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Efeitos da exposição ao fumo passivo nos estágios iniciais do desenvolvimento ósseo.

Effects of the secondhand smoking exposure in the early stages of the bone development.

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Dissertação apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Mestre em Biologia Buco Dental, na Área de anatomia.

Dissertation presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Master in Oral Biology, in Anatomy area.

Orientador: Prof. Dr. Paulo Henrique Ferreira Caria.

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA DISSERTAÇÃO DEFENDIDA PELO ALUNO CRISTIANO FITTIPALDI ALVES E ORIENTADO PELO PROF. DR. PAULO HENRIQUE FERREIRA CARIA

Piracicaba
2019

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Odontologia de Piracicaba
Marilene Girello - CRB 8/6159

AL87e

Alves, Cristiano Fittipaldi, 1975-
Efeitos da exposição ao fumo passivo nos estágios iniciais do desenvolvimento ósseo / Cristiano Fittipaldi Alves. – Piracicaba, SP : [s.n.], 2019.

Orientador: Paulo Henrique Ferreira Caria.
Dissertação (mestrado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Poluição por fumaça de tabaco. 2. Osso esponjoso. 3. Microtomografia por raio-X. I. Caria, Paulo Henrique Ferreira, 1972-. II. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: Effects of the secondhand smoking exposure in the early stages of the bone development
Palavras-chave em inglês:
Tobacco smoke pollution
Cancellous bone
X-ray microtomography
Área de concentração: Anatomia
Titulação: Mestre em Biologia Buco-Dental
Banca examinadora:
Paulo Henrique Ferreira Caria [Orientador]
Cezar Augusto Souza Casarin
Michelle Franz Montan Braga Leite
Data de defesa: 15-02-2019
Programa de Pós-Graduação: Biologia Buco-Dental

Identificação e informações acadêmicas do(a) aluno(a)
- ORCID do autor: https://orcid.org/0000-0002-5649-4594
- Currículo Lattes do autor: http://lattes.cnpq.br/5371379589112986

UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Odontologia de Piracicaba

A Comissão Julgadora dos trabalhos de Defesa de Dissertação de Mestrado, em sessão pública realizada em 15 de Fevereiro de 2019, considerou o candidato CRISTIANO FITTIPALDI ALVES aprovado.

PROF. DR. PAULO HENRIQUE FERREIRA CARIA

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A Ata da defesa, assinada pelos membros da Comissão Examinadora, consta no SIGA/Sistema de Fluxo de Dissertação/Tese e na Secretaria do Programa da Unidade.

DEDICATÓRIA

Dedico este trabalho a todos os homens de Boa Vontade que neste momento fazem de sua vida uma verdadeira utilidade pública, a serviço de um Nobre e Grande Objetivo colocado neste planeta: a construção de um Mundo Bem Melhor.

AGRADECIMENTOS

Ao Prof. César Alexandre Fabrega Carvalho na FMJ e toda sua equipe. Ao departamento de morfologia da FOP-UNICAMP, professores e equipe técnica, e em especial ao meu amigo e orientador Prof. Paulo Henrique Ferreira Caria.

RESUMO

O objetivo deste estudo foi avaliar os efeitos do tabagismo passivo na microarquitetura óssea trabecular mandibular de ratos descendentes de matrizes tabagistas passivas. Cinquenta e cinco ratos, *Rattus norvegicus albinus* - *Wistar*, descendentes de matrizes tabagistas e não tabagistas, foram divididos em três grupos: descendentes tabagistas contínuos (DTC), descendentes tabagistas interrompidos (DTI) e descendentes não tabagistas (DNT / controle). Após 21º, 42º, 63º e 128º dias, suas mandíbulas foram analisadas por microtomografia computadorizada (micro-CT). Imagens do osso trabecular inter-radicular dos primeiros molares inferiores foram submetidas à reconstrução microtomográfica tridimensional e foram analisadas. A fração volumétrica óssea (BV/TV, volume ósseo / volume total), a espessura trabecular (Tb.Th), o espaçamento trabecular (Tb.Sp), o número trabecular (Tb.N) e o índice de modelo de estrutura (SMI) foram analisados. A análise BV/TV revelou aumento dos valores médios no grupo DTC, no 21º e 42º dias ($p = 0,0124$), diminuindo a partir do 42º dia. Os animais do grupo DTI não apresentaram diferença significativa em BV / TV, em relação ao grupo controle ($p = 0,9751$). Os resultados de Tb.Th foram, durante todo o período avaliado, maiores nos animais do grupo DNT, seguido dos DTI e DTC, com diferença entre os três grupos ($p<0,005$). Para os parâmetros Tb.Sp e Tb.N não foram encontradas diferenças entre os grupos avaliados (com $p=0,6680$ e $p=0,3308$, respectivamente). O SMI mostrou valores maiores para os DTC, seguido dos DTI e DNT, com diferença significativa entre os DTC e DNT, DTC e DTI, ambos com ($p <0,0001$). A diferença entre o DNT e DTI não foi significativa ($p = 0,1253$). A inalação da fumaça de cigarro de segunda mão pelos descendentes das matrizes tabagistas passivas, mostrou efeitos prejudiciais na microarquitetura do osso trabecular da mandíbula dos ratos em desenvolvimento. Diminuiu a elasticidade óssea, enfraquecendo o osso durante o amadurecimento da prole. Sobre os grupos interrompidos, a recuperação da microarquitetura ocorreu parcialmente.

Palavras Chave: Fumo passivo, Descendentes tabagistas, Osso trabecular mandibular, Microtomografia computadorizada

ABSTRACT

Purpose. To evaluate the effect of secondhand passive smoking on the trabecular mandibular bone micro-architecture in the rats, offspring of passive smoking matrices. **Materials and Methods.** Fifty-five rats, *Rattus norvegicus albinus*, offspring of passive smoking and non-passive smoking matrices, were divided into three groups: continuous smoking offspring (CSO), interrupted smoking offspring (ISO) and non-smoking offspring (NSO/control). After the 21st, 42nd, 63rd and 128th days, the mandibles were analyzed by microcomputed tomography (micro-CT). Images of inter-radicular alveolar bone of the mandibular lower first molars were submitted to three-dimensional microtomography reconstruction and were analyzed. The bone volume fraction (BV/TV, bone volume/total volume), trabecular thickness (Tb.Th), trabecular spacing (Tb.Sp), trabecular number (Tb.N) and structure model index (SMI) were analyzed. **Results.** The BV/TV analysis revealed an increase of the values average in the CSO group, at 21st and 42nd days ($p=0,0124$), tending to decrease from the 42nd day. The animals of the ISO group did not showed a significant difference in BV/TV, regarding the control group ($p=0,9751$). During the experimental period, the results of Tb.Th were higher in the NSO group, followed by the ISO and CSO, with differences among the three groups ($p<0,005$). For the parameters Tb.Sp and Tb.N were not found differences between the groups evaluated (with $p=0,6680$ and $p=0,3308$, respectively). The SMI showed greater values for CSO, followed by ISO and NSO, with significant differences between the CSO and NSO, CSO and ISO, both with $p<0,0001$. The difference between NSO control and ISO groups were not significant ($p=0,1253$). **Conclusion** The secondhand smoking inhalation of cigarette by the offspring of passive smoking matrices had a harmful effect in the microarchitecture of the trabecular bone mandible of the rats under development. Reduced bone elasticity, weakening the bone during aging of the offspring. About the ISO group, the recovery of the microarchitecture occurred partially.

