Clin Oral Invest (2018) 22:733–744
DOI 10.1007/s00784-017-2148-5

ORIGINAL ARTICLE

Chemical composition and morphology study of bovine enamel submitted to different sterilization methods

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Received: 30 March 2016 / Accepted: 2 June 2017 / Published online: 30 June 2017

Abstract
Objectives The morphology and chemical composition of enamel submitted to different sterilization methods was studied.
Methods X-ray photoelectron spectroscopy (XPS), field emission gun scanning electron microscopy (FEG-SEM), and energy-dispersive X-ray spectroscopy (EDS) were performed to evaluate 50 bovine enamel specimens sterilized using four methods: microwaving (MI), gamma irradiation (GI), ethylene oxide (EO), and steam autoclave (SA). Non-sterilized specimens were used as control.
Results XPS indicated that the concentration of P (phosphorus), CO_3 (carbonate), and CO_3/P was not changed in all groups. GI produced no significant change on elemental composition. SA produced the major decrease in calcium (Ca), Ca/P ratio, and increase in N (nitrogen). MI was found to decrease Ca, Ca/P ratio and O (oxygen), and increase in C (carbon) and N. EO produced decrease in Ca and O with increased C concentration. FEG-SEM revealed surface and in-depth morphological changes on SA specimens. Minor surface alterations were observed for EO and for MI groups, and no alteration was observed on GI group. EDS indicated no difference on elemental composition of enamel bulk among groups.
Conclusions SA produced mineral loss and morphological alterations on surface and in depth. MI and EO sterilization caused mineral loss showing only slight alteration on enamel surface. GI sterilization preserves the morphological characteristics of enamel. The sterilization methods could be classified from lower to high damage as GI < MI < EO < SA.
Clinical relevance This is a comprehensive comparative study where different methods for enamel sterilization were investigated in terms of chemical changes. The results presented here may help researchers to choose the most appropriate method for their research setting and purpose.

Keywords Dental enamel · Sterilization · Chemical analysis · XPS · SEM · EDS

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Introduction

Human and bovine enamel specimens have been widely used in dental research [1, 2]. Considered as a source of potential pathogenic microorganisms, enamel specimens must be sterilized before being used, in order to avoid contamination in in vitro experiments and minimize transfer of pathogenic microorganisms in in situ studies [1, 3].

The sterilization process must not affect the enamel properties [1, 3, 4]. The most common sterilization methods in use are gamma irradiation, ethylene oxide, and autoclave steam. Previous studies demonstrated that gamma irradiation and ethylene oxide gas had no effect on surface microhardness and response to demineralization of enamel [5–7]. However, sterilization by these methods is time consuming and relatively expensive and must be carried out by specialist companies and personnel. In addition to these limitations, gamma irradiation has shown to cause color change of enamel [6, 8] and ethylene oxide may not be effective for whole teeth sterilization [9].

Steam autoclave could be considered the most accessible sterilization method, and thus is widely used by researchers. However, structure of the enamel can be altered by autoclaving process [5–7, 10–13] and can influence the outcomes of the studies, which occasionally show conflicting results [2].

In a previous study [14], encouraging results for enamel sterilization were obtained using microwave irradiation. Microwaving was effective for complete disinfection of bovine enamel specimens without affecting the surface microhardness and the response to demineralization or remineralization. This procedure was microbiologically effective against both aerobic and anaerobic microorganisms. It is a fast method (3 min), requires only a domestic microwave oven and water, is less expensive than the usual sterilization methods, and does not use radioactivity [14]. Despite the positive results, complementary studies are necessary, as the preliminary study provided only indirect information about the chemical composition and surface integrity of enamel. Further, comparisons with other sterilization methods should provide a more comprehensive overview on the applicability of the microwaving method.

Considering that the properties of enamel are strictly related to chemical composition and microstructure [15], it is possible that the effects on enamel caused by sterilization process are associated to changes on enamel composition and morphology. According to the literature, numerous studies have evaluated the sterilization effects on enamel properties using different approaches [1, 2]. Nevertheless, the morphological and compositional information obtained from these studies are mostly indirect and limited. In particular, morphological analysis by FEG-SEM as well XPS and EDS chemical composition evaluation have not yet been investigated. The aim of this study was to evaluate the influence of four enamel sterilization methods, including microwave irradiation, on the elemental chemical composition and the morphology of bovine enamel using spectroscopic methods and scanning electron microscopy.

Materials and methods

Specimen preparation

Ten freshly extracted bovine incisors free from macroscopic cracks and staining as assessed by visual examination were used in this in vitro study. Five dental specimens (5 × 5 mm) were obtained from central portion of each crown, using a water-cooled diamond saw and a cutting machine (Isomet; Buehler, Lake Bluff, Ill., USA). The labial enamel surface was preserved and the dentin tissue was reducing by serially polishing using a water-cooled mechanical grinder (Metaserv 2000, Buehler) and 400-grit silicon carbide paper (Buehler) in until the specimen reach approximately 2 mm thick. One specimen from each tooth was randomly assigned to each one of five groups (n = 10): microwave irradiation (MI), gamma irradiation (GI), ethylene oxide (EO), and steam autoclave (SA) and control group. The specimens were stored individually in eppendorf tubes containing deionized water at room temperature until use.

