Micrographia

Effect of Heavy Metals Contamination from Cigarette Smoke on Sound and Caries-Like Enamel

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Abstract

In this study, we sought to evaluate the influence of cigarette smoke and pH cycling on the chemical composition and surface/cross-sectional enamel microhardness. A total of 40 dental blocks obtained from bovine incisors were divided into four groups (n = 10): no treatment (control); exposure to cigarette smoke (CS); exposure to pH cycling (PC); and exposure to cigarette smoke and pH cycling (CS-PC). The samples were analyzed by synchrotron radiation micro X-ray fluorescence, bench mode X-ray fluorescence, as well as surface microhardness (SMH) and cross-sectional microhardness (CSMH) testing. The SMH results were submitted to analysis of variance (ANOVA) and Tukey’s test. The CSMH results were evaluated using split-plot ANOVA and Tukey’s test. A high amount of Cd and Pb and traces of Ni and As were observed in enamel and dentin after exposure to cigarette smoke (CS and CS-PC). The SMH and CSMH of CS were statistically higher when compared with the control. The PC and CS-PC showed lower SMH and CSMH. We conclude that exposure to cigarette smoke promoted heavy metal deposition in enamel/dentin. In addition, it increased the enamel microhardness but did not promote a protective effect on the in vitro development of caries. The clinical significance of this work is that there is significant bioaccumulation of heavy metals from cigarette smoke on the surface and in the enamel and dentin.

Key words: tobacco, dental enamel, dental caries susceptibility, heavy metals

(Received 12 December 2016; revised 29 September 2018; accepted 15 October 2018)

Introduction

Tobacco is one of the main toxic agents of civilization, and smoking is a highly dynamic process involving a complex matrix consisting of aerosol with more than 3,800 compounds, most of which have negative effects on the oral tissues (Klus, 2005; Vellappally et al., 2007). Cigarette smoke is composed of thousands of toxic substances, such as carbon monoxide (CO), ammonia (NH3), nickel (Ni), arsenic (As), and heavy metals such as lead (Pb) and cadmium (Cd). There is a significant correlation between smoking and increased incidence of periodontal disease and caries (Fujinami et al., 2011). Active smoking is a well-known risk factor for these diseases, as there is evidence that exposure to cigarette smoke enlarges the demineralized area (Fujinami et al., 2011). A relationship has also been shown between passive smoking and caries (Fujinami et al., 2011). An extreme temperature (between 200 and 600°C), changes the arrangement of hydroxyapatite crystals and consequently alters the crystal morphology, phase change and chemical modification can occur, which thereby increases the enamel hardness. Although cigarette smoke does not reach an extreme temperature, studies have suggested that the increase of the temperature caused by smoke, in addition to the heavy metal contamination could influence the microhardness of tooth enamel (Bertoldo et al., 2011). The aim of this study was to evaluate whether cigarette smoke and pH cycling modify the enamel or affect enamel microhardness. The null hypothesis is that cigarette smoke does not affect the dental composition (enamel and dentin) or microhardness of the enamel surface (ES).
Materials and Methods

Sample Preparation

A total of 40 bovine incisors were obtained and stored in a 0.1% thymol buffered solution. The coronal portion was separated from the root using a double-faced diamond disc (KG Sorensen Ind. Com Ltda, Barueri, SP, Brazil). A total of 40 enamel/dentin (ED) blocks, with a surface area of 25 mm² and 3 mm of height/thickness with 1 mm enamel, were obtained using a metallographic cutting machine (Isomet 1000; Buehler, Lake Bluff, IL, USA) and a diamond disc (4'' × 012 × 1/2; Buehler, IL, USA). The ED was flattened using silicone carbide abrasive paper (SiC) of 600 and 1,200 granularity under constant irrigation in a polishing machine (AropolE; Arotec, Cotia, SP, Brazil) and polished with felt disks (Arotec, Cotia, SP, Brazil) and diamond pastes (1, 1/2, and 1/4) using a graphic cutting machine (Isomet 1000; Buehler, Lake Bluff, IL, USA). The ED was also performed with the same program using the fundamental elemental maps (qualitative analysis) were built using the PyMCA program (Solé et al., 2007). The number of pixels (spectra) per area of the specimen was between 3,000 and 4,000. Quantitative analyses were also performed with the same program using the fundamental parameters method.