Key Words : Secondhand smoke, smoking offsprings, bone, Microcomputed tomography**.**

SUMARIO

1. INTRODUÇÃO

A fumaça da ponta do cigarro aceso, além daquela exalada pelo fumante ativo, constitui aproximadamente 85% da fumaça inalada por um não fumante. É, também, quatro vezes mais tóxica por grama de material particulado total que a fumaça principal inalada pelo fumante ativo (Schick e Glantz, 2005). Assim, os efeitos tóxicos da fumaça do cigarro comprometem a reparação tecidual (Guo e Dipietro, 2010) em não-fumantes, devido à inalação passiva da fumaça do cigarro (Ajiro et al, 2010) (Santiago et al, 2017). Estima-se que 40% das crianças, 33% meninos e 35% meninas, estão expostas ao tabagismo passivo em todo o mundo (Ko et al, 2015). No Brasil, houve a redução de 36% na prevalência de fumantes ativos no país, dos iniciais 15,7% em 2006, para 10,1% em 2017. Contudo, a gravidade e o risco de surgimento de doenças ainda estão relacionados a fatores como o tempo de exposição à fumaça e a genética (http://www.actbr.org.br/post/cigarro-eletronico-entenda-se-o-polemico-aparelho-faz-mal-a-saude-ou-nao/17767). A toxicidade da fumaça do cigarro pode levar à diminuição da divisão celular, indução de danos ao DNA, aumento do tempo de cicatrização e a diminuição da capacidade de regeneração tecidual por reduzir a nutrição sanguínea em vários tecidos (Guo e Dipietro, 2010) (Prieme et al, 1998).

O dano arterial é irreversível (Guo X, et al, 2006) e aumenta o risco do desenvolvimento de doenças e de distúrbios como a osteoporose (Kanis et al, 2005) (Lee et al, 2013). Considerada uma desordem osteometabólica (Bouxsein, 2003) a osteoporose caracteriza-se pela redução da massa óssea e alterações microestruturais que causam fragilidade e risco de fraturas (Genant et al, 1999) (Legrand et al, 2000). A nicotina presente no cigarro inibe a função dos glóbulos vermelhos, causa vasoconstrição e reduz os níveis de oxigênio no sangue (Benowitz et al, 1983), além de reduzir os níveis de cálcio e estrogênio (*American Academy of Orthopaedic Surgeons*, 2019), substâncias importantes no formação e manutenção dos ossos (Liu et al, 2015). Também modula vários fatores de diferenciação, proliferação e crescimento de osteoblastos, podendo diminuir a expressão osteogênica durante a neoformação óssea (Wahl et al, 2016) (Marinucci et al, 2014), na cicatrização e na regeneração de fraturas (Glowacki et al, 2008). Os efeitos causados pela toxicidade da fumaça do cigarro na remodelação óssea sugerem

alterações na organização microestrutural e diminuição da densidade mineral óssea em fumantes passivos (Ajiro et al, 2010). O conceito de qualidade óssea evoluiu da densidade, apenas, para uma abordagem da microarquitetura estrutural (Wirth et al, 2011). A alteração da microarquitetura tridimensional e a redução do conteúdo mineral ósseo indicam um aumento do risco de fraturas (Inyang et al, 2014).

No entanto, existem controvérsias quanto à possibilidade e ao tempo de recuperação do tecido ósseo, tendo em vista os efeitos adversos da exposição passiva (Cesar Neto et al, 2005; Hapidin et al, 2011). A microtomografia é uma ferramenta precisa, rápida e não destrutiva que permite a medição de microestruturas em biópsias não-processadas e até mesmo em pequenos ossos, determinando automaticamente índices histomorfométricos tridimensionais (Müller et al, 1998). Assim, tornou-se um método eficaz para avaliar a influência da fumaça do cigarro sob a estrutura óssea (Santiago e Zamarioli, 2017). Considerados os elementos apresentados, o objetivo deste estudo foi avaliar os efeitos tóxicos da fumaça do cigarro sobre a microarquitetura do osso trabecular mandibular e sua recuperação após a interrupção da inalação em ratas de matrizes passivas de fumo.

2. ARTIGO: Effects of the secondhand smoking exposure in the early stages of the bone development.

Artigo submetido ao periódico PLOS ONE (anexo 1)

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Abstract

Purpose

To evaluate the effect of secondhand passive smoking on the trabecular mandibular bone micro-architecture in the rats, offspring of passive smoking matrices.

Materials and Methods

Fifty-five rats, Rattus norvegicus albinus, offspring of passive smoking and non-passive smoking matrices, were divided into three groups: continuous smoking offspring (CSO), interrupted smoking offspring (ISO) and non-smoking offspring (NSO/control). After the 21st, 42nd, 63rd and 128th days, the mandibles were analyzed by microcomputed tomography (micro-CT). Images of inter-radicular alveolar bone of the mandibular lower first molars were submitted to three-dimensional microtomography reconstruction. The bone volume fraction (BV/TV, bone volume/total volume), trabecular thickness (Tb.Th), trabecular spacing (Tb.Sp), trabecular number (Tb.N) and structure model index (SMI) were analyzed.

Results

The BV/TV analysis revealed an increase of average values in the CSO group, at 21st and 42nd days (p=0,0124), and decrease from the 42nd day. The animals of the ISO group did not showed a significant difference in BV/TV, regarding the control group (p=0,9751). During the experimental period, the results of Tb.Th were higher in the NSO group, followed by the ISO and CSO, with differences among the three groups (p<0,005). For the parameters Tb.Sp and

Tb.N were not found differences between the groups evaluated (with p=0,6680 and p=0,3308, respectively). The SMI showed greater values for CSO, followed by ISO and NSO, with significant differences between the CSO and NSO, CSO and ISO, both with p<0,0001. The difference between NSO control and ISO groups were not significant (p=0,1253).

Conclusion

The secondhand smoking inhalation from cigarette by the offspring of passive smoking matrices had a harmful effect in the microarchitecture of the trabecular bone mandible of the rats under development. Reduced bone elasticity, weakening the bone during aging of the offspring. About the ISO group, the recovery of the microarchitecture occurred partially.

Introduction

It is estimated that 40% of the world's children (being 33% of boys and 35% of girls) are exposed to secondhand smoke [1]. The cigarette smoke inhaled in a passive way by a non-smoking, called secondhand smoke, consists in 85% of the sidestream smoke, which is the result of the burning of the burning tip of the cigarette. The sidestream smoke, coming from the lighted cigarette, is considered four times more toxic than the mainstream smoke that is inhaled by the active smoker [2]. The secondhand smoke is considered the third largest cause of avoidable death worldwide and it is the main polluting agent in indoor environments [3]. Most cases of lung cancer deaths were observed in a study that tracked 9,1540 non-smoker spouses of active smokers for 14 years [4]. The cigarette smoke toxicity reduces the blood nourishment and it can cause a cell division reduction, DNA damage induction increased wound healing time and decreased tissue regenerative capacity [5-6]. Such reduction causes permanent artery damages [7] that increase the risk of diseases development and alterations such as osteoporosis [8-9]. The osteoporosis is an osteometabolic disorder [10] characterized by the bone mass loss and by microstructure alterations, which cause weakness and fracture risks [11-12]. The nicotine, main alkaloid present in cigarettes, suppresses the function of the red blood cells, causes vasoconstriction and reduces the blood oxygen levels [13], and also it reduces the calcium and estrogen levels [14], important substances in growth and bone maintenance [15]. The nicotine also modulates several factors of the osteoblasts differentiation, proliferation and growth and it can decrease during the bone neoformation, osteogenic expression [16-17], healing and fracture regeneration [18]. The effects caused by the cigarette smoke toxicity, inhaled in a passive way during the bone remodeling, suggest reduction of the bone mineral density and the alteration in the microstructural bone organization in passive smokers. [19]. The concept of bone quality, based only in density, has evolved towards the structural microarchitecture [20]. A recent study, showed that the trabecular bone microarchitecture weakness, besides the bone mineral content, indicates an increased risk of fractures [21].

