CSI on mandibular bone may be reversible since there was no significant difference between groups 1 (control) and 2 (cessation). However, although the effect of smoke inhalation on bone density was clear on day 150, caution should be used here since the experimental design used in the present study does not provide absolute evidence that CSI affected bone density on day 83. In addition, as shown in figure 2, the readers should be aware that the present study radiographically examined loss of mandibular basal bone rather than bone loss around the teeth. Despite smoking being considered one of the main risk factors for osteoporosis, most of the studies did not report a correlation between nicotine administration and lower bone density[38-42]. Akhter et al.[38] suggested that tobacco agents other than nicotine are responsible for the decreased bone density and increased fracture risk observed in smokers. Similarly, it has been reported that smoke exposure may affect mineralized tissue in healing areas[25,27] and around titanium implants placed in rat tibiae[26]. Therefore, the results of the present investigation support the hypothesis that smoking may decrease bone density not only in areas of new bone formation, and suggests that the mandibular bone may also be affected. Additional studies should be considered in order to further investigate the interactions between smoking and mandibular bone density, especially because it may be of great clinical significance in the dental implants field; and whether the influence of smoking on mandibular bone density may also account for the increased bone loss resulting from ligature-induced periodontitis.

In conclusion, within the limits of the present study, data analysis suggests that smoking cessation should be considered when dealing with individuals diagnosed with periodontitis or when mandibular bone density is an important issue for clinical procedures.

Acknowledgment

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Figure 1 – Schematic illustration of the experimental design.

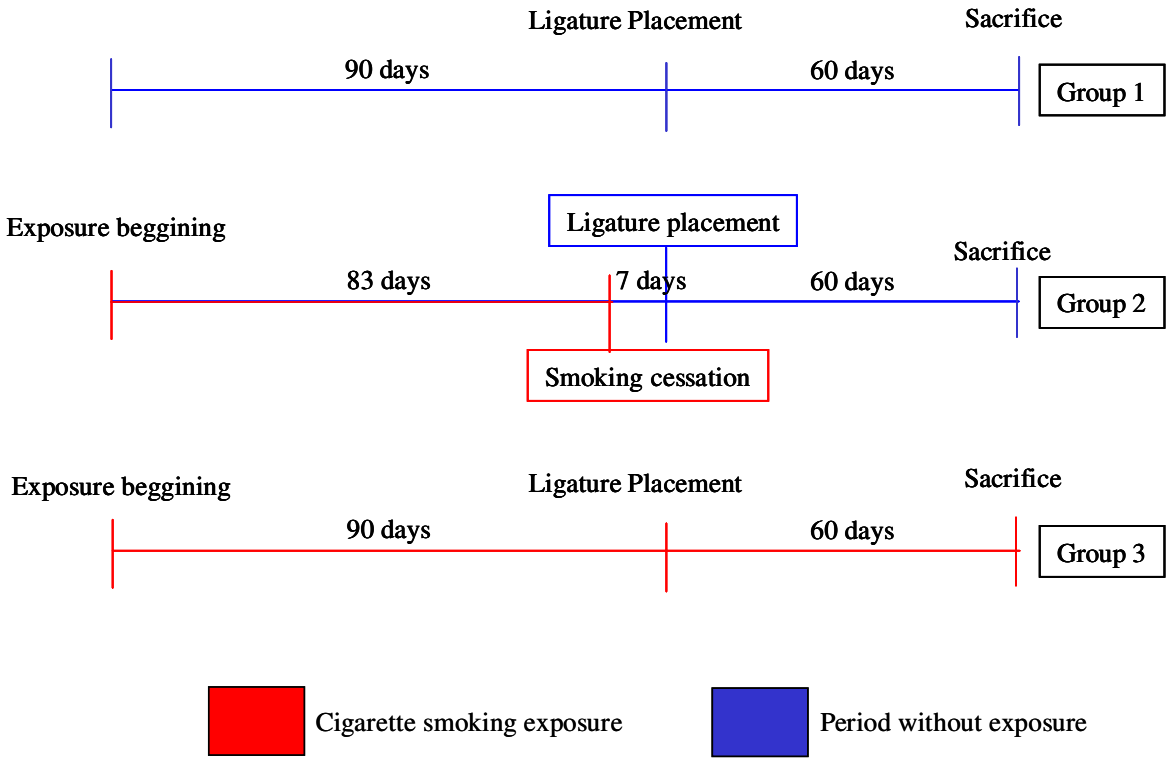


Figure 2 - Radiographic image illustrating the mandibular area where photodensitometric measurements were performed.

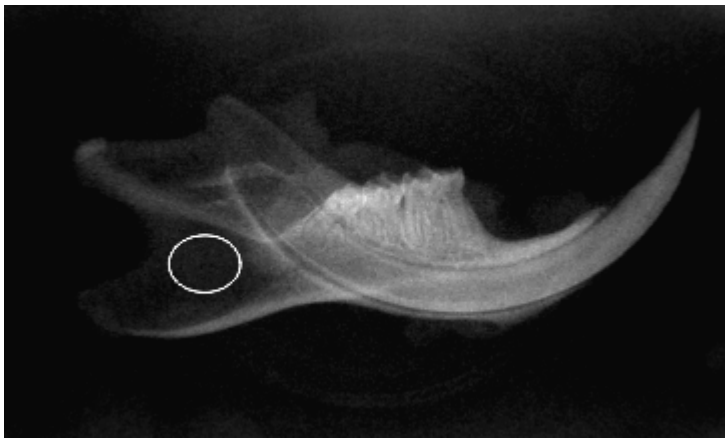
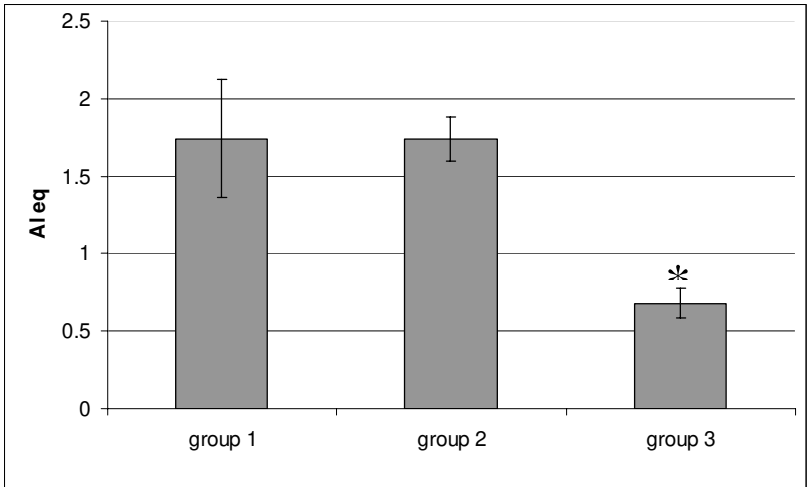
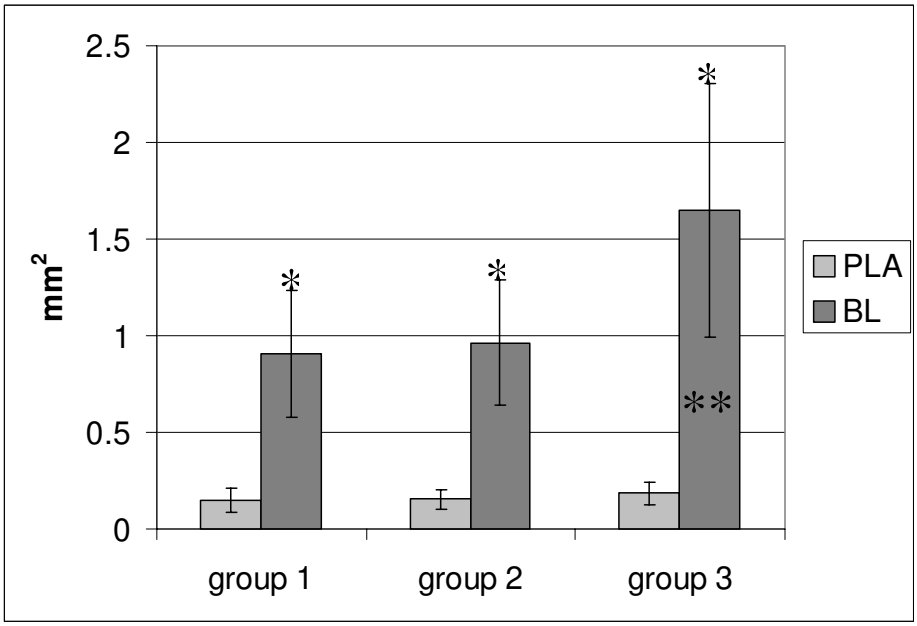