Exposure to Cigarette Smoke

For the CS and CS-PC groups (n=10), a smoke machine developed by the Department of Restorative Dentistry, Operative Dentistry Area, Piracicaba Dental School—University of Campinas—2011 (Brazilian patent protocol #01810012043 INPI—Brazilian National Institute of Intellectual Property) was used. The cycle is scheduled over a time interval, which simulates the smoke aspiration often performed by a smoker, with duration of 3 s. The temporizer allows the ambient air to be inhaled every 10 s, thus simulating smoke exhaustion and subsequent elimination. Each specimen was subjected to smoke from 20 cigarettes (Marlboro; Philip Morris Brazil Industry and Trade, Santa Cruz do Sul, RS, Brazil) per day, for a total of 5 days (Bertoldo et al., 2011). In the interval between each simulation, the samples were stored in artificial saliva at 37°C, and every 24 h, the samples were washed with distilled water and re-immersed in a fresh solution of artificial saliva to prevent sedimentation (Bertoldo et al., 2011). Before exposure to cigarette smoke, all samples were isolated with mild acid resistant varnish (Colorama, São Paulo, Brazil), with the exception of the polished enamel area.

pH Cycling

The samples of the PC and CS-PC groups were subjected to pH cycling for 8 days, thereby simulating a cariogenic challenge, as described by Moi et al. (2008). The cycle consisted of enamel immersion in a demineralizing solution for 4 h, followed by immersion in a remineralizing solution for 20 h. Samples were previously isolated using mild acid resistant varnish and then individually immersed in 75 mL of demineralizing solution (3 mL/mm²) (Queiroz et al., 2008) at 37°C (0.1 M acetate buffer; 1.28 mM Ca, 0.74 mM Pi, and 0.03 μg F/mL) (pH 5.0). After 4 h, the samples were washed with deionized water for 30 s and dried with absorbent paper. The individual samples were immersed in 37.5 mL of remineralizing solution (1.5 mL/mm²) at 37°C [1.5 mM Ca, 0.9 mM Pi, 150 mM KCl, 0.05 μg F/mL, 0.1 M Tris buffer (pH 7.0)] (Queiroz et al., 2008).

Analysis of Enamel Surface and Cross-sectional Microhardness

The microhardness tests were carried out on all groups, to evaluate demineralization and changes of the ES after exposure to cigarette smoke and pH cycling, with a microhardness tester (Knoop indenter, 50 g, 5 s) and each indentation was spaced by 100 μm. The arithmetic mean of five indentations in the central region of the specimen was obtained.

For the CSMH evaluation, the samples were sectioned in half using a high precision metallographic cutter, with one half embedded under controlled pressure in a thermoplastic resin for CSMH testing (Arotec Pre 30; Cotia, SP, Brazil) and the other half submitted to μ-X-ray fluorescence (XRF) analysis. The ED was flattened using SiC abrasive paper of 600 and 1,200 granularity under constant irrigation in a polishing machine (AropolE) and polished with felt disks and diamond pastes (1, 1/2, and 1/4 μm). Samples were ultrasonically cleaned for 15 min between each application of sandpaper and felt and at the end of the polishing. Samples were stored in artificial saliva at 37°C until used.

The samples were randomly divided into four groups (n = 10) and subjected to the following treatments: no treatment (control); exposure to cigarette smoke (CS); exposure to pH cycling (PC); and exposure to both cigarette smoke and pH cycling (CS-PC). Changes in composition of enamel structure by exposure to cigarette smoke were assessed, as well as the effect of pH changes through pH cycling on the surface and CSMH enamel structure.

X-ray Microfluorescence Analysis

For μ-XRF analysis the sample was half-sectioned using a high precision metallographic cutter. The inner surface (ED surface) of the sample (tooth) was analysed. μ-XRF measurements were carried out at D09B-XRF beamline (Pérez et al., 1999) of the Brazilian Synchrotron Light Laboratory (LNLS) located in Campinas, São Paulo, Brazil. The experiments were performed under pressure of 1 atm and controlled temperature (23°C). A mirror-based XRF microprobe provides an intense X-ray microbeam of 20 μm in diameter. The experimental setup also includes an optical microscope (∼500×) to place the samples on the focus plane and select the region of interest for analysis. The x, y, z, and θ motorized positioning stages enable raster scanning of the samples through the X-ray microbeam. An energy dispersive silicon drift detector (KETEK GmbH, Munich, Germany), placed at 90° from the incoming beam direction, was used to collect the XRF and scattered radiation coming from the samples. Aluminium foil was used in front of the detector to reduce the high intensity of the Ca-K emission lines from the sample matrix.