XPS analysis

The enamel surface elemental chemical composition quantification was performed by XPS before and after the sterilization procedures, using four specimens from each sterilization group. XPS measurements were carried out using a spectrometer (UNI-SPECS UHV) equipped with a monochromatic Mg K_α X-ray source (1253.6 eV) operated at 250 W, under ultra-high vacuum conditions (10^{-7} Pa). XPS total spectra (pass energy of 45 eV) and high-resolution spectra from all detected elements were taken at pass energy of 10 eV. The entire enamel labial surface (5 × 5 mm) was analyzed for each specimen, and the composition of the surface layer (<5 nm) was determined from the ratio of the relative peak areas corrected by sensitivity factors of the corresponding elements. The analysis of chemical elements states was performed by deconvolution of spectral intensities using a Voigt function, and the quantitative data were obtained from peak areas of high-resolution spectra for each element. The element quantification in atomic concentrations was carried out for carbon (C) 1s, nitrogen (N) 1s, oxygen (O) 1s, calcium (Ca) 2p, and phosphorus (P) 2p. Ca/P ratio was calculated from the area of Ca 2p and P 2p spectra. The components related to each element were also identified, and their contents have been accounted and used for qualitative evaluation.

Enamel sterilization procedures

Specimens from MI group were individually immersed in 200 ml of sterile distilled water and submitted to microwave irradiation for 3 min at 70% of power in an unmodified domestic microwave oven at 650 W (Model Sensor Crisp 38, Double Emission System; Brastemp SA, Manaus, Brazil). The microwave oven was calibrated before experiments, as described elsewhere [14]. In the GI group, the specimens were irradiated at room temperature (27 °C) in a 60 Cobalt gamma irradiator (Gammacell 220 N, Atomic Energy of Canada Ltd., Ottawa, Ontario, Canada). The irradiation was carried out at an average dose rate of 1.55 kGy/h for 16 h and 8 min to achieve the targeted dose of 25 kGy [6]. The EO specimen's sterilization was carried out in three steps (ACECIL, Comércio e Esterilização a Óxido de Etileno). First, specimens were pre-conditioned for 1.5 h under 50–60% relative humidity. In the second step, specimens were exposed to ethylene oxide gas with a gas concentration of 600 mg/L at low pressure and temperature (45–55 °C) for 3.5 h. The last step was a 1.5 h degassing period with nitrogen [9], following by the aeration period of 48 h. Specimens from SA group were

subjected to a steam autoclaving (AV-50, Phoenix, Araraquara, Brazil) at 121 °C for 30 min followed by 10 min air-drying at sub-atmospheric pressure. Finally, the temperature was gradually decreased to room temperature [4].

FEG-SEM and EDS analysis

All specimens from each sterilization group (n = 10) and control group (non-sterilized; n = 10) were used for superficial FEG-SEM analysis. Specimens were mounted on metallic stubs, sputter-coated with a 5-nm layer of carbon by vapor deposition (sputter coater, BAL-TEC model SCD 050, Balzers, Germany), and finally stored in a desiccator for 24 h at 17 °C. The enamel surface of samples without any other special treatment was analyzed and images corresponding to the representative areas were taken using a field emission gun scanning electron microscope (FEG-SEM, Jeol model 7500F) operated at 2 kV and equipped with X-ray energy-dispersive spectroscopy (ThermoNoran Superdry, Thermo Scientific, Walthan, MA).

For performing FEG-SEM analysis of cross-sectional surface, after enamel surface analysis, three enamel specimens from each group were selected and frozen in liquid nitrogen and fractured manually using tweezers. One cross fracture surface of each specimen was, then, mounted on metallic stubs, sputter-coated with carbon, and stored in a desiccator as above described. During the morphological analyses, for each magnification, the entire surface of enamel was evaluated and two representative areas were selected for micrograph acquisition.

In order to evaluate qualitatively the chemical composition of samples, EDS analyses were performed for all groups (n = 10) using the acceleration voltage of 12 kV and counting time (live time) of 180 s. Two areas corresponding to approximately 0.25 mm^2 were analyzed for each specimen, and the elements present on enamel specimens were identified. The advantage of EDS analysis is to be more volumetric (depth analysis of about 1 μm) than the XPS one, so it was used complementary to the quantitative XPS analysis.

Statistical analysis

The contents of C, N, O, Ca, P, and Ca/P were expressed by means of % atomic concentration (at.%) and standard deviations (SD). Statistical analysis was performed at a standard p value of 0.05, using SPSS software (version 19) and Microsoft Excel Macro (available at: http://www.ime.usp.br/~jmsinger/). Assuming non-normal distribution of the data, non-parametric statistical tests were applied. Comparisons inter-group were conducted using Kruskall-Wallis test on pre- and on post-sterilization. The interaction effect, group and time (pre- and post-sterilization), was also evaluated, using Brunner nonparametric analysis [16].

Results

XPS

XPS analysis showed that oxygen (O), carbon (C), calcium (Ca), phosphorus (P), and nitrogen (N) elements were present in higher concentrations, while sodium (Na), magnesium (Mg), and chlorine (Cl) elements were detected in minor quantities (<0.5 at.%). All intensity distributions are characterized by typical enamel-binding energies, where 531.1 for O 1s, 347.3 for Ca 2p 3/2, 133.4 for P 2p, 285.0 for O 1s, and 399.6 for N 1s (Fig. 1). The atomic concentrations of O, C, Ca, P, N, and Ca/P ratio before and after sterilization process are shown in Table 1.