Figure 3: Mean and standard deviation of mandibular radiographic bone density (Al eq), according to each group.



* Statistically significant - Intergroup analysis showing a significant lower radiographic bone density for group 3 when compared to groups 1 and 2 (ANOVA - α=0.05).

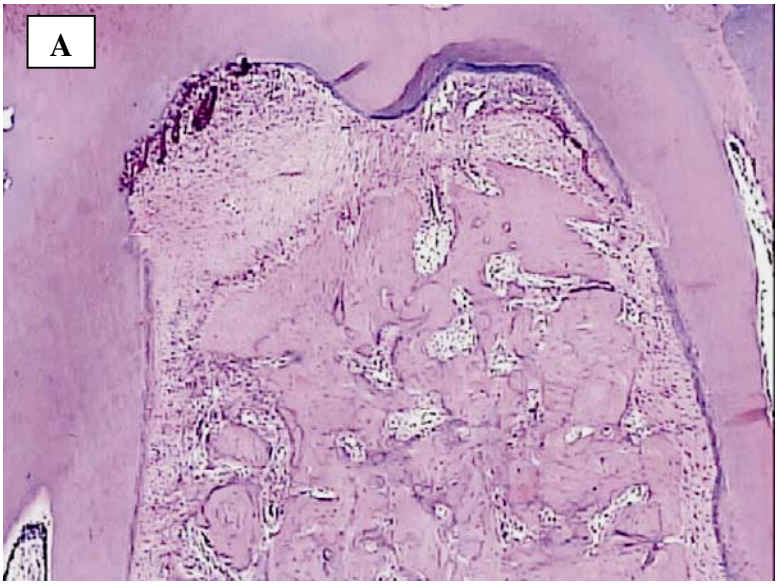
Figure 4: Mean and standard deviation of periodontal ligament area (PLA) and bone loss (BL) area (mm^2) for unligated and ligated teeth, respectively, according to each group.

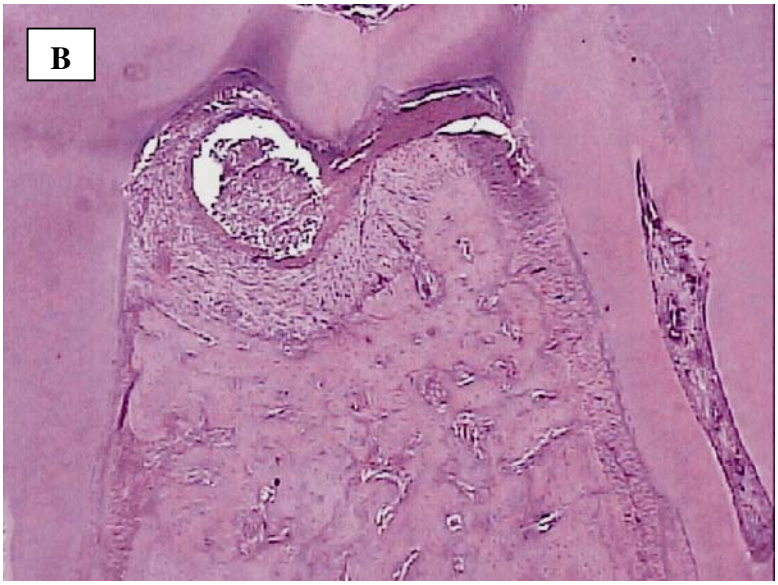


* Statistically significant - Intragroup analysis by Student t-test - α=0.05.

** Statistically significant - Intergroup analysis (ligated teeth) by Kruskal-Wallis test - α=0.05.

Figure 5: Photomicrograph illustrating periodontal ligament area and bone loss in the furcation region of mandibular molars, which were quantitatively analyzed. Figure A, B and C are representatives of ligated teeth from group 1, 2 and 3; respectively (Original Magnification 12.5x - H & E).

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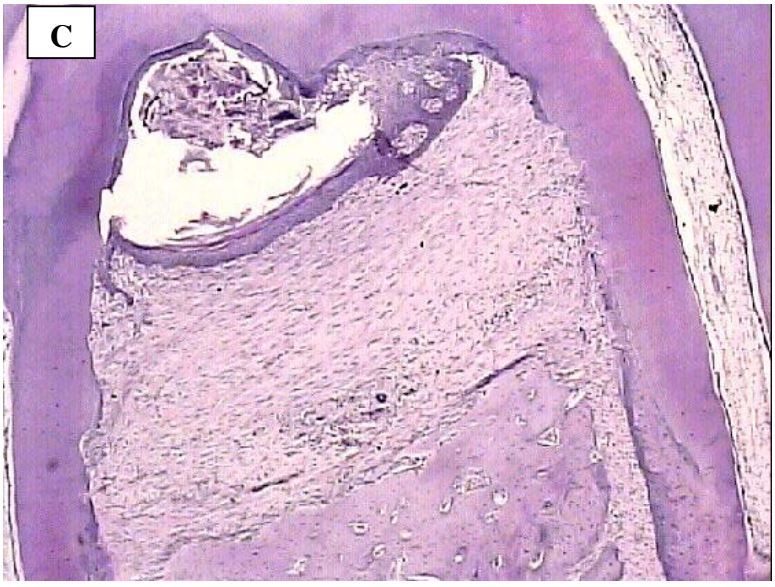
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3.8 Capítulo 8

THE INFLUENCE OF CIGARETTE SMOKE INHALATION AND ITS CESSATION ON THE TOOTH-SUPPORTING ALVEOLAR BONE. A HISTOMETRIC STUDY IN RATS.

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ABSTRACT

Objective: The aim of the present investigation was to histometrically evaluate the influence of cigarette smoke inhalation (CSI) and its cessation on tooth-supporting alveolar bone quality.

Design: Sixty male Wistar rats were randomly assigned to one of the following groups: Group 1 – control (n=15), Group 2 **–** Two months of CSI (n=13), Group 3 **–** Three months of CSI and two months without exposure to CSI (n=16) and Group 4 – Five months of CSI (n=16). Five months after the beginning of CSI regime (2 months for Group 2), the animals were sacrificed, the mandible was removed and prepared for histological sections. Bone density in the furcation area (i.e., the proportion of mineralized tissue in a 1000µm-zone under the furcation and between the roots – BD) was obtained.