However, there are controversies related to the possibility and the time of the bone tissue recovery due to the adverse effects of passive exposure to cigarette smoke [22-23]. Thus, the computed microtomography (mCT) is an effective tool to evaluate the cigarette smoke influence in the bone structure [24]. It

is an accurate, fast and non-destructive method that allows the measurement of the microstructures in non-processed biopsies and even in small bones, establishing automatically tridimensional histomorphometry rates [25].

Considering the reasons here presented, the purpose of this study was to evaluate the cigarette smoke toxic effects in the microarchitecture of mandibular trabecular bone in development and its possible recovering after the cessation of the inhalation, in passive smoker rats, offspring of smoking matrices, in different periods.

Materials and methods

Treatment of the matrices

This study was approved by the Animal Use Ethics Committee from the Jundiaí Faculty of Medicine, number 285/2013. Twenty matrices were composed of Rattus norvegicus albinus (Wistar), at 8-week-old, weighting 340 ± 10g. There were four male rats and four female rats in the control group, and there were six male rats and six female rats in the smoking group. They were kept at controlled environment temperature (22 ± 2 °C) and in a light and dark cycle of 12 hours, with water and food *'ad libitum'*.

Exposition to the Cigarette Smoking and Formation of the Experimental Groups

Commercially available cigarettes was used for the adaptation to smoke (tar: 10mg, and nicotine: 0,8mg). The matrices were exposed to the cigarette smoke gradually, until complete a total of 20 cigarettes units per day, for eight weeks. Then, couples were formed with the mating purpose. The lighted cigarettes were placed in the influx system of a vented shelf (smoking area) (fig.1) and homogeneously, the smoke of the lateral flux was spread into the sealed chambers [24]. During the gestation period, the offspring of passive smoking matrices were exposed to the smoke. After the weaning, 35 male offspring were selected and kept under the smoke exposition at the same initial conditions of their mothers, constituting 4 groups of continuous smoking offspring (CSO). The offspring submitted to the cessation of the inhalation – the group of interrupted smoking offspring (ISO), was formed from the CSO subdivision in a period equivalent to half of the exposure time to the cigarette smoke (Fig 2). It was not formed an ISO group in the first 21 days because it was a breastfeeding period, and the milk abstinence would be harmful to the bone development of the offspring. The non-smoking offspring (control group or NSO) were kept in a cigarette smoke-free exposure environment (Fig 2).

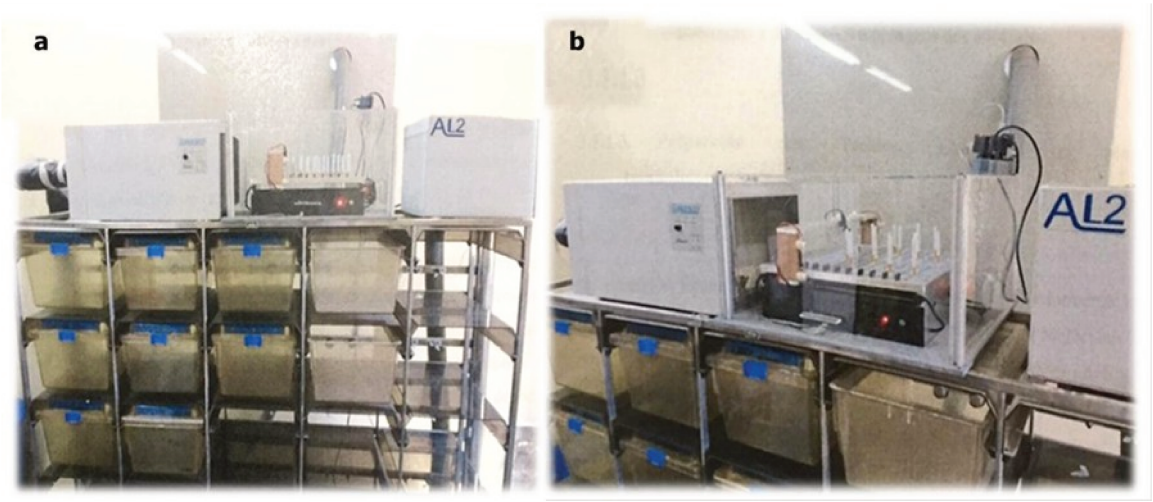

Fig 1. Vented shelf. a: frontal aspect, b: cigarettes put in the area of the influx system of the smoke for the sealed chambers.

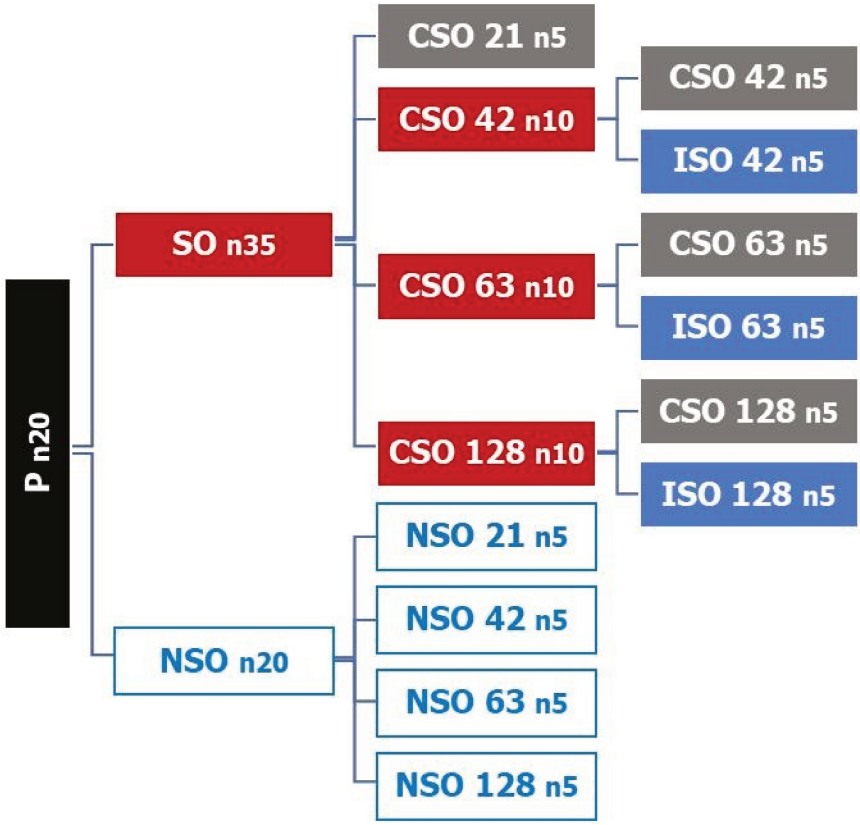

Fig 2. Organogram of the method used to obtain the three experimental groups: 1)CSO, Continuous Smoking Offspring (in gray); 2)ISO, Interrupted Smoking Offspring (in blue); and 3)NSO, Non-Smoking Offspring or control (in white). Where is P, progenitors (in black) and SO, Smoking Offspring (in red).

Microtomography bone analysis (mCT)

After the 21st, 42nd, 63rd and 128th days, the animals were sacrificed by deepening anesthesia (ketamine/xylazine) in accordance with the animal testing principles established by Brazilian College of Animal Experimentation and by the National Council for Control of Animal Experimentation (COBEA/CONCEA). After that, their mandibles were removed and stored in 10% buffered formaldehyde solution. Fifty-five samples were obtained (20 CSO, 20 NSO, and 15 ISO) and scanned in a computed microtomography (mCT) (SkyScan 1174, Kontich, Belgium), with 800 mA, 50kVp and exposure time of 3800ms, for each image. The image reconstruction was obtained from isotropic voxel, with a nominal resolution of 23μm, and an aluminum filter was used (thickness 0,5mm) according to the literature by Bouxsein et. al. [26]. The scanning procedure lasted around 20 minutes, resulting in 270 images per sample. A global thresholding technique was used to binarize the mCT images on a grayscale. The minimum values, between the apices in the gray value histogram, the bone marrow, and bone were chosen as limit values. The region of interest (ROI) of the alveolar bone was established manually in each image in the inter-root septum of the mandibular first molar (M1), on the right side, due tension concentration in this region, already used in the histomorphometry analysis of the trabecular bone. [27]. The ROI was obtained according to the method used by Liu et al. (2015) that enabled the evaluation and visualization of the alveolar bone in an integral way [28].