According to Table 1, the initial analysis of surface composition among groups showed significant differences only in nitrogen concentrations of pre-sterilization data (Kruskal-Wallis test, $P < 0.05$). Following the sterilization procedures, no significant differences on atomic concentration were

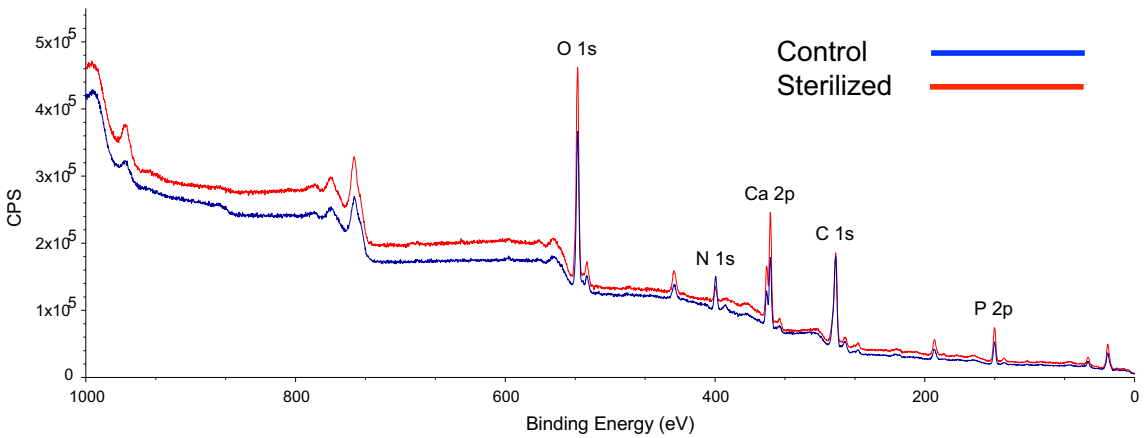

Fig. 1 An illustrative example of superimpositions of survey scans for enamel on pre-sterilization (control) and post-sterilization (sterilized). Graph correspondent to counts per second (CPS) × binding energy (eV) for the elements C 1s, N 1s, O 1s, P 2p, and Ca 2p

Table 1 Means (SD) of atomic concentration (at.%) of enamel surface elements measured by XPS, according to groups ($n = 4$). Relative effects (RE) and its corresponding interaction effects are presented

Element	Time		Group				*P* value		
			MI	GI	EO	SA	Group	Time	Interaction
C	Pre	Mean (SD)	30.75 (1.38) A,**a**	31.73 (3.58) A,a	33.28 (4.32) A,**a**	34.30 (7.42) A,a	0.204^{NS}	**0.003**	**<0.001**
		RE	0.305	0.363	0.457	0.484			
	Post	Mean (SD)	37.03 (5.80) A,**b**	30.78 (2.50) A,a	36.38 (5.58) A,**b**	40.68 (4.37) A,a			
		RE	0.645	0.305	0.617	0.824			
P	Pre	Mean (SD)	9.35 (0.62) A,a	9.28 (0.91) A,a	9.18 (1.08) A,a	9.00 (1.56) A,a	0.654^{NS}	0.051^{NS}	0.051^{NS}
		RE	0.602	0.582	0.531	0.500			
	Post	Mean (SD)	8.33 (1.54) A,a	9.43 (0.67) A,a	9.23 (1.26) A,a	7.78 (1.31) A,a			
		RE	0.383	0.621	0.555	0.227			
Ca	Pre	Mean (SD)	12.75 (0.59) A,**a**	12.58 (1.16) A,a	12.33 (1.14) A,**a**	12.55 (1.86) A,**a**	0.699^{NS}	**0.001**	**0.010**
		RE	0.629	0.594	0.566	0.578			
	Post	Mean (SD)	10.83 (2.21) AB,**b**	12.70 (0.85) **A**,a	11.83 (1.27) AB,**b**	10.28 (1.85) **B**,**b**			
		RE	0.320	0.645	0.438	0.230			
N	Pre	Mean (SD)	4.30 (1.14) ***A**,**a**	4.23 (0.51) ***A**,a	4.45 (0.87) ***A**,a	3.18 (0.28) ***B**,a	0.601^{NS}	**0.001**	**0.001**
		RE	0.434	0.512	0.535	0.098			
	Post	Mean (SD)	5.70 (1.69) A,**b**	4.18 (0.41) A,a	4.28 (0.66) A,a	5.58 (2.62) A,**b**			
		RE	0.758	0.504	0.512	0.648			
O	Pre	Mean (SD)	42.40 (1.64) A,**a**	42.20 (2.09) A,a	40.83 (2.77) A,**a**	40.93 (4.05) A,a	0.113^{NS}	**<0.001**	**<0.000**
		RE	0.696	0.664	0.512	0.551			
	Post	Mean (SD)	38.15 (3.72) **B**,**b**	42.98 (1.44) **A**,a	38.58 (3.59) **B**,**b**	35.70 (3.85) **B**,a			
		RE	0.324	0.727	0.348	0.180			
Ca/P	Pre	Mean (SD)	1.37 (0.04) A,**a**	1.36 (0.04) A,a	1.35 (0.05) A,a	1.40 (0.04) A,**a**	0.861^{NS}	**<0.001**	**0.022**
		RE	0.652	0.578	0.535	0.805			
	Post	Mean (SD)	1.30 (0.05) **B**,**b**	1.35 (0.02) **A**,a	1.32 (0.08) AB,a	1.32 (0.02) **B**,**b**			
		RE	0.254	0.496	0.395	0.285			