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

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Conclusion: Within the limits of the present study, it can be concluded that: 1- CSI may affect the tooth-supporting bone as early as 2 months after the initial exposure; and 2- smoke exposure cessation may revert its negative impact on the alveolar bone.

Keywords: smoking, bone density, alveolar bone, rats.

INTRODUCTION

The influence of systemic factors on bone has been widely investigated. Several factors like cigarette smoking, low body weight, estrogen deficiency, alcoholism, caucasian race, inadequate physical activity, calcium intake and poor health were reported to influence bone quality and osteoporosis incidence in both skeletal and oral bones[1-6].

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, post-menopausal women who smoke loose significantly more cortical bone and have more spinal osteoporosis than nonsmoking counterparts[4], and were 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[7-8]. A 15 patient clinical study revealed that 80% of the individuals with impaired osseous healing were smokers[9]. Additionally, it is well recognized that cigarette smoking is associated with impaired wound healing after surgical treatment in the oral cavity[10], reduced bone height[11], increased bone loss rate and resorption of the alveolar ridge[12], higher incidence of periodontitis[13] and type iv bone[14].

Evidences have now been provided showing 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[15]. In oral tissues, Liede et al.[16] observed that periodontal status and mucosal health were better in individuals who had quit smoking when compared to current smokers. It has been shown that gingival microcirculation recovers its normal function on the early stages of smoking cessation[17], and the changes in the inflammatory response of the periodontium can also be reversible on quitting smoking[18]. Bain[19] clinically examined the influence of smoking cessation on implant outcomes, and did not find 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 and periodontitis-related bone loss, very limited information is

available with respect to the influence of both smoking and its cessation on the tooth-supporting alveolar bone under general and local clinically healthy conditions. It probably happens because the identification and adjustment for risk and confounding factors is 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, at the histological level in rats, the influence of cigarette smoke inhalation and its cessation on the tooth-supporting alveolar bone around clinically healthy teeth.

MATERIALS AND METHODS

ANIMALS

Sixty male Wistar rats (300-400g) 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 (CSI) at any time during the experimental period (n=15), **Group 2 –** 2 months of CSI (n=13), **Group 3 –** 3 months of CSI and 2 months without exposure to CSI (n=16) and **Group 4** – 5 months of CSI (n=16). The animals of groups 2, 3 and 4 were intermittently housed in a cigarette smoke exposure chamber as previously described[20-23]. Briefly, the device consisted of a 45 X 25 X 20 cm^3 clear acrylic chamber, an air-pump and two inflow/outflow tubes[20]. Five animals were housed in the chamber at the same time, and the cigarette smoke of 10 cigarettes, containing 1.3 mg of nicotine, 16.5mg of tar, and 15.2mg of carbon monoxide each, was pumped into the chamber. Thus, the animals were exposed to cigarette smoke that contaminated the air for 8 minutes, 3 times daily during the CSI 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 CSI 2 months before sacrifice. The serum levels of nicotine and cotinine obtained by using this model has been previously reported[22].

HISTOMETRIC PROCEDURE:

After the experimental period (5 months for Groups 1, 3 and 4; and 2 months for Group 2), the animals were sacrificed by decapitation. The jaws were removed and fixed in 4% neutral formalin for 48h, 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), bone density (BD) in the furcation area (i.e. the proportion of mineralized tissue in a 1000µm-zone under the furcation and between the roots) was obtained by a blinded examiner.

STATISTICAL ANALYSIS

Mean values of alveolar bone density were determined for each group and compared statistically using the one-way ANOVA test (α=0.05). Pair wise multiple comparisons were carried out by the Tukey test (α=0.05) in case the ANOVA test showed significant differences.

RESULTS

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

DISCUSSION

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

studies that suggest a negative influence of smoking on bone quality in areas also investigated for osteoporosis[15,24-26].

This investigation is part of a series of studies that initially intended to evaluate, in rats, the influence of CSI on periodontitis progression and on bone healing around titanium implants. It was observed that CSI decreased bone healing around the implants[21,22], and promoted a higher rate of periodontal breakdown[23]. It was also observed a negative effect of CSI on the pre-existing bone in the tibiae[20]. It was further radiographically investigated the impact of smoking on the skeletal and oral bone quality (tibiae and mandible, respectively) in rats submitted to CSI in comparison to non-exposed animals[27,28]. 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[20,27,28], and additionally show that the tooth-supporting bone might also be significantly affected by cigarette smoke inhalation.

Although clinical studies have previously reported a negative influence of smoking on skeletal bone, in regions most affected by osteoporosis[15,24-26] as well as in a group at low risk for osteoporosis[25], and in alveolar bone height and quality in a group of post-menopausal women under a periodontal maintenance program[5]; the results of the present study are very original information on the impact of cigarette smoke on the tooth-supporting alveolar bone quality. Our findings here agree with a previous study[29] that showed that high concentrations of nicotine might present a direct effect on the tooth-supporting alveolar bone.

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 nonsmokers concerning hormonal profile[5,15,30-32]. In vitro studies have shown that cigarette smoke compounds have citotoxic 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[33]. Henemyre et al.[34] observed that, at the 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 (CSE) was evaluated on human osteoprogenitor cells and osteoblast-like cells. CSE inhibited the proliferation of osteoprogenitor cells and the differentiation of osteoprogenitor cells toward osteoblast-like cells[35]. The chemotactic response of

both cell types for fibronectin and PDGF-BB, important molecules for bone repair and remodeling, was inhibited by CSE[36]. CSE also inhibited the production of fibronectin by both cell types[36]. Since, the process of bone remodeling is the mechanism by which bone renews itself and remains structurally competent, alterations on the cellular events involved in remodeling may be one of the means by which smoking affects bone density.

The present investigation additionally reported a beneficial effect of CSI cessation on bone. The reversible effect of smoking on bone has been previously documented in medicine with respect to its density[15,24,32,37,38] and fracture risk[37,39]. A meta-analysis study demonstrated that current smokers presented a significantly reduced bone density when compared to former and never smokers[15] and, that former smokers presented bone density that is intermediate or similar to never smokers[15,24]. The influence of smoking on fracture risk was investigated in 59232 men and women from 10 prospective cohort studies[39]. 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[39]. 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 periodontal cells[40,41]. Smoking cessation also exerted a beneficial effect on periodontal risk, which decreased with the number of years since quitting[42]. A prospective study over 20 years showed that patients who stopped smoking lost significantly less marginal bone than current smokers[43]. Furthermore, it was demonstrated that CSI cessation positively impacted on the bone loss rate resulting from ligature-induced periodontitis and it also benefited mandibular bone quality[28]. In the implant field Bain[44] was the first to report that a smoking cessation protocol would improve success rates for osseointegration in smokers who follow it. The results of the present investigation are in agreement 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 CSI on the alveolar bone around clinically healthy teeth, and reinforce the hypothesis that like skeletal bone, oral bone may be influenced. However, it remains to be investigated whether smoking influences bone loss resulting from periodontitis and affects the bone healing around titanium implant by the same mechanism that it affects the tooth-supporting

bone around healthy teeth. Based on previous findings[29], nicotine may at least partially take part, but further studies should be considered in order to better elucidate the mechanisms by which smoking affects alveolar bone.

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Figure1: Schematic illustration of the analyzed area.