At the beginning was identified, in bidimensional images, the coronal surface which passes through the midst of the intermediate buccal and lingual roots (Fig 3-a). After that, was chosen two horizontal surfaces, separated each other, crossing the alveolar crest and the apex of the intermediate root buccal (lines 1 and 2, Fig 3 - a) parallel to the occlusion plane. The red portion shows the ROI in a bidimensional image (Fig 3-a and 3-b). Finally, in a horizontal plane which showed the M1, was chosen the inter-root alveolar bone drawing a bone outline from the midst of a root canal to another, avoiding the roots and other structures in the bidimensional image. (Fig 3-b). After the microtomography reconstruction of the images in the software connected to the system (NRecon SkyScan, Kontich, Belgium), the histomorphometry of the alveolar bone was obtained by the straight evaluation from the ROI 3D model, by the system software (CTAnalyser SkyScan, Kontich, Belgium). The following histomorphometric parameters were analyzed: the bone volume fraction (BV/TV), the trabecular thickness (Tb.Th), the trabecular spaces (Tb.Sp), the trabecular number (Tb.N) and the Structure Model Index (SMI).

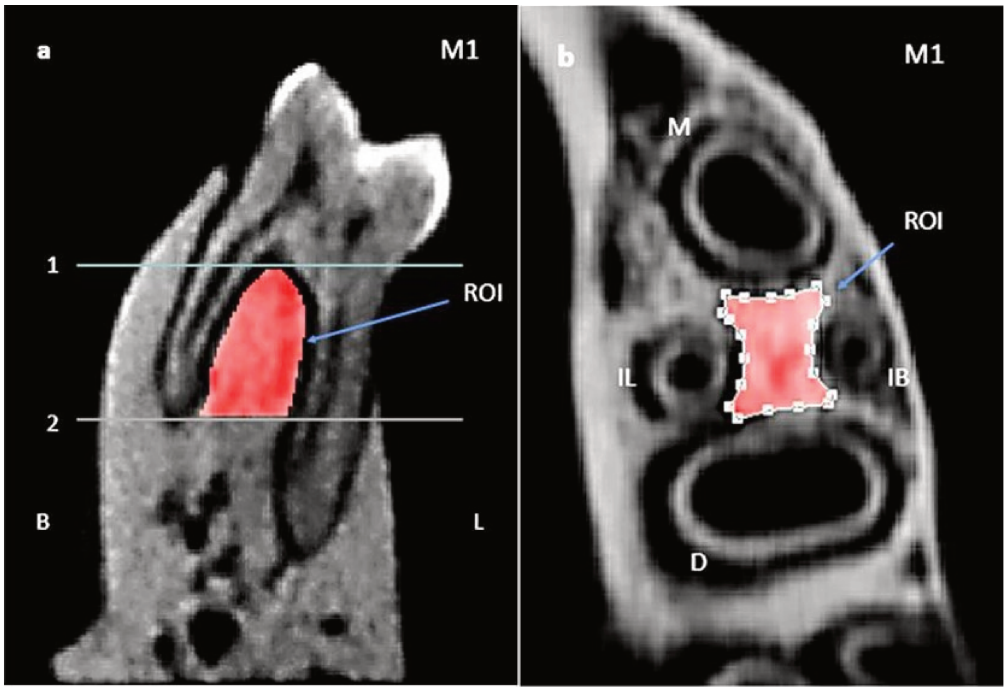

Fig 3. Micro-CT images of the rat mandibular alveolar bone and the mandibular first molar (M1); a) it shows the coronal surface passing through the midst of the buccal roots and lingual intermediates. The lines 1 and 2 passes by the alveolar crest and by the buccal apex, respectively, parallel to the occlusion plane and delimiting the red area which represents the region of interest (ROI). B, buccal, L, lingual; b) horizontal surface parallel to the occlusion plane and the ROI between the midst of the four roots from M1; M, mesial root; IB, intermediate buccal root; D, distal root; IL, intermediate lingual root.

Statistical Analysis

Mean and standard deviation (SD) of the data generated by the software CTAnalyser were calculated by the software IBM-SPSS, version 17.0 (SPSS Inc., Chicago, IL, USA). It was also used the Shapiro-Wilk normality test and the Levene's test of homogeneity variances, with $p<0,005$. It was used Fisher's variance analysis through the Gauss -Markov's linear model and the multiple comparisons through the Tukey HSD's criterion. It was evaluated in the different microtomographic analyzes, the error in the systematic intra-observer in measures by repetition, made by the same examiner through the Dahlberg error formula (apud HOUSTON, 1983) [29].

Results

Microcomputed tomography

The microcomputed tomography was used to investigate the microstructural alterations in the mandibular bone of all the offspring in development. Dahlberg's formula about the intra-examiner systematic error (apud HOUSTON, 1983) [29] indicated a significance level that validated the data collected. The trabecular

parameters of the alveolar bone, such as BV/TV, Tb.Th and SMI, showed significant differences in the CSO group comparing to the values of the control group (Fig 4). Parameters such as Tb.Sp and Tb.N did not show significant differences between the groups (Fig 4). In the CSO group, were observed significant reductions in BV/TV, with p=0,0124, in Tb.Th, p<0,0001, associated with the significant increase of the SMI, with p<0,0001, in relation to the control group (Table 1).

About the ISO group, there was no significant difference in the relation BV/TV, with p=0,9751 when compared to the control group (Table 1). However, in the parameter Tb.Th, the differences among the three groups were significant, showing higher values in the control group, followed by the ISO group with p=0,0020, and by the CSO group with p=0,001, showing the lower values (Table 2). The average values of the SMI for the ISO group, followed the values of the control group, with p=0,1253, during the three periods studied (Table 3). The Tb.Sp showed no differences between the groups evaluated, and the effect during the period of exposure to cigarette smoke was not significant, with p = 0.6680 (Table 4). The Tb.N parameter, during all the exposure period to the cigarette smoke, there was no statistical difference between the groups, with p=0,3308 (Table 4). The values related to the trabecular bone alterations during all the experimental period, reveals about the CSO and NSO groups: difference of 18% in the BV/TV, 38,7% in the Tb.Th and 51% in the SMI. Between CSO and ISO groups, 19,2% in the BV/TV, 24% in the Tb.Th and 24,6% in the SMI. The difference between NSO and ISO was 1,4% in the BV/TV, 19,3% in the Tb.Th and 14% in the SMI. The chart in Fig. 4 illustrates the tendencies, on average, of the three groups analyzed during the four periods. At figures 5 to 8, it is possible to observe cross-sectional slices from the mCT of the inter-radicular bone of M1 and representative images illustrating figure analysis in the inter-radicular region.

Table 1. The microarchitectural parametric values, BV/TV, of the mandibular bone in the inter-radicular region (n=55)

Group	Mean(%) and SD	Contrasts	P
NSO n20	69,35±16.69	CSO / NSO	0.0124
CSO n20	56,81±13,33	NSO / ISO	0.9751
ISO n15	70,33±6,60	CSO / ISO	0.0126

BV/TV: bone volume/ total volume; between CSO and NSO, and CSO and ISO p<0,05; between NSO and ISO, no significant values (p>0,05); CSO: continuous smoking offspring; NSO: non-smoking offspring; ISO: interrupted smoking offspring.