Interaction effect (Brunner nonparametric analysis): For each element, different capital letters in the same row indicate significant difference among groups. Different lowercase letters in the same column indicate significant difference between pre- and post-sterilization. Interaction effects evaluation was based on residuals evaluation. Bold entries indicate significant difference

NS not significant

*Significant difference among groups (Kruskal-Wallis; $P = 0.03$)

observed among groups for all elements. From interaction effect evaluation (Brunner nonparametric analysis), phosphorus showed no significant effects ($P > 0.05$). According to Brunner nonparametric analysis, significant interaction effects were observed on concentration of the elements carbon, calcium, nitrogen, oxygen, and Ca/P ratio, indicating that these alterations after sterilization were dependent to the sterilization method.

Before sterilization, SA group showed lower N concentration than other groups. A significant increase in nitrogen concentration was found in SA and MI groups after sterilization, with no changes on GI and EO groups. While carbon concentration was not changed after sterilization with GI and SA. a significant increase was observed after sterilization with MI and EO. Oxygen concentration showed significant decrease after sterilization with groups EO and MI, while the GI and SA groups showed no significant alteration. After sterilization, significantly higher concentrations of O were observed on GI group when compared to the EO, MI, and SA groups. While the Ca concentration presented a significant decrease after sterilization with SA, EO, and MI, GI group did not show any significant change. The Ca concentration was significantly higher on GI than on SA group after sterilization. In GI and EO groups, the Ca/P ratio was not changed after sterilization procedures, whereas a significant decrease was observed on SA and MI groups. After sterilization, Ca/P showed significant higher values on GI group when compared to the SA and MI groups.

The components $CaHPO_4/CO_3$ and C-O were identified for O 1s. C 1s was composed of C-H, C-O, C = O, O-C = O, and CO_3. Both Ca 2p and P 2p were attributed to $CaHPO_{4\ 3/2}$ and $CaHPO_{4\ 1/2}$. The components $C\text{-}NH_2$ and C-N were attributed to N 1s. The components of each element were expressed in percentage and plotted in graphs, according to groups (Fig. 2).

After sterilization, O 1s plot shows decreased $CaHPO_4$ and/or CO_3 percentage on SA, MI, and EO groups. The component C–O was slight increased on MI group and slight