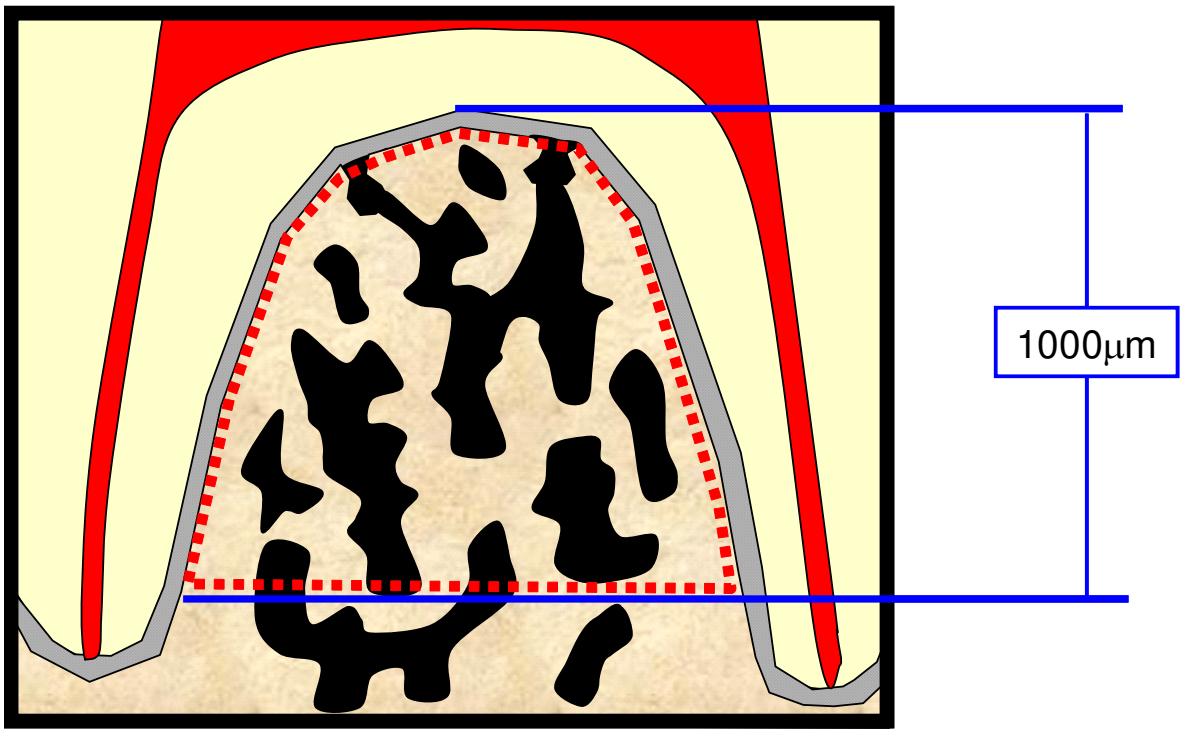
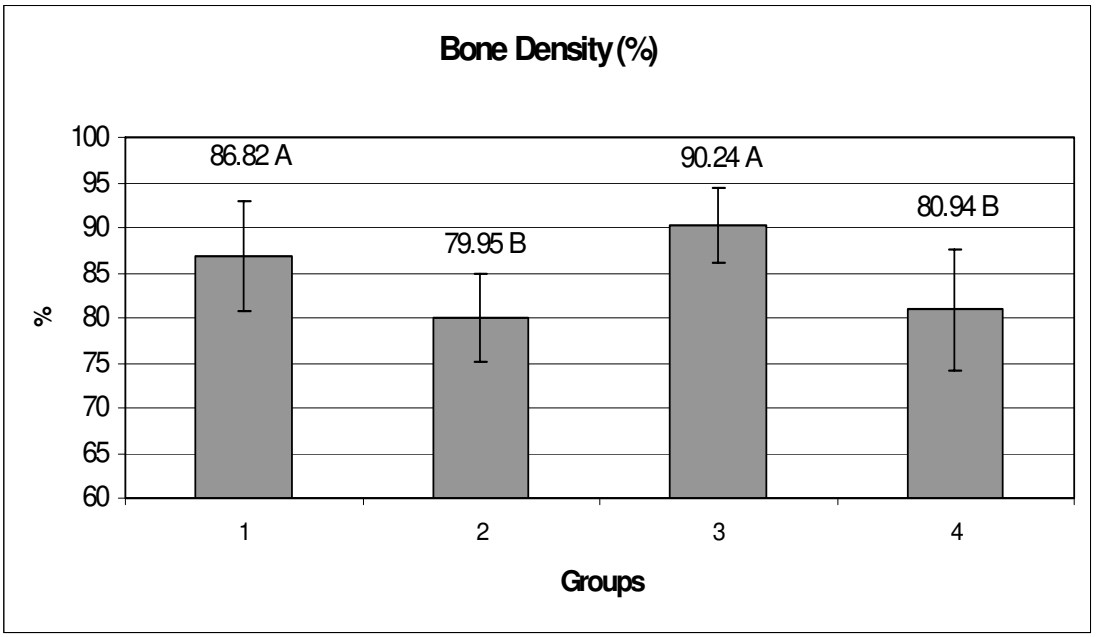
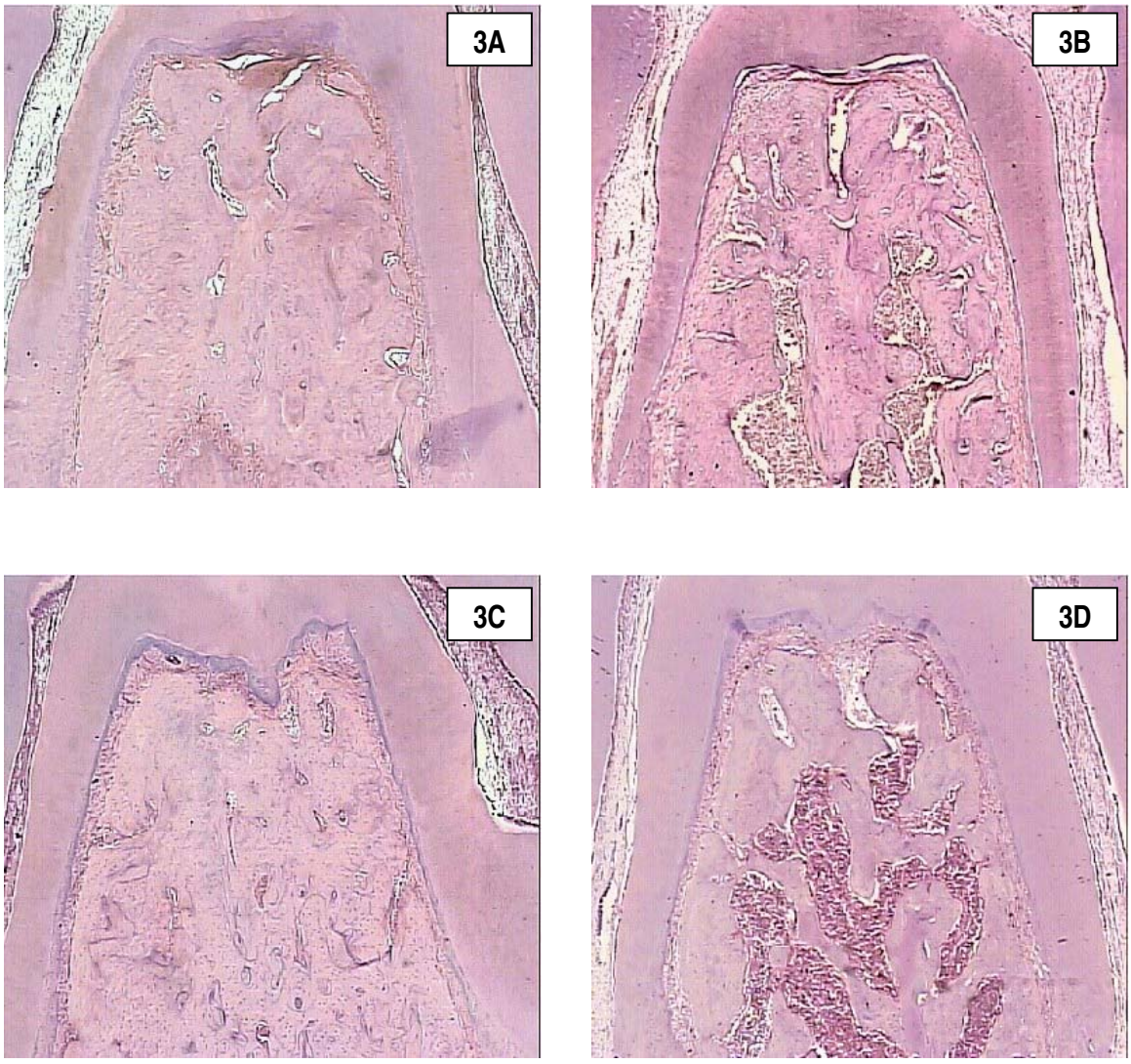