Table 2. The microarchitectural parametric values, Tb.Th, of the mandibular bone in the inter-radicular region (n=55)

Group	Mean(mm) and SD	Contrasts	P
NSO n20	0.31±0,05	CSO / NSO	< 0.0001
CSO n20	0.19±0,04	NSO / ISO	0.0030
ISO n15	0.25±0,05	CSO / ISO	0.0020

Tb.Th: Trabecular thickness; statistically significant differences among the three groups, with p<0,005. CSO: continuous smoking offspring; NSO: non-smoking offspring; ISO: interrupted smoking offspring.

Table 3. The microarchitectural parametric values, SMI, of the mandibular bone in the inter-radicular region (n=55)

Group	Mean and SD	Contrasts	P
NSO n20	1.8±0,4	CSO / NSO	< 0.0001
CSO n20	2.7±0,2	NSO / ISO	0.1253
ISO n15	2.0±0,3	CSO / ISO	< 0.0001

SMI: Structure Model Index; between CSO and NSO, and CSO and ISO, with p<0,05. Between NSO and ISO no significant values, with p>0,005. CSO: continuous smoking offspring; NSO: non-smoking offspring; ISO: interrupted smoking offspring.

Table 4. The microarchitectural parametric values, Tb.Sp and Tb.N, of the mandibular bone in the inter-radicular region (n=55)

Group	Tb.Sp Mean(mm) and SD	Tb.N Mean(mm) and SD
NSO n20	0.2173±0,06	2,51±0,76
CSO n20	0.2198±0,02	2,51±0,76
ISO n15	0.1998±0,03	2,5707±0,51

Tb.Sp: trabecular spacing; TbN: trabecular number; no significant values to Tb.Sp, with p=0,6680 and to Tb.N with p=0,3308. CSO: continuous smoking offspring; NSO: non-smoking offspring; ISO: interrupted smoking offspring.

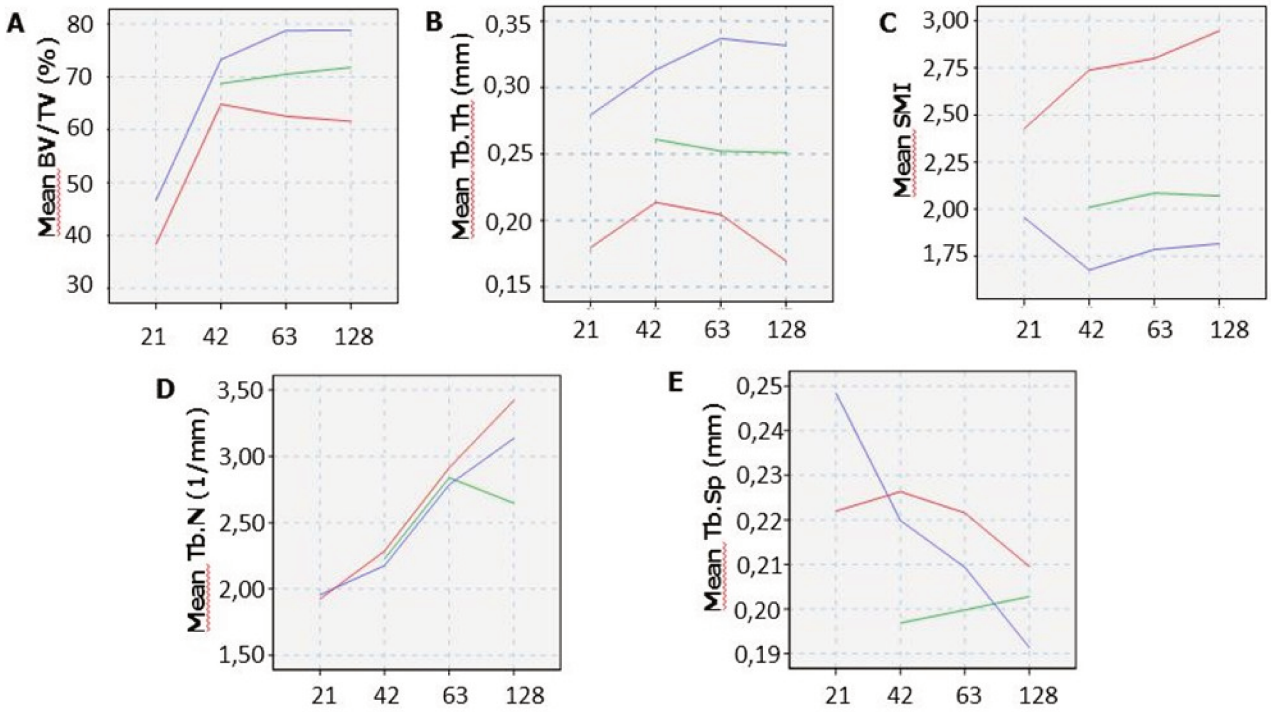

Fig 4. Micro-tomographic histomorphometric comparisons between the CSO (red), ISO (green) and NSO (blue) groups in the 21st, 42nd, 63rd and 128th days. A: BV/TV = bone volume fraction (bone volume / total volume); B: Tb.Th = trabecular thickness; C: SMI = Structure Model Index; D: Tb.Sp = trabecular spacing; E: Tb.N = trabecular number. CSO: continuous smoking offspring; NSO: non-smoking offspring; ISO: interrupted smoking offspring.

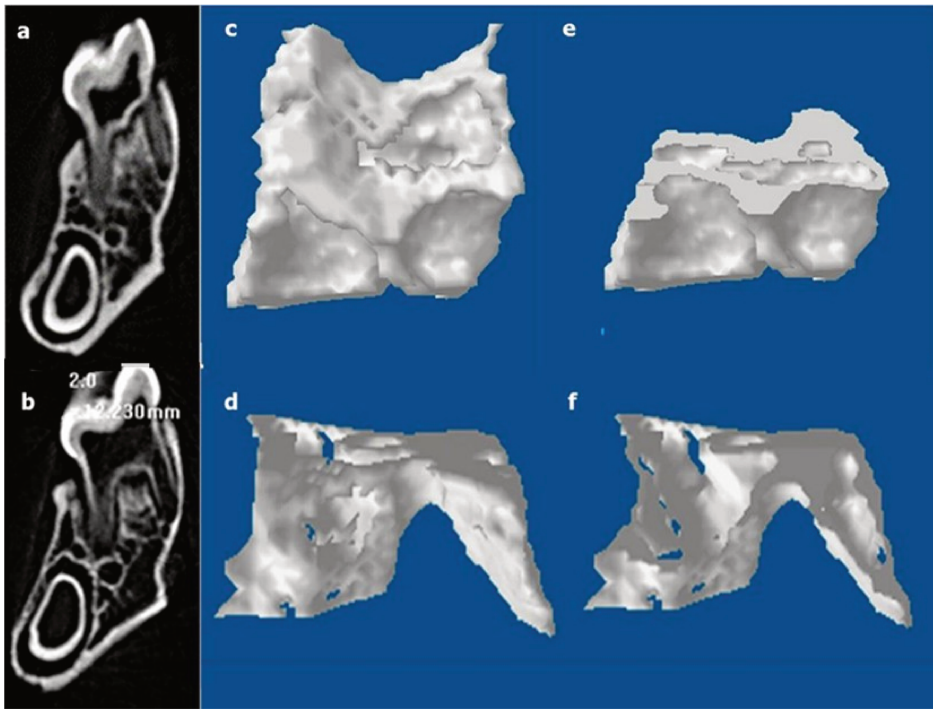

Fig 5. Tomographic cross-sectional slices of M1 area (a, b) and representative images illustrating micro-CT image analysis in the inter-radicular region of the mandibular bone (c, d, e, f). a, c and e were obtained from a 21st-day NSO rat; b, d, and f were obtained from the 21st day CSO rat. C and d showed in the 3D reconstruction images, while e and f, the same images after section slice, to observe the internal aspect. Images generated after the segmentation of osseous and non-osseous tissues demonstrated the ability to quantitatively analyze trabecular bone morphology.