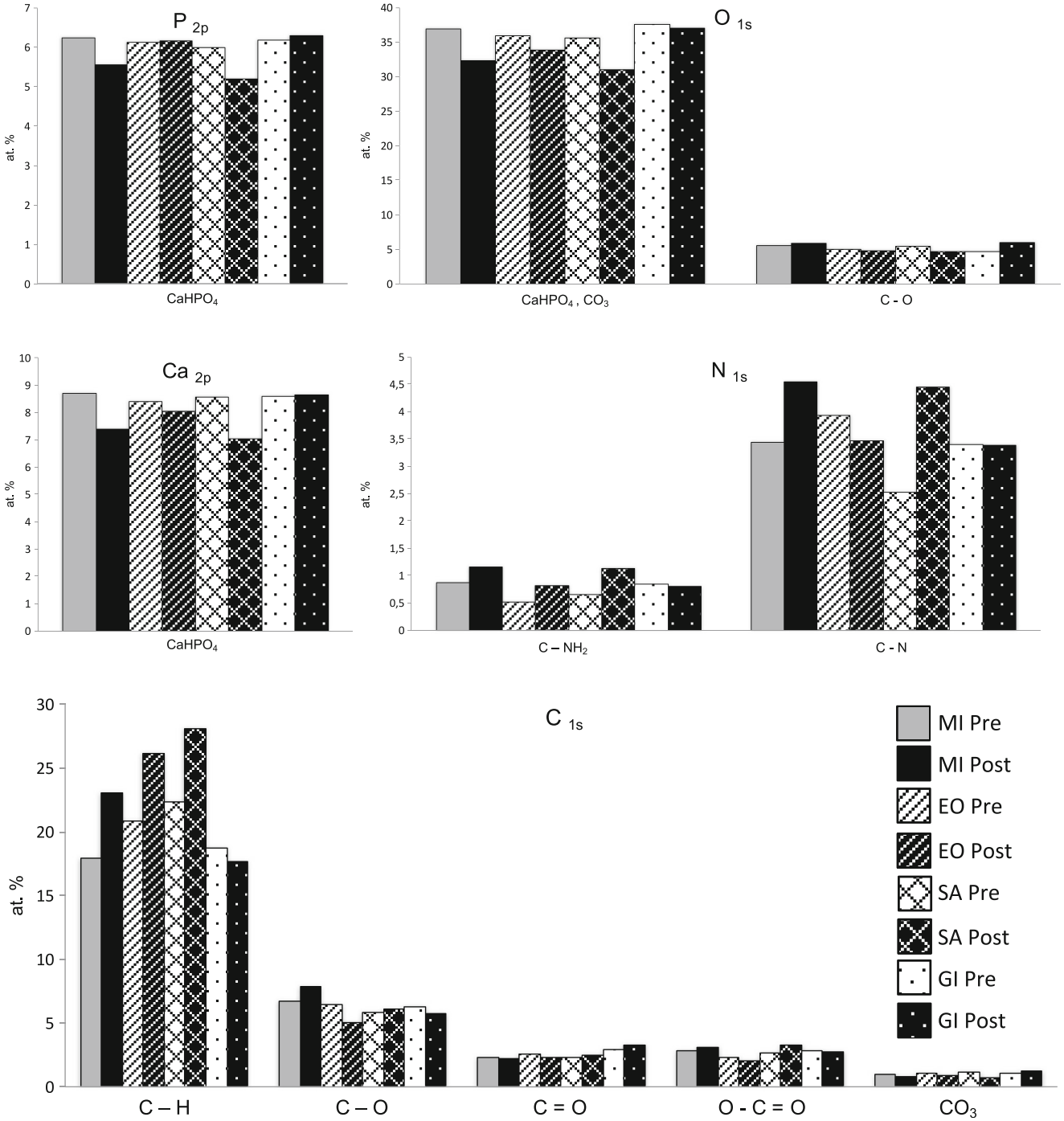

Fig. 2 Graphs correspondent to at.% of different components for the elements C 1s, N 1s, O 1s, P 2p, and Ca 2p in each group on pre- and post-sterilization

decreased on SA and EO group after sterilization. GI group shows only discrete alteration. As shown on C 1s graph, an increase in C–H component was observed after sterilization on SA, MI, and EO groups. MI group also shows increased porcentage on C–O component. C = O, O–C = O, and CO_3 components showed only slight alterations for all groups. GI group showed only minor alteration on C components. $CaHPO_4$ components from Ca 1s and P 2p were decreased after sterilization on MI and SA groups (graph Ca 1s and P 2p). EO group showed that $CaHPO_4$ decreased only on Ca 2p component. GI group did not show any considerable alteration on $CaHPO_4$ component after sterilization. Lower percentage of C–N component was detected on SA group before sterilization (N 1s plot). MI and SA groups showed increased C–NH_2 and mostly C–N component after sterilization. A discrete increase on C–NH_2 and decrease on C–N was observed on EO group after sterilization. N 1s components showed no alteration on GI group after sterilization.

EDS

From EDS analyses, C, O, Ca, and P elements were identified in all control and sterilized specimens. The elements Na, Mg, Cl, and zinc (Zn) were also present in some specimens, although in lower concentrations. As a qualitative evaluation, EDS supports the volumetric chemical composition of enamel obtained by XPS and could not identify significant differences in elemental composition of enamel specimens among sterilization groups, as well as between controls and their respective experimental groups. Representative EDS spectra of all groups are shown in Fig. 3.

FEG-SEM and EDS

Representative FEG-SEM micrographs showing the enamel surface of specimens of control and sterilization groups are shown in Fig. 4a–e (left and middle). Non-sterilized surface topography shows a relatively homogeneous appearance, predominantly with smooth areas and scratches (Fig. 4a, left and middle). Different degrees of alteration, ranging from minor to severe damage, were observed in the experimental groups. In GI group, no significant morphological change was observed, as seen in Fig. 4b (left and middle). It was also observed minor changes on specimens from MI group, with specimens showing a slight etch (stars) on the surface when compared to control (Fig. 4c, left and middle). From the micrographs of EO group (Fig. 4d, left and middle), cracks (arrows) and pores with etching surface (stars) were systematically observed. Degradation and loss of substance of the enamel could also be observed on the micrographs from SA group (Fig. 4e, left and middle). It was observed cracks (stars) in all specimens, with pores becoming larger with intensification of etching (stars). The representative micrographs showing the cross section characterization of enamel are shown in Fig. 4a–e (right). Figure 4a (right) is representative of the morphological appearance of control group, revealing a homogeneous, predominantly dense and smooth area. The images obtained from GI, MI, and EO groups were similar, with no significant changes on enamel morphology when compared to control group (Fig. 4b–d, right). However, morphological changes were observed for SA group, characterized by roughness and porosity aspect along the cross section surface (Fig. 4e, right).