Figure 2: Mean and standard deviation (%) representative of the furcation bone density, for groups 1, 2, 3 and 4.



Different letters represent significant differences (Intergroup analysis; ANOVA - $\alpha = 5\%$)

Figure 3: Photomicrographs 2A to 2D illustrate the histological aspect of the furcation bone for groups 1, 2, 3 and 4, respectively. Original Magnification 12.5x - Hematoxylin and Eosin.



4 DISCUSSÃO GERAL

O presente trabalho avaliou histologicamente o efeito da fumaça de cigarro e a sua interrupção sobre o osso da tíbia após a colocação de implantes, o osso alveolar de suporte e a progressão da periodontite induzida. Esse conjunto de estudos objetiva documentar em modelo animal, a existência de fenômenos histológicos que possam contribuir para a elucidação dos resultados observados clinicamente. Os estudos avaliando o tecido ósseo ao redor de implantes de titânio demonstraram que a inalação da fumaça de cigarro exerce um efeito negativo no osso preexistente e neoformado, promovendo uma menor densidade óssea na região medular adjacente ao implante, um menor preenchimento das roscas e um menor contato osso-implante. Esses resultados estão em concordância com estudos clínicos que demonstraram a influência do tabaco na taxa de sucesso de implantes osseointegráveis (BAIN & MOY, 1993; BAIN, 1996), na reparação de fraturas (PORTER & HANLEY, 2001; ADAMS *et al.*, 2001) e na fusão espinhal cirúrgica (GLASSMAN *et al.*, 2000). Além disso, corroboram com os estudos de UENG et al. (1997 e 1999) que observaram, através de uma análise histológica e de testes de resistência à torção, um impacto negativo da fumaça de cigarro (utilizando um modelo semelhante ao do presente estudo) sobre a reparação óssea de tíbias de coelhos após distração osteogênica.

Outro objetivo de nossos estudos foi avaliar o efeito da nicotina, em comparação a fumaça de cigarro, sobre o reparo ósseo ao redor de implantes. Os resultados mostraram que a nicotina é responsável por parte dos efeitos adversos do fumo sobre o osso ao redor de implantes e sugere que outros componentes da fumaça podem ter importância, uma vez que os valores de contato osso-implante e preenchimento das roscas do grupo nicotina foram intermediários entre o grupo que recebeu fumaça de cigarro e o grupo controle. Este resultado diverge dos estudos iniciais realizados em nosso laboratório os quais não observaram um efeito negativo da nicotina sobre o reparo ósseo ao redor de implantes inseridos em tíbias de coelhos (STEFANI *et al.*, 2002; NOCITI *et al.*, 2002). Entretanto esses estudos prévios utilizaram outro modelo experimental (coelhos) e uma menor concentração de nicotina (0,93 mg/kg vs. 3 mg/kg do presente estudo) administrada em menor freqüência (1vez/dia vs. 2vezes/dia do presente estudo). Estudos utilizando outros desenhos experimentais também apresentaram resultados diversos (SALDANHA *et al.*, 2004; PINTO *et al.*, 2002; HOLLINGER *et al.*, 1999; AKHTER *et al.*, 2003; IWANIEC *et al.*, 2001; IWANIEC, *et al.*, 2000) e alguns deles ressaltaram a importância da concentração da droga e freqüência de administração

para o efeito da nicotina (PINTO *et al*., 2002; HOLLINGER *et al*., 1999). Esses estudos corroboram com nossos achados e reforçam a hipótese de que, além do modelo, a dose e freqüência de administração também podem ter contribuído para as diferenças entre os resultados de estudos prévios de nosso laboratório e a presente investigação (STEFANI *et al*., 2002 e NOCITI *et al*., 2002 vs. Capítulo 2).

Durante a segunda fase das investigações sobre implantes, avaliou-se o impacto da interrupção permanente e temporária do consumo de cigarros sobre os parâmetros descritos anteriormente (densidade do osso preexistente, contato osso-implante e preenchimento das roscas). Observou-se que ambos os regimes de interrupção foram efetivos para diminuir os efeitos negativos do fumo ao redor dos implantes. Este resultado está de acordo com o estudo clínico de BAIN (1996), no qual ex-fumantes e pacientes que seguiram um protocolo de interrupção do consumo de cigarros (interrupção uma semana antes da colocação de implantes, voltando a fumar apenas 8 semanas após a cirurgia) apresentaram índices de sucesso semelhantes a pacientes que nunca fumaram. Os resultados do presente estudo e de BAIN (1996) sugerem que tanto a interrupção temporária quanto definitiva influenciam de maneira positiva a taxa de sucesso de implantes. Entretanto, estes resultados devem ser interpretados com cuidado, uma vez que não existem estudos clínicos de longa duração avaliando pacientes que seguiram o protocolo proposto por BAIN (1996). Assim não se sabe se os bons resultados obtidos em curto prazo são mantidos ao longo do tempo nos pacientes que voltaram a fumar após a colocação de implantes. Resultados semelhantes (aos obtidos com implantes) foram observados na avaliação sobre o osso alveolar, onde os animais que receberam fumaça de cigarro apresentaram uma menor densidade óssea na região da bifurcação e este efeito negativo era revertido após a interrupção da inalação de fumaça. Dentro dos limites de nosso conhecimento, este é o primeiro estudo que mostra, em nível histológico, o efeito da IFC sobre o osso alveolar saudável e confirma a hipótese de que o consumo de cigarros afeta tanto o osso esquelético quanto os ossos da face. Estes resultados estão de acordo com investigações clínicas sobre o efeito do tabagismo na densidade óssea (WARD & KLESGES, 2001; ORTEGO-CENTENO *et al*., 1997; HOLLENBACH *et al*., 1993), incidência de osteoporose e osteopenia (WARD & KLESGES, 2001; HOLLENBACH *et al*., 1993) e risco a fraturas (KANIS *et al*., 2004), os quais mostraram um efeito reversível do consumo de cigarros sobre essas condições.