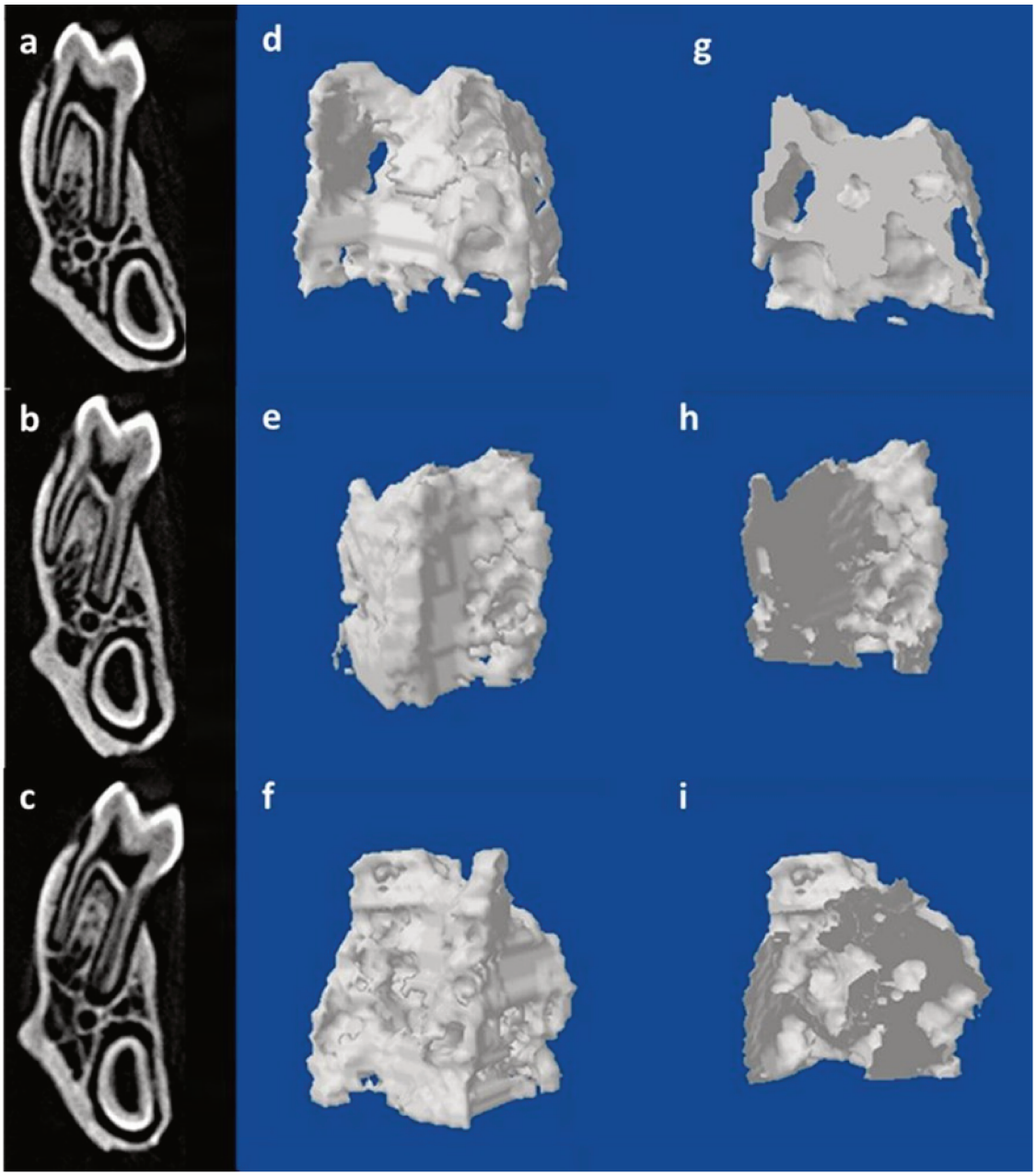

Fig 6. Tomographic cross-sectional slices of M1 area (a,b,c) and representative images illustrating micro-CT image analysis in the inter-radicular region of the mandibular bone(d,e,f,g,h,i). a,d,g were obtained from a 42-day NSO rat, b,e,h were obtained from a 42-day ISO rat, and c,f,i were obtained from a 42-day CSO rat. d,e,f showed the 3D reconstruction images, while g,h,i the same images after section slice to observe the internal aspect. Images generated after the segmentation of osseous and non-osseous tissues demonstrated the ability to quantitatively analyze trabecular bone morphology.