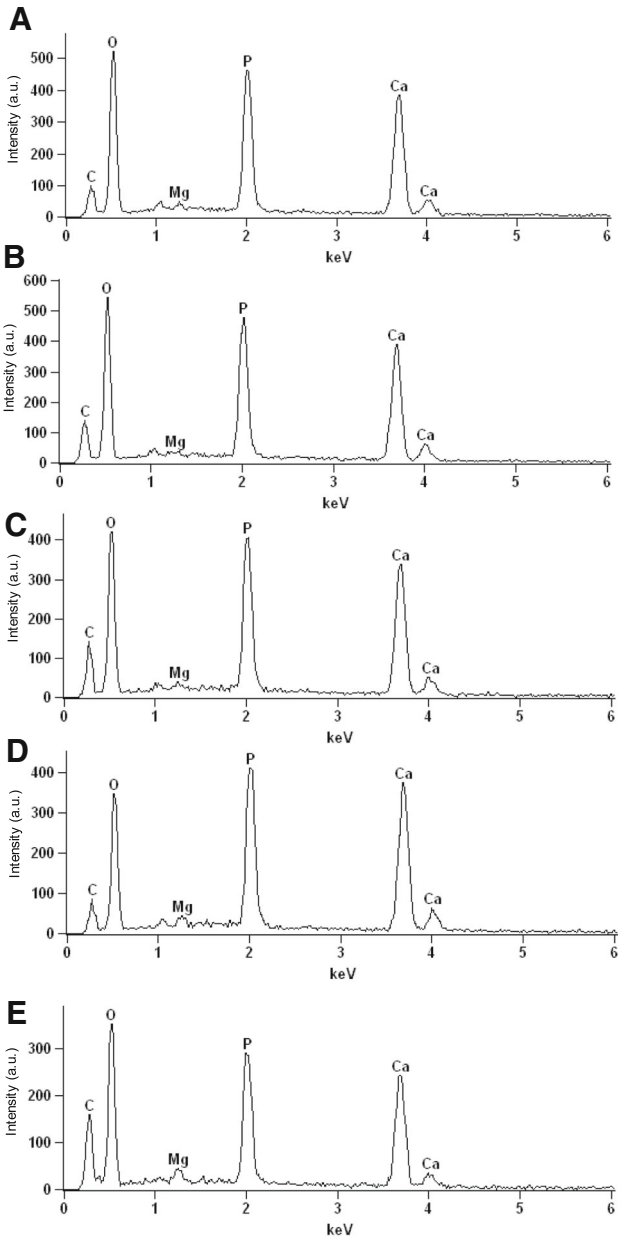

Fig. 3 Graphs illustrating the obtained results of to EDS analyses. **a** Control. **b** Gamma irradiation. **c** Microwave irradiation. **d** Ethylene oxide. **e** Steam autoclave. Graph correspondent to intensity (arbitrary units a.u.) × energy (keV)

Discussion

Since the first comparative study of sterilization effects on enamel properties in the mid-1980s [17], many researches have evaluated the sterilization effects on enamel properties [5–10, 13, 14, 17–19]. Besides these previous studies, the recommendations of sterilization methods have been made without a comprehensive understanding of their effects on the chemical composition of the enamel surface and their influence on the morphology of both surface and cross-sectional surface of enamel. Alterations on enamel elemental composition have been described to present a profound effect on the enamel properties and microstructure [15]. Thus, the present study used highly specific methods to determine whether the sterilized enamel could be related to possible changes in the elemental composition and morphology.

The chemical composition of dental enamel is well-known for its inherent variation, observed even within the same tooth [20]. It was suggested that enamel specimens from the same