Além das análises com implantes de titânio esta série de estudos pesquisou os efeitos do consumo de cigarros e sua interrupção sobre a perda óssea resultante da periodontite induzida em ratos. Estes resultados confirmaram histologicamente os resultados de estudos clínicos (TOMAR & ASMA, 2000) e radiográficos (JANSSON & LAVSTEDT, 2002), em humanos, que demonstraram um efeito potencializador do tabagismo sobre a perda dos tecidos de suporte resultante da periodontite. Além disso, nossos resultados sugerem que a MMP-2 pode ser uma das moléculas associadas com a maior destruição periodontal observada nos fumantes. A MMP-2 é uma das principais enzimas envolvidas na degradação de tecidos moles (SHAPIRO *et al.*, 1998) e, embora o papel da MMP-2 na doença periodontal não esteja totalmente estabelecido, evidências têm mostrado que esta enzima está aumentada no tecido gengival de pacientes com periodontite (KOROSTOFF *et al.*, 2000). Alguns estudos também observaram níveis aumentados de TNF-α no fluido gengival de fumantes com periodontite (BOSTROM *et al.*, 1998 e 1999) e uma maior expressão de IL-1 nos monócitos do sangue periférico de tabagistas (RYDER *et al.*, 2002). Estes resultados reforçam a hipótese de que a MMP-2 pode estar relacionada à patogênese da DP em fumantes, uma vez que a IL-1 e o TNF-α são as principais moléculas pró-reguladoras da produção de MMPs. Observou-se ainda, nos estudos sobre a periodontite, que os animais que deixaram de receber fumaça de cigarro comportaram-se de maneira similar aos animais do grupo controle (que não receberam fumaça em nenhum momento durante o período experimental), apresentando níveis de perda óssea semelhante a este grupo. Alguns estudos anteriores já haviam sugerido que os efeitos do consumo de cigarros sobre o periodonto podem ser reversíveis. Em um estudo sobre culturas de fibroblastos do ligamento periodontal observou-se que a nicotina (PEACOCK *et al.*, 1993), a acroleína e o acetaldeído (CATTANEO *et al.*, 2000) (componentes da fumaça de cigarro) exerciam um efeito tóxico sobre estas células, afetando sua adesão e proliferação. Entretanto, este efeito era revertido quando estas moléculas eram removidas do meio de cultura. Dados do maior estudo epidemiológico sobre consumo de cigarros e doença periodontal (onde foram avaliados 12329 adultos) mostraram que os fumantes apresentaram um risco de ter periodontite 4 vezes maior que os não-fumantes, enquanto os ex-fumantes mostraram um risco 1,68 vezes maior (em comparação aos não-fumantes) (TOMAR & ASMA, 2000). Além disso, entre os ex-fumantes, o risco diminuía de acordo com o número de anos desde que o paciente deixou de fumar (3,22 após 2 anos e 1,15 após 11 anos). Em um estudo prospectivo, 507 indivíduos foram acompanhados através de radiografias por 20 anos e os

resultados deste estudo mostraram que os pacientes que deixaram de fumar durante o período experimental perderam significantemente menos osso marginal quando comparados aos que fumaram durante todo o período de acompanhamento (JANSSON *et al*., 2002). A interrupção do consumo de cigarros também tem demonstrado um benefício sobre os resultados da terapia periodontal. Grossi *et al.* (1997) observaram que ex-fumantes e não-fumantes apresentaram uma maior melhora nos parâmetros clínicos e uma maior redução de *bacteroides forsythus* e *porphyromonas gingivalis* do que os fumantes atuais. Portanto, os resultados do presente estudo estão de acordo com os resultados de estudos clínicos e indicam que o efeito negativo do tabagismo sobre o periodonto parece ser uma condição reversível.

Os mecanismos pelos quais o tabagismo afeta o tecido ósseo não estão totalmente entendidos. Estudos *in vitro* têm mostrado que os componentes da fumaça de cigarro têm efeitos citotóxicos nas células responsáveis pelo metabolismo e remodelação óssea. A nicotina tem apresentado um efeito bifásico sobre culturas de osteoblastos, mostrando efeitos antiproliferativos em altas concentrações e estimulatórios em níveis muito baixos (WALKER *et al*., 2001). HENEMYRE *et al.* (2003) observaram que, em níveis clinicamente relevantes, a nicotina não apresenta toxicidade sobre osteoclastos. Ela parece estimular a diferenciação osteoclástica e a reabsorção de cálcio e fosfato, importantes componentes do tecido ósseo. A influência da fumaça de cigarro (FC) sobre células osteoprogenitoras humanas e células tipo-osteoblasto também tem sido avaliada. A FC inibiu a proliferação das células osteoprogenitoras e sua diferenciação em células tipo osteoblasto (LIU *et al*., 2001). A resposta quimiotática de ambas as células à fibronectina e ao PDGF-BB, moléculas importantes para o reparo e a remodelação óssea, foi inibida pela FC (LIU *et al*., 2003). A FC também inibiu a produção de fibronectina por ambos os tipos celulares (LIU *et al*., 2003). Uma vez que o processo de remodelação óssea é o mecanismo pelo qual o osso se renova e mantém-se estruturalmente competente, as alterações nos eventos celulares envolvidos na remodelação pode ser uma das maneiras pelo qual o tabagismo influencia a densidade óssea. Estes resultados também podem ser relevantes para o reparo ósseo, uma vez que, muitas das células e moléculas envolvidas na remodelação participam da formação óssea. Além disso, é importante destacar que o consumo de cigarros de tabaco pode gerar uma menor nutrição dos tecidos e este fenômeno pode ter influência, principalmente nos tecidos em reparação. Essas áreas apresentam alta atividade metabólica necessitando de um grande suprimento sanguíneo e uma alta

disponibilidade de oxigênio. O monóxido de carbono da fumaça de cigarro liga-se à hemoglobina nos capilares pulmonares e forma um composto altamente estável. Nessa forma, a hemoglobina não transporta oxigênio (O_2), pois ambos os gases reagem com os mesmos grupamentos da molécula (KLAASSEN, 1996). Como a afinidade pelo CO é cerca de 220 vezes maior que pelo O_2, o CO apresenta seus efeitos mesmo em baixas concentrações (KLAASSEN, 1996). A quantidade de O_2 disponível para os tecidos é ainda menor pela influência inibitória da carboxihemoglobina na dissociação de qualquer molécula de oxihemoglobina ainda disponível (KLAASSEN, 1996). Este mecanismo pode ser de particular relevância para os osteoblastos que precisam de altas taxas de oxigênio para a produção de tecido osteóide e são altamente sensíveis a variações na disponibilidade deste gás. Os fenômenos descritos acima podem, em conjunto ou isoladamente, fornecer bases biológicas para os resultados do presente estudo.