The μ-XRF analyses were used to determine the presence and distribution of Pb, Ni, Cd, and As. The overall time required for analyzing each sample was between 3 and 6 h. Two-dimensional elemental maps (qualitative analysis) were built using the PyMCA program (Solé et al., 2007). The number of pixels (spectra) per map was between 3,000 and 4,000. Qualitative analyses were also performed with the same program using the fundamental parameters method.
X-ray Fluorescence Analysis

The XRF measurements were performed in the Minipal 4.0-Panalytical EDXRF spectrometer at the Chemical Analysis Center of the Institute of Chemistry of São Carlos, University of São Paulo, under helium atmosphere and ambient temperature, with an analysis time of 420 s. The analysis protocol was set using Omniam software based on fundamental parameters.

Energy-dispersive X-ray Spectrometry Analysis

Five samples of the group were randomly selected for Energy-dispersive X-ray Spectrometry (EDS) analysis. The samples were sputtering coated with carbon under vacuum (Denton Vacuum Desk II; Denton, Moorestown, NJ, USA). The EDS point analysis (Vantage, Acquisition Engine Company, Tokyo, Japan) was performed on the ES to determine the elemental presence of phosphorus (P) and calcium (Ca). For each sample, five points were randomly selected (300 \( \mu \text{m}^2 \) for each point) and the mean values were calculated (Vieira-Junior et al., 2018). The elemental levels (wt%) of Ca and P and Ca:P ratio were determined.

Statistical Analysis

The SMH data were subjected to repeated measures analysis of variance (ANOVA) and Tukey’s test (\( \alpha = 0.05 \)). For the CSMH data, split-plot ANOVA and Tukey’s test were used (\( \alpha = 0.05 \)). The EDS data (Ca and P concentration levels (wt%) and the Ca:P ratio) were subjected to a one-way ANOVA and Tukey’s test (\( \alpha < 0.05 \)).

Results

Figure 1 shows the spatial distribution of several elements on the ED surface of samples exposed to cigarette smoke, and Figure 2 shows the group exposed to cigarette smoke and pH cycling. Calcium from the matrix and other elements, Cd, Ni, Pb, and As, absorbed by the dental structure from the cigarette smoke are observed. For the control and PC groups (samples not exposed to cigarette smoke), XRF images are not available because these elements were not detectable.

A semi-quantitative analysis by \( \mu \)-XRF was performed to determine the chemical composition of detected elements over a region around the ED of the samples exposed to cigarette smoke and to cigarette smoke and pH cycling (Fig. 3). A distribution of each element can be observed on all ED surfaces.

Table 1 shows the results of the semi-quantitative analysis by \( \mu \)-XRF and XRF using the fundamental parameter method. For the CS group, it is possible to observe higher concentrations of elements Ni and Cd, and lower concentrations of As and Pb. For the CS-PC group, we can observe an increase of concentration of elements Ni and Cd on the ES as well as the ED.

The initial microhardness values did not differ statistically among the groups. After the treatments, the ES microhardness of the CS group was statistically higher than that of the control group (\( p < 0.0001 \)). The samples exposed to pH cycling (PC and CS-PC) showed lower mean surface values of microhardness than the groups not exposed to pH cycling (CS and control), and they did not differ between themselves (\( p > 0.05 \)) (Table 2).

The enamel CSMH of the CS group was statistically higher when compared with the control group at the depths of 10, 40, 60, 80, 100, 140, 160, and 180 \( \mu \text{m} \) (\( p < 0.0001 \)). The samples exposed to pH cycling (PC and CS-PC) showed lower sub-surface mean...
values of microhardness than the groups not exposed to pH cycling (CS and control) (Table 3).

Table 4 shows the results of EDS analysis for elemental levels (wt%) of Ca, P and Ca:P ratio. For the Ca values, CS and PC groups presented lower values statistically differing from control group ($p < 0.05$), and the CS-PC groups did not differ from the groups. For the $p$ values, PC group differs statistically ($p < 0.05$) from the others, presenting from the lower values. The group exposed to cigarette smoke (CS) was statistically higher ($p < 0.05$) when compared with the others.