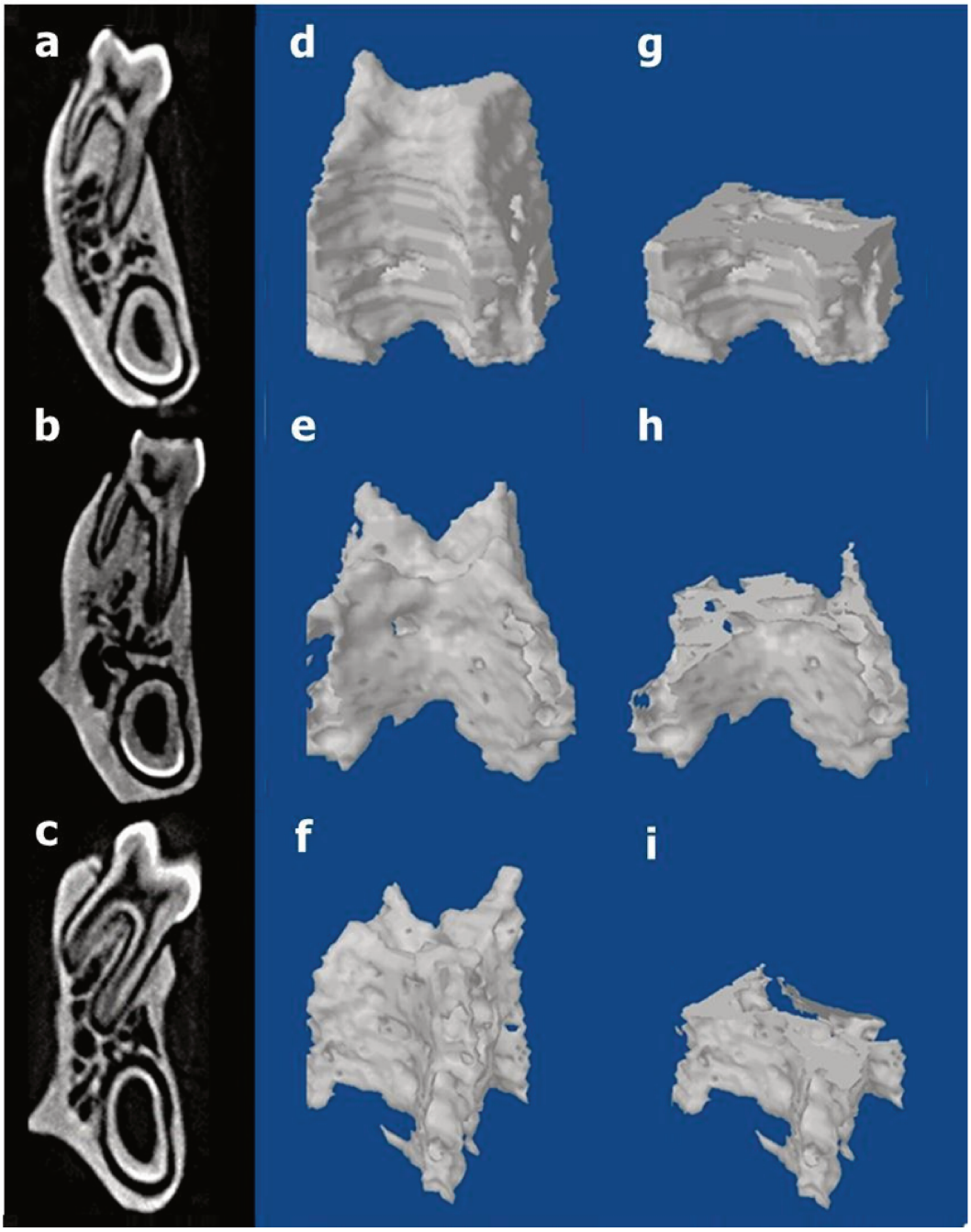

Fig 7. Tomographic cross-sectional slices of M1 area (a,b,c) and representative images illustrating micro-CT image analysis in the inter-radicular region of the mandibular bone (d,e,f,g,h,i). a,d,g were obtained from a 63-day NSO rat, b,e,h were obtained from a 63-day ISO rat, and c,f,i were obtained from a 63-day CSO rat. d,e,f showed the 3D reconstruction images, while g,h,i the same images after section slice to observe the internal aspect. Images generated after the segmentation of osseous and non-osseous tissues demonstrated the ability to quantitatively analyze trabecular bone morphology.

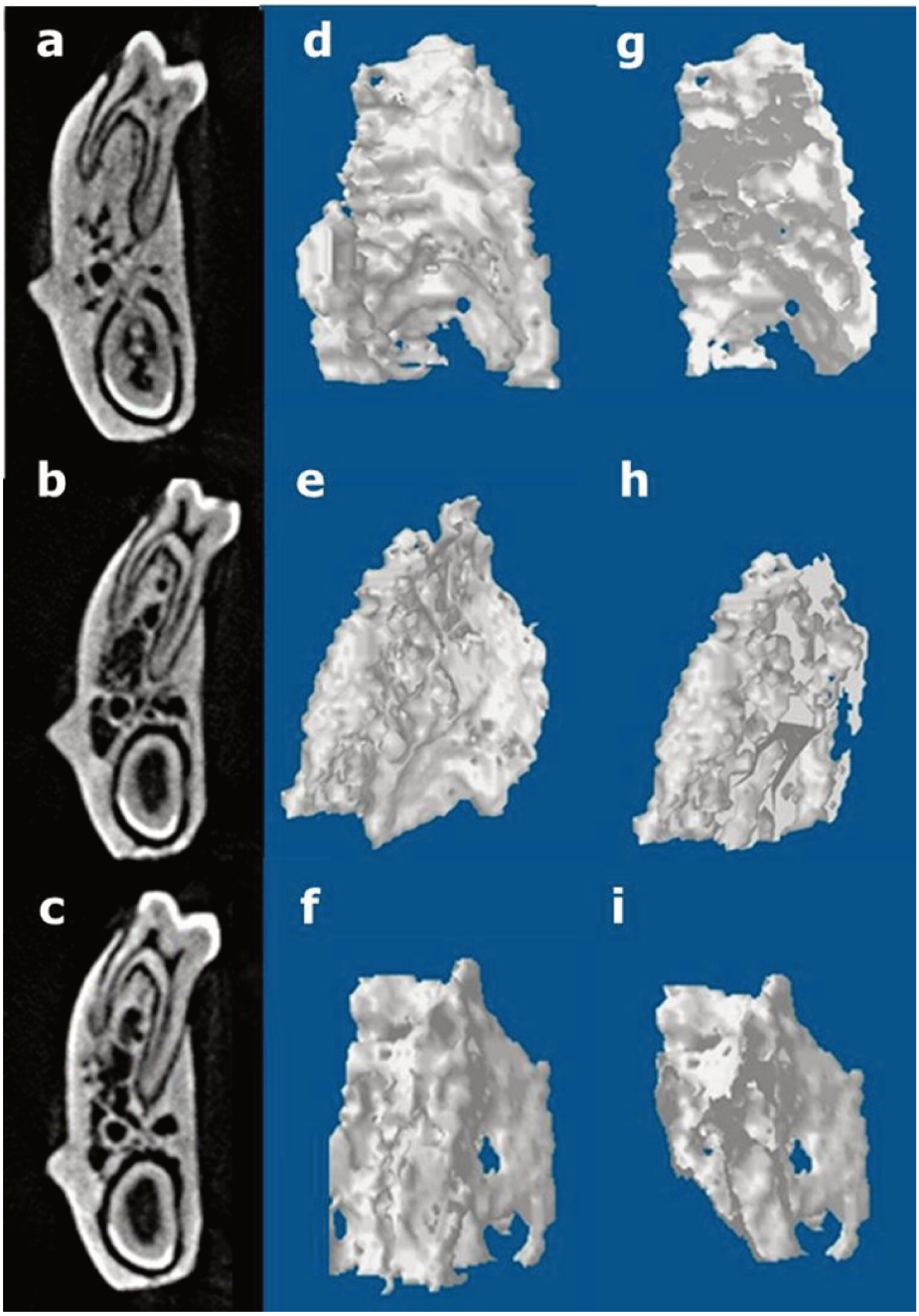

Fig 8. Tomographic cross-sectional slices of M1 area(a,b,c) and representative images illustrating micro-CT image analysis in the inter-radicular region of the mandibular bone(d,e,f,g,h,i). a,d,g were obtained from a 128-day NSO rat, b,e,h were obtained from a 128-day ISO rat, and c,f,i were obtained from a 128-day CSO rat. d,e,f showed the 3D reconstruction images, while g,h,i the same images after section slice to observe the internal aspect. Images generated after the segmentation of osseous and non-osseous tissues demonstrated the ability to quantitatively analyze trabecular bone morphology.

Discussion

The present study simulated the environment of human beings in early stages, when exposed to secondhand cigarette smoke until the maturity. Similar to what happens in public smoking areas or at home, where one or more family members are smokers. Our model was able to identify the secondhand smoke effects in the bone tissue of the animals evaluated in this study. The bone maturity of the Wistar rats happens from 119th to 147th days of life, that is equivalent, respectively, to 18 and 24 years old in human beings [30]. Our study, evaluated animals with 21 days of life (that means 3 years old in humans), animals with 42 days of life (equivalent 7 years old in humans), animals with 63 days of life (equivalent 10 years old in humans), animals with 128 days of life (equivalent 21 years old in humans). Only the present study, until this time, evaluated the characteristics of the bone tissue from the initial development phase to the beginning of adulthood, in offspring of secondhand smoking matrices, considering also their effects after the passive inhalation cessation of the cigarette smoke.

The parameters BV/TV and Tb.Th, indicators of the trabecular bone density, showed growth during development, of the bone volume and of the trabecular thickness, in the animals of the CSO group, from 21st to 42nd days of life. Revealing a growing development, however, with lower values if compared with the control group. From 42nd to 63rd days of life, the rats going from puberty to sexual maturity [31], presented tendency to lose bone density that was intensified until the 128th day. Dong et al. (2011) [32] showed general morphological inhibition in the development of the hard-dental tissues in the offspring of passive smoking rats, with lower values of thickness and volume. Corroborating with our results, other studies have shown a significant similar reduction in the bone volume and in the trabecular bone thickness, indicating the suppression of the formation and the increased bone resorption in rats exposed to secondhand smoke [19,33,34]. Although, the offspring in those studies were not born from smoking matrices, as in the present study.