Embora um extenso número de estudos tenha avaliado a influência do tabagismo sobre o tecido ósseo, a taxa de sucesso de implantes e os tecidos periodontais, alguns pontos ainda necessitam de mais investigações. Em relação à doença periodontal mais estudos são necessários para esclarecer os mecanismos de progressão da doença em fumantes. Estudos futuros também são necessários no intuito de investigar se o tabagismo afeta a perda óssea resultante da periodontite e o reparo ósseo ao redor dos implantes de titânio pelos mesmos mecanismos pelos quais afeta diretamente o osso alveolar e se essa influência direta no osso alveolar poderia ser mais um fator que favorece a perda óssea durante a periodontite. Em um estudo recente, Bain *et al.* (2002) observaram que quando os fumantes eram reabilitados com implantes de superfície tratada (osseotite-3I) suas taxas de sucesso foram semelhantes a dos não-fumantes. Logo, estudos histológicos são necessários para investigar se evidências obtidas neste nível podem dar suporte a este resultado clínico. Além disso, não existem evidências, em longo prazo, sobre pacientes que seguiram um protocolo de interrupção do tabagismo no período peri-operatório e depois voltaram a fumar, ou antigos não-fumantes que iniciaram o consumo de cigarros após a colocação de implantes, portanto não se sabe o comportamento dos implantes frente a estas situações. Embora algumas dúvidas ainda persistam, os resultados do presente estudo são uma importante contribuição, pois demonstram histologicamente que os efeitos negativos do fumo sobre o periodonto e o tecido ósseo são reversíveis. Esses achados podem refletir diretamente sobre os resultados clínicos, uma vez que o consumo de cigarros pode ser considerado um fator de risco

“controlável” para falha de implantes e doença periodontal. Assim, o aconselhamento dos pacientes em relação aos prejuízos que o consumo de cigarros pode promover na saúde geral, periodontal e para o resultado dos implantes, sugerindo o abandono desse hábito, deve fazer parte do tratamento odontológico.

5 Conclusões Gerais

Baseado nos objetivos deste estudo e dentro de suas limitações pode-se concluir que:

1- A inalação da fumaça de cigarro influencia negativamente o osso preexistente e o osso neoformado ao redor de implantes de titânio;

2- A nicotina é responsável por parte dos efeitos do tabagismo sobre o tecido ósseo ao redor de implantes;

3- O tabagismo exerce um efeito negativo direto sobre o tecido ósseo que não está em reparo e este efeito pode ser observado tanto nos ossos do esqueleto quanto orais;

4- Tanto a interrupção temporária quanto definitiva, da inalação da fumaça de cigarro, exercem um efeito positivo sobre o tecido ósseo ao redor de implantes de titânio;

5- A interrupção definitiva da inalação da fumaça de cigarro exerce efeitos positivos sobre os ossos do esqueleto e orais, sugerindo que os efeitos negativos do fumo sobre os ossos são reversíveis;

6- A inalação da fumaça de cigarro potencializa a perda óssea produzida pela doença periodontal induzida por ligadura e promove um aumento dos níveis de MMP-2 no tecido gengival adjacentes a sítios com periodontite;

7- A interrupção da inalação da fumaça de cigarro exerce um efeito positivo sobre a progressão da periodontite induzida.

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ANEXOS

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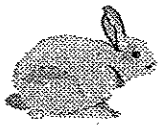

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Comissão de Ética na Experimentação Animal
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C E R T I F I C A D O

Certificamos que o Protocolo nº **467-2**, sobre "**Efeito de um Protocolo de Interrupção da Inalação da Fumaça de Cigarro sobre o Reparo Ósseo ao Redor de Implantes de Titânio: Estudo Histomérico em Ratos**", sob a responsabilidade de **Prof. Dr. Enilson Antonio Sallum** está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal (CEEA)-IB-UNICAMP em reunião de **21 de Março de 2003**.

C E R T I F I C A T E

We certify that the protocol nº **467-2**, entitled **"Effect of a Protocol of Cigarette Smoking Inhalation Interruption on Bone Healing Aroud Titanium Implants: a Histometric Study in Rats"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - UNICAMP) on **March 21, 2003**.

Campinas, 21 de Março de 2003.

Profa. Dra. Liana Verinaud
Presidente
CEEA/IB/UNICAMP

Fátima Alonso
Secretária Executiva
CEEA/IB/UNICAMP

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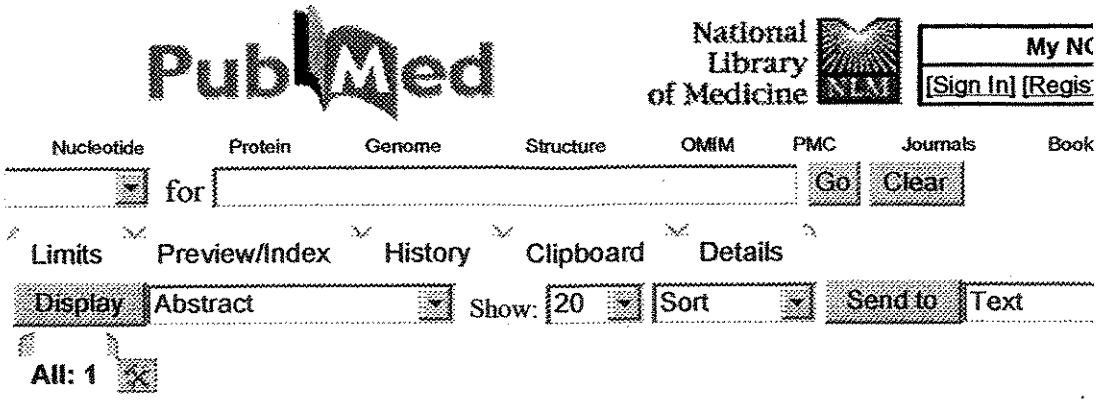

☐ 1: Int J Oral Maxillofac Implants. 2002 May-Jun;17(3):347-52. Related Articles, Li

Bone density around titanium implants may be influenced by intermittent cigarette smoke inhalation: a histometric study in rats.

Nociti FH Jr, Cesar NJ, Carvalho MD, Sallum EA.

Department of Prosthodontics and Periodontics, School of Dentistry at Piracicaba, Sao Paulo, Brazil. nociti@fop.unicamp.br

PURPOSE: This study investigated the influence of cigarette smoke on bone healing around titanium implants placed in rats. MATERIALS AND METHODS: After administration of anesthesia, the tibia surface was expose and screw-shaped titanium implants (4.0 mm in length and 2.2 mm in diameter) were placed bilaterally (1 each side). The animals (n = 32) were randomly assigned to either group 1 (control, n = 18) or group 2 (intermittent cigarette smoke inhalation, n = 14). After 60 days, the animals were sacrifice and undecalcified sections obtained. Bone density (the proportion of mineralized bone in a 500-microm-wide zone lateral to the implant) was measured in the cortical (zone A) and cancellous bone (zone B) areas. RESULTS: In zone A, a slight difference in bone density was noted between the groups (96.18% +/- 1.08% and 95.38 +/- 1.17% in groups 1 and 2, respectively; P > .05) but was not statistically significant. In contrast, bone density was significantly decreased in zone B in the animals that were expos to cigarette smoke (17.57 +/- 6.45% and 11.30 +/- 6.81% for groups 1 and 2, respectively; P < .05). DISCUSSION: Whether different results would be observed if animals were exposed to cigarette smoke for a longer period of time and/or before implant placement remains to be investigated. CONCLUSION: Although intermittent cigarette smoke exposure may not seriously affect cortical bone density, it may jeopardize bone quality around titanium implants in the cancellous bone area.

PMID: 12074449 [PubMed - indexed for MEDLINE]

☐ 1: J Periodontol. 2003 Oct;74(10):1454-9. Related Articles, Li

A comparative study on the effect of nicotine administration and cigarette smoke inhalation on bone healing around titanium implants.

Cesar-Neto JB, Duarte PM, Sallum EA, Barbieri D, Moreno H Jr, Nociti FH Jr.

Department of Prosthodontics and Periodontics, Division of Periodontics, School of Dentistry at Piracicaba, UNICAMP, Piracicaba, Sao Paulo, Brazil.