Discussion
In the present study, the feasibility of determining heavy elements at trace concentration levels was demonstrated due to the high

Figure 2. X-ray microfluorescence analysis of a region similar to Figure 1 but exposed to cigarette smoke and pH cycling. (a): Enamel/dentin (ED) block section—enamel (E) and dentin (D); elements: (b) calcium (Ca); (c) cadmium (Cd); (d) nickel (Ni); (e) lead (Pb); (f) arsenic (As); 2D XRF images showing the co-distribution of; (g) calcium (Ca) and cadmium (Cd); (h) calcium (Ca) and nickel (Ni); and (i) calcium (Ca) and lead (Pb).

Figure 3. (a) X-ray microfluorescence analysis of a region containing the enamel/dentin surface exposed to cigarette smoke. Quantitative XRF analysis of cadmium (white); lead (blue); nickel (green); arsenic (red). (b) X-ray microfluorescence analysis of a region similar to (a) but exposed to cigarette smoke and pH cycling.
sensitivity of the synchrotron radiation (SR) assisted μ-XRF technique. SR achieves lower detection limits by several orders of magnitude compared with those obtained by conventional X-ray sources used in XRF (Pérez et al., 2004). Nevertheless, conventional analysis (Minipal 4 spectrometer; PANalytical, Almelo, The Netherlands) was necessary to use to confirm the semi-quantitative data obtained for Cd and Ni. Despite the use of filters, a high concentration of calcium in the dental structure may interfere with the analysis of extremely sensitive techniques, such as SR assisted μ-XRF.

According to Table 1, As and Pb concentrations were observed in the groups exposed to cigarette smoke (CS and CS-PC), without affecting the caries-like conditions. Moreover, an increase in Ni and Cd concentrations was observed in the CS-PC group after pH cycling. The increase in Ni and Cd concentrations due to caries-like treatment is probably related to surface area increase after pH cycling. The increase in Ni and Cd concentrations due to cigarette smoke (CS and CS-PC), exposure to cigarette smoke; PC, exposure to pH cycling. Heavy metal absorption by teeth from smoking is a heterogeneous process, and possible internal diffusion of these metals. Heavy metal absorption by teeth from smoking is a heterogeneous process, and possible internal diffusion of these metals. Heavy metal absorption by teeth from smoking is a heterogeneous process, and possible internal diffusion of these metals. Heavy metal absorption by teeth from smoking is a heterogeneous process, and possible internal diffusion of these metals.

Interestingly, the results showed no influence of the heavy metal incorporation on the demineralization process in vitro (Tables 2 and 3). The PC and CS-PC groups did not present statistical differences; therefore, teeth exposed to smoke showed a similar hardness to those not exposed to smoke. It is possible to infer that the incorporation of heavy metals at the concentration levels reported in the study did not alter the hydroxyapatite structure to increase or protect against caries development. However, it is not known whether prolonged exposure to cigarette smoke can cause some effect in the carious process because in this study, we exposed the dental structure to cigarette smoke for a short time.

In relation to the SMH and CSMH (Table 3) at depths of 40, 60, 80, 100, 140, 160, and 180 μm, the CS group showed the highest values, which were significantly different from the control group. However, the results of Ca:P ratio (Table 4) showed that group CS presented statistically lower ratio than the control group. The increase of the hardness associated to the decrease of Ca/P suggest that the incorporation of heavy metals was responsible for the increase in microhardness in the depth of the...
enamel. In addition, teeth heating by smoking can also play a role in the change of microhardness. Zhou & Hsiung (2007) reported that the surface roughness could also interfere with the measurement of surface hardness, but only at the very early stages, because the uniform alignment of enamel prisms is more important than the relative orientation between the prism axis and the indenter. It is assumed that this effect may have occurred due to a possible temperature variation of enamel over time when exposed to cigarette smoke (chronic effect), which, in turn, might have altered the space disposition of hydroxyapatite crystals within the enamel. Therefore, the null hypothesis was rejected, because cigarette smoke altered the composition and microhardness of the dental structure. However, these alterations were not sufficient to increase the susceptibility to demineralization by pH cycling.

Finally, the exposure of enamel to cigarette smoke promoted Ni, Cd, Pb, and As bulk and surface contamination. In addition, smoke exposure increased the enamel superficial and bulk microhardness without contributing to demineralization.

Acknowledgments. This work has been supported by the LNLS under proposal XAFS1-17100 and XAFS1-17101. The authors thank the agency FAPESP (#2013/15225-8). The authors acknowledge the help of Dr. Carlos Pérez for his help with data analysis from μ-XRF measurements. The collaboration between Piracicaba Dental School Group and Institute of Chemistry of São Carlos Group was supported by the CAPES/DAAD program ProBral I. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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