The ISO group showed bone volume recovery comparing to the CSO group, while the mean trabecular thickness value remained between the CSO and NSO groups, from the 42nd to the 128th day. That result suggests a weakened trabecular bone density. According to Hapidin et al. (2011), the cessation of nicotine usage during two months in rats did not allow the recovery of the adverse effects in the bone tissue, neither the return of the histomorphometry parameters at the control level group [22]. Our results are according to the results of Ward et al (2001), who concluded that the cigarette smoke, in active human smokers, have a harmful effect, dose-dependent and causes bone loss with an increase of fracture risk. Such effects, can be partially reversed after the cessation/interruption of the inhalation [35]. The SMI is a parameter to indicate the relative prevalence of the elements in cylindrical forms and/or of plates in a 3D structure, as the trabecular bone [36]. It is important in the evaluation of the osteoporotic degradation of trabecular bone, characterized by a transition from a plate architecture shape to a cylindrical architecture shape. The similar plate-like structure contributes to the elastic behavior of the bone, while the cylindrical shape results in a less rigid microarchitecture and less able to withstand mechanical tensions coming from several directions [37].

In our study, there was prevalence of cylindrical bone structural elements in the CSO group, that evolved animals from 21st to 128th day, representing an increase of 51,1% in relation to the control. Ko et al. (2015), showed similar result indicating that the secondhand smoke exposure in rats, increased in 46% the value of the SMI

in comparison to the control, confirming also the osteopenia condition [1]. According to the findings in the present study, Sasaki M, et al. (2018) showed in rats exposed to secondhand smoke, from gestation and birth until the 28th day of life, increase of bone volume and induction to osteoporosis, due to the spatial disorientation of the bone microstructure [38].

The SMI analysis in this study indicated that the animals of the ISO group presented a surprising recovery in relation to the CSO groups during the three periods evaluated. We can consider by this parameter, that the microstructure has recovered its elastic property and, therefore, can be able to withstand natural mechanical challenges of beings in full growth process. This shows that cessation of inhalation causes positive signaling to osteoblastic cells responsible for bone formation. However, the trabecular thickness in intermediate levels, between the CSO and control groups, still suggests a fragile structure. Hapidin et al. (2011), showed that nicotine abstinent rats presented a significant reduction in Tb.Th [21] being in accordance with our data [22].

In this study, we evaluated the effects of passive smoking on the trabecular bone micro-architecture in the mandible of rats, children of passive smoking matrices and who remained with their mothers until the beginning of sexual maturity. The offspring of smokers presented loss of quality in the first molar interradicular bone (in the region of interest - ROI) determined by the low values of BV/TV and Tb.Th, besides the high values of the SMI. In previous studies, [1-39] the values obtained were different from the values presented in this study, however, the trends observed here were consistent and the trabecular bone parameters in the smoking group were also lower than in the control group, indicating the formation of bone tissue with low elasticity that presents a greater risk of fractures as it matures. Besides that, we also showed that the offspring removed from exposure, in half the time of the animals exposed to passive inhalation from cigarette smoke, presented a favorable recovery, but under the control level. This demonstrates that these offspring removed from the toxic action of cigarette smoke may still be subject, thanks to the weakening of microarchitecture, to the same bone health problems as the animals continuously exposed to secondhand smoke. Until the conclusion of this study, there are no reports about the evaluation of the toxic effects of passively inhaled cigarette smoke in skeletal sites of organisms under development from its gestation through adulthood.

In children of smokers, this natural loss of bone elasticity, observed in our study, can lead to the development of skeletal structure and an increased adverse effect on bone surgeries, as well as increased hospitalization time, and even failure to adequately repair the bone fracture. The risks to bone health may be greater in the children of smokers than in their smoking parents. Thus, anti-smoking campaigns and laws prohibiting smoking indoors should be encouraged, as well as, the need for greater awareness of society about such toxic effects resulting from passive exposure to secondhand smoke in the bones of children and young people who live with smokers.

Author Contributions

Conceptualization: CFA, CAFC, PHFC.
Data curation: CFA.
Formal analysis: CFA, FHN, AFI.
Investigation: CFA, PHFC, CAFC, AFI.

Methodology: CFA, CAFC, FHN, PHFC.
Project administration: CFA, PHFC, CAFC.
Supervision: PHFC, CAFC.
Validation: PHFC, AFI, FHN.
Visualization: CFA, PHFC, AFI, FHN.
Writing ± original draft: CFA, PHFC.
Writing ± review & editing: CFA, PHFC, CAFC.

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3. CONCLUSÃO

A inalação da fumaça de cigarro de segunda mão pelos descendentes das matrizes tabagistas passivas, mostrou efeitos prejudiciais na microarquitetura do osso trabecular da mandíbula dos ratos em desenvolvimento. Diminuiu a elasticidade óssea, enfraquecendo o osso durante o amadurecimento da prole. Sobre os grupos interrompidos, a recuperação da microarquitetura ocorreu parcialmente.

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* De acordo com as normas da UNICAMP/FOP, baseadas na padronização do International Committee of Medical Journal Editors - Vancouver Group. Abreviatura dos periódicos em conformidade com o PubMed.

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ANEXO 1: Protocolo para uso de animais em pesquisa.

Fls. nº 49
Proc. nº 285/13
Rub.:

FACULDADE DE MEDICINA DE JUNDIAÍ

R. Francisco Telles, 250 - CEP 13202-550 - Cx. Postal 1109
Fone/Fax: (11) 4587-1580 - Jundiaí - SP - site: www.fmj.br - e-mail: fmj@fmj.br

COMITÊ DE ÉTICA PARA USO DE ANIMAIS

Jundiaí, 03 de dezembro de 2013

Ilustríssimo Senhor
CESAR ALEXANDRE FABREGA CARVALHO

Ref.: Aprovação de Projeto de Pesquisa

TÍTULO: "Avaliação morfoquantitativa e funcional dos sistemas orgânicos de ratos expostos ao tabagismo passivo"

Prezado Pesquisador

O Comitê de Ética para Uso de Animais – CEUA desta Faculdade, em reunião no dia 03 de dezembro de 2013, no cumprimento de suas atribuições e após revisão ao seu protocolo de pesquisa em epígrafe, emitiu o parecer APROVANDO o seguinte documento:

>> Protocolo para Uso de Animais em Pesquisa nº 285/2013

Lembramos a V.Sa. que é necessário enviar a este CEUA relatórios de eventos adversos, caso estes venham a ocorrer, assim como relatório final com os resultados da pesquisa, para finalização do processo. Quaisquer dúvidas estamos à disposição.

Atenciosamente,

Prof. Dr. Marcelo Rodrigues da Cunha
Coordenador do Comitê de Ética para Uso de Animais

Declaro ter recebido uma via do parecer emitido pela CEUA.

Data ___/___/___ Assinatura(*) _____

ANEXO 2: Protocolo de submissão ao Periódico PLOS ONE.

De: em.pone.0.613aee.100c18dd@editorialmanager.com
<em.pone.0.613aee.100c18dd@editorialmanager.com> em nome de PLOS ONE
<em@editorialmanager.com>
Enviado: terça-feira, 12 de fevereiro de 2019 10:47
Para: Cristiano Fittipaldi Alves
Assunto: Submission Confirmation for PONE-D-19-04201 - [EMID:f0d8aadbf222fb2a]

PONE-D-19-04201
Effects of the secondhand smoking exposure in the early stages of the bone development
PLOS ONE

Dear Mr. Alves,

Thank you for submitting your manuscript entitled 'Effects of the secondhand smoking exposure in the early stages of the bone development' to PLOS ONE. Your assigned manuscript number is PONE-D-19-04201.

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ANEXO 3: Relatório "Turn it in".

Efeitos da exposição ao fumo passivo nos estágios iniciais d desenvolvimento ósseo.

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1	Cristiano Fittipaldi Alves, Cesar Alexandre Fabrega Carvalho, Antônio Francisco lemma, Francisco Haiter Neto et al. "Effects of the secondhand smoking exposure in the early stages of the bone development", Cold Spring Harbor Laboratory, 2019 Publication	64
2	repositorio.unicamp.br Internet Source	2
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