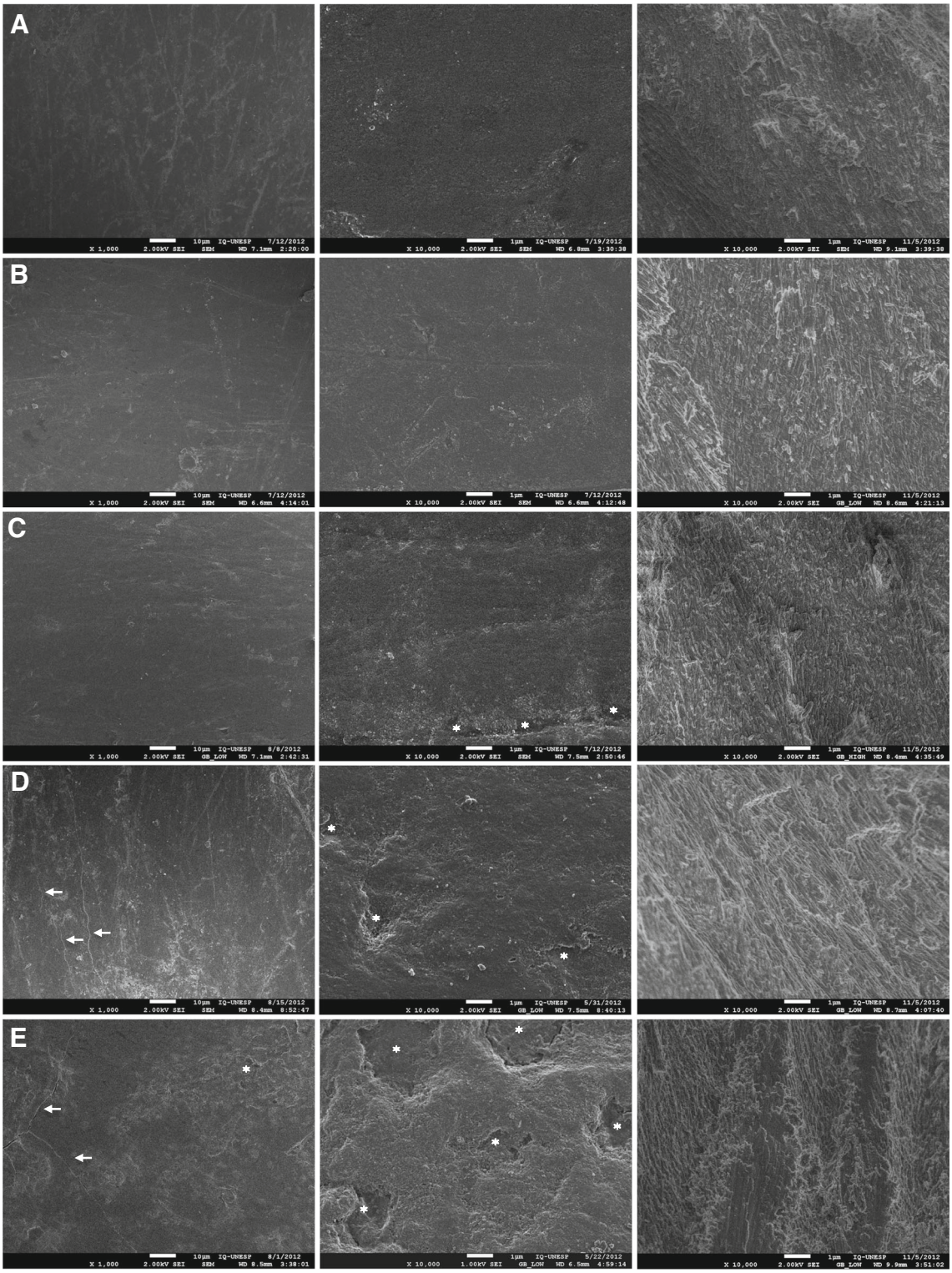

Fig. 4 FEG-SEM micrograph of enamel surface at magnifications of ×1000 and ×10,000 (*left and middle columns*) and cross-sectional enamel at magnifications of ×10,000 (*right column*). **a** Control. **b** Gamma irradiation. **c** Microwave irradiation. **d** Ethylene oxide. **e** Steam autoclave. **c** Specimens from MI group showing a slight etch (*stars*) on the surface when compared to control (*left and middle*). **d** Specimens from EO group showing cracks (*arrows*) and pores with etching surface (*stars*) (*left and middle*). **e** Specimens from SA group showing cracks (*stars*), with pores becoming larger with intensification of etching (*stars*) (*left and middle*)

tooth present lower variation on enamel composition than specimens from different teeth [20]. Considering the more uniform composition of bovine enamel when compared to human enamel [2], the present study evaluated enamel

specimens from bovine teeth. To make possible a proper comparison of the effect of different sterilization methods on enamel chemical composition and morphology, with minimal variation between specimens, a specific experimental protocol was designed so that all groups presented specimens from the same tooth sample, and in each group, the same specimens were used in the three non-destructive evaluation tests (XPS, FEG-SEM, and EDS). XPS is a very sensitive technique for surface analysis [21]; hence, the enamel surface was preserved in all specimens and no polishing procedures were carried out, avoiding contamination and misinterpretation of results.

The present XPS results are in agreement with previous study, where the elements detected with highest concentrations were O, C, Ca, P, and N, which are characteristic to unpolished and ungrounded enamel surface [22]. Studies using different specimen preparations also reported these elements as main components of enamel at similar binding energies [21, 23–25]. The difficulty in obtaining enamel samples with similar composition has been described for human [23] and bovine enamel [22]. Comparing the control groups, it can be seen that the experimental design was successful in achieving homogeneity so that, with the exception of N on SA group, all the elements showed similar concentrations among control groups. In a detailed observation of SA group, lower C-N component, which is related to the inherent proteins and peptides composition of enamel surface [23], appears to be the major responsible for lower N concentration. Thus, the detected difference on N concentration could be attributed to regular variation of enamel composition.

After sterilization, SA specimens showed significant decrease on Ca and Ca/*P* values. Decrease on Ca/P has been related to adsorption of phosphates on the crystal surfaces, substitution of Ca by Na or Mg or incorporation of impurities [26]. Significant increased concentration of N was also observed and assigned to C–NH2 and mostly C–N component. In addition, increased N and decreased Ca and Ca/P have also been documented for etched enamel [22]. As observed on Ca 2p and P 2p component's graphs, the decreased Ca/P could be attributed to reductions on both Ca and P content. Thus, the present findings suggest that SA sterilization method promoted loss of minerals and consequently increased exposure of inherent organic/protein components of enamel.

The specimens from MI group showed a slight, but significant increased C and N content and decreased concentration of O, Ca, and Ca/P ratio. Carbon content has been associated to the inherent organic composition of enamel and may also indicate the presence of adventurous organic contamination [21, 22]. In a detailed observation of C 1s components, MI group showed apparent increased C-H and C-O, where the first is related to the smear layer and the second to the organic composition of enamel matrix [21]. As part of organic composition of enamel, both N components C-N and C–NH_2 were increased after microwaving. Both components C-O (from C 1s) and C-N (from N 1s) were increased after MI sterilization. Nevertheless, due to concomitant increase on N concentration and decrease on inorganic components, it is more likely that mineral loss and exposition of specific organic components of enamel have occurred along the surface. The present MI effect on carbon content is much lower than those observed for Er:YAG laser irradiated enamel surface, in which decreased C-O, C = O, O-C = O, and CO_3 were observed and attributed to organic component carbonization [21]. The observation of MI C 1s components suggests that carbonization effect was not present on MI specimens.

XPS results from EO group indicated significant increase on C and decrease on Ca and O concentration, suggesting surface changes that included mineral loss and exposure of organic components of enamel. The effect of increased C content observed for EO group could be considered solely due to increase on C-H components, once C-O, C = O, O-C = O, and CO_3 appear to be slightly decreased. Decreases on both water and the above cited organic components have also been reported for enamel irradiated with Er:YAG laser [21]. The present study does not explain whether EO affected the water content or organic components; nevertheless, it could be observed that EO and MI groups showed different effects on C 1s components.