BACKGROUND: A series of isolated studies has focused on the influence o smoking on bone around titanium implants. This study proposes to investigat the impact of two conditions, i.e., nicotine administration and cigarette smok inhalation, on the healing around implants. METHODS: Forty-five Wistar ra were used. After anesthesia, the tibiae surface was exposed and a screw-shap titanium implant was placed bilaterally. The animals were randomly assigned to one of the following groups: Group 1: control, n = 19; Group 2: intermitte cigarette smoke inhalation, n = 15; and Group 3: subcutaneous administratio of nicotine (3 mg/kg) twice daily, n = 11. After 60 days, the animals were sacrificed. The degree of bone-to-implant contact (BIC) and the bone area (BA) within the limits of the threads of the implant were measured in the cortical (zone A) and cancellous bone (zone B) areas. RESULTS: In zone A, cigarette smoke presented a significant negative influence on BIC and BA (Kruskal-Wallis test, $P < 0.05$). In contrast, the administration of nicotine did not influence either parameter ($P > 0.05$). In zone B, cigarette smoke inhalati also resulted in a decreased percentage of BIC compared to the control group ($P < 0.05$). In addition, the BA was significantly decreased in groups 2 and 3 when compared to controls ($P > 0.05$). CONCLUSION: The negative impact smoking on implant outcomes may be related to more than one molecule present in the cigarette smoke and nicotine seems to partially contribute, especially in the cancellous bone.

PMID: 14653391 [PubMed - indexed for MEDLINE]

1: J Periodontol. 2004 Jul;75(7):995-1000.

Matrix metalloproteinase-2 may be involved with increased bon loss associated with experimental periodontitis and smoking: a study in rats.

Cesar Neto JB, de Souza AP, Barbieri D, Moreno H Jr, Sallum EA, Noci FH Jr.

Department of Prosthodontics and Periodontics, Division of Periodontics, School of Dentistry at Piracicaba, University of Campinas, Piracicaba, Sao Paulo, Brazil.

BACKGROUND: Smoking has been associated with periodontitis severity a is considered a risk factor for its development. It has been reported that matri metalloproteinase (MMP) produced by host cells plays a major role in periodontal tissue destruction. Thus, the present study tested, in rats, the hypothesis that local increased levels of MMP-2 would be associated with th enhanced periodontitis-related bone loss after intermittent cigarette smoke inhalation (CSI). METHODS: Twenty-seven adult male Wistar rats were use A ligature was placed around one of the mandibular first molars of each anin and they were randomly assigned to the following control (N = 13) or CSI (N 14) group. Sixty days later, the animals were sacrificed, the gingival tissues harvested, and the specimens processed for decalcified sections. Extracts fro the gingival tissues were prepared and assayed for MMP-2 expression. RESULTS: Intergroup comparisons (unligated sites) showed that CSI might directly affect alveolar bone (0.16 +/- 0.03 mm2 versus 0.24 +/- 0.09 mm2 fc non-smokers and smokers, respectively; P = 0.001). Moreover, CSI significantly enhanced bone loss resulting from experimental periodontitis (0.64 +/- 0.36 mm2 versus 1.50 +/- 0.50 mm2 for non-smokers and smokers, respectively; P<0.05). In addition, zymography demonstrated that CSI also enhanced both MMP-2 levels and activity in the gingival tissues around ligat teeth. CONCLUSION: Within the limits of the present investigation, it can b assumed that the effect of CSI on MMP-2 levels and activity may account fo the increased periodontitis progression rate observed in smokers.

PMID: 15341358 [PubMed - indexed for MEDLINE]

1: J Periodontol. 2005 Apr;76(4):520-5.

Smoking cessation may present a positive impact on mandibular bone quality and periodontitis-related bone loss: a study in rats.

Cesar-Neto JB, Benatti BB, Neto FH, Sallum AW, Sallum EA, Nociti FH.

Department of Prosthodontics and Periodontics, Division of Periodontics, School of Dentistry at Piracicaba, University of Campinas, Piracicaba, Sao Paulo, Brazil.

Background: It has been previously shown that cigarette smoke inhalation (CSI) enhances bone loss in ligature-induced periodontitis. In this study, the hypothesis that the interruption of smoke exposure would reverse the impact of CSI on mandibular bone quality and periodontitis-related bone loss was tested. Methods: Fifty-three Wistar rats were randomly assigned to one of the following groups: group 1: control, N = 16; group 2: 83 days of CSI prior to ligature placement, N = 17; or group 3: 90 days of CSI before and 60 days after ligature placement, N = 20. Animals were sacrificed 60 days after ligature placement, the jaws removed and immediately radiographed for photodensitometry analysis. Bone loss was histometrically evaluated. Results: CSI did not affect unligated sites in either condition (P >0.05); however, smoke inhalation during the whole experimental period significantly enhanced bone loss in ligated teeth (P <0.05). Moreover, similar levels of bone loss were observed for ligated teeth between the control and cessation groups (0.90 +/- 0.33 mm(2); 0.96 +/- 0.32 mm(2); 1.64 +/- 0.65 mm(2); groups 1, 2 and 3, respectively). Radiographically, continuous exposure to cigarette smoke promoted a significantly reduced bone density (1.74 +/- 0.38 aluminum equivalence [Al eq]; 1.74 +/- 0.14 Al eq; and 0.68 +/- 0.10 Al eq for groups 1, 2, and 3, respectively). Conclusions: Within the limits of the present investigation, it can be assumed that CSI may enhance bone loss in ligatureinduced periodontitis, and negatively impact mandibular bone quality. Additionally, smoke exposure cessation seems to reverse its impact on mandibular bone, and, therefore, may be of clinical relevance. J Periodontol 2005;76:520-525.

03 25 2005
>
> Dear Prof. Nociti,
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> I am pleased to inform you that your manuscript, BONE FILLING
> AROUND TITANIUM IMPLANTS MAY BENEFIT FROM SMOKING CESSATION. A
> HISTOLOGIC STUDY IN RATS (JOP-04-0352.R1), is accepted for
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----- Original Message -----
From: QuinJOMI@aol.com
To: nociti@fop.unicamp.br
Sent: Monday, April 04, 2005 1:33 PM
Subject: Re: Ms # 04-800

Dear Dr. Nociti:
Correction: the MS # is 04-8400. The revision has been received & likely will be published later this year.
Sincerely,
Dr. W. R. Laney

São Paulo, 28 de abril de 2005.

Prezados autores João Batista César-Neto, Bruno Braga Benatti, Flávio Ricardo Manzi, Enilson Antônio Sallum, Antônio Wilson Sallum, Francisco Humberto Nociti Junior,

Seu artigo "The influence of cigarette smoke inhalation on bone density. A radiographic study in rats" encontra-se em fase de revisão para publicação na Revista Brazilian Oral Research.

Para que possamos dar continuidade ao processo editorial, solicitamos o esclarecimento das seguintes dúvidas:

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3/18/2005

Dr. Francisco Humberto Nociti Jr
Faculdade de Odontologia de Piracicaba
Av. Limeira 901
Caixa Postal: 052, CEP: 13414-903
Piracicaba, SP BRAZIL

Dear Dr. Nociti:

Many thanks for your manuscript entitled "The influence of cigarette smoke inhalation and its cessation on the tooth-supporting alveolar bone. A histometric study in rats". As soon as the Editorial Board has reached a decision on it, you will receive further notice.

Sincerely yours,

Jørgen Slots