GI specimens showed completely preserved elemental surface composition after sterilization, as no significant changes of enamel elements and Ca/P content were observed. When compared to the groups, it can be seen that EO, MI, and SA revealed surface with less oxygen, MI and SA showed lower Ca/P and SA presented lower calcium than GI specimens. These results suggest that, while no changes on enamel surface were observed for enamel sterilized using GI, progressive lower mineralization of enamel was detected when EO, MI, and SA sterilization methods were used. Besides the detected mineral loss observed on SA, MI, and EO groups after sterilization, changes on the peaks corresponding to inorganic elements were very small. Regarding the increased organic elements observed on SA, MI, and EO specimens, it must be stated that the present study choose to preserve the enamel surface. Ruse et al. [22], described the presence of an organic-rich layer on preserved enamel surface. Thus, the detected increase on organic composition could also be related to this organic-rich layer, characteristic of the enamel outer surface.

The Ca/P ratio is considered an indicator of mineralization degree [27]. Values of Ca/P ratio ranging from 1.48 to 1.29 have been reported for dental enamel [21, 23, 24, 26], which is in the range of the findings in the present study. It has been demonstrated that the decrease in Ca and P content in enamel resulted in decreased mechanical properties [28]. Also considered a relevant component of enamel, the carbonate can influence its mechanical properties [24], where increasing the carbonate amount was associated with decrease in crystallinity,

hardness, and Young's modulus [28]. In the present study, no substantial changes in CO_3 concentration were observed after sterilization, regardless of the method used.

The enamel below the surface is more densely and more uniformly mineralized than the enamel on the surface [22, 29], and the mineral content of enamel and the concentration of minor components differed between the surface and inner portions of enamel [15]. The present EDS results showed that the composition of enamel specimens was found to be similar among groups, where the same main elements C, O, Ca, and P were detected in all specimens. Previous EDS studies using dental enamel detected as main elements Ca and P [30–33], O, Ca, and P [34], and also O, Ca, P, and Cl [35, 36]. Such variation on detected elements among these studies could be attributed to differences on EDS detection system. In the present study, the use of a quantitative analysis for EDS performed on FEG-MEV was considered not convenient. Therefore, it is difficult to make direct comparison with other studies, as the total number of elements examined was different among the previous studies and chemical compositions were quantitatively reported in percentage. Nevertheless, the present EDS evaluation was important to show that no aggressive damage occurred on enamel bulk of all groups evaluated.

In this study, SEM technique, which is widely used in materials science for surface characterization and morphological enamel evaluation in dentistry studies [23, 36–38], was chosen to evaluate the morphology of both surface and cross section of enamel, using a high-resolution FEG-SEM microscope. According to the results, the morphological characteristics observed for the control group are in agreement with the untreated enamel features observed in SEM studies using bovine enamel [39] and human enamel [40]. While preserved characteristics were observed on GI sterilized specimens, different levels of morphological changes were observed when enamel samples were sterilized by MI, EO, and SA.

Thermal effects have been described as responsible for morphological damages such as cracking and melting on enamel submitted to laser irradiation [41]. The present outcomes showed similar surface etch on MI and EO specimens; however, surface cracking was most common on enamel of specimens sterilized using EO. Considering the differences on sterilization protocols of MI and EO, the temperature cannot be considered the only factor involved on the present enamel alterations. On MI process, the specimens are immersed on water, irradiated for 180 s at 70% of power of 650 W, which implies about 126 s of effective intermittent irradiation and 54 s of no irradiation; therefore, there is time for the enamel specimen be cooled between irradiation restart. The water temperature reached nearly 100 °C at approximately 130 s from start of procedure and remained at this temperature for only 50 s during microwave irradiation [14]. The thermal and nonthermal effects of microwave irradiation are considered proportional to exposure time [42, 43], and the temperature range achieved during microwave irradiation of enamel was considered insufficient to cause extensive damage on enamel microhardness [14]. The enamel changes observed on MI specimens were attributed to both temperature and water immension factors. Considering that the diffusion rate through the organic barrier is an important factor in determining the degree of enamel erosion [23], it can be suggested that the diffusion process may had occurred on enamel surface of MI. The water may have facilitated the mineral diffusion and consequent decrease on inorganic components. On the other hand, uniform distribution of energy and temperature around the irradiated specimen decreases cracks occurrence. The effect of this method on the enamel morphology was very similar to GI and control specimens, consisting in an alternative method for enamel decontamination in studies where morphological characteristics are to be evaluated.

The ethylene oxide sterilization process comprises steps where the specimens are submitted to environmental conditions without water immersion. Specimens are submitted to relative humidity of 50 to 60%, 1.5 h of gas exposition under temperature ranging from 45 to 55 °C, and aeration and degassing phases, totaling 54.5 h. The prolonged exposure with high water vapor pressure possibly resulted in leaching effect on minerals from the enamel, which implies in greater carbon exposure on the surface. Other possibility comprises the continuous environment conditions of gas exposition with no cooling effect of water, possibly resulting in excess heat into the enamel specimen and consequent cracking by thermal damage. The use of laser with no cooling water and under continuous wave, which means no pulsed laser, have been related to thermal damages on enamel [41]. The enamel water content has been described as sufficient for diffusion of acids and other components into the tooth and leaching of calcium and phosphate during the erosion process [44, 45], especially in enamel specimens without a intact pellicle, i.e., only protein/lipid coating of the individual crystals. Thus, loss of minerals of EO specimens possibly occurred due to diffusion out of enamel surface during sterilization.

The observed changes on autoclaved specimens could be considered more severe when compared with other evaluated methods. Several authors have suggested that the autoclaving process can affect the enamel structure [5–7, 10–13, 18]. Contrasting results concerning autoclaving effect on enamel morphology have also been reported. SEM evaluations carried at low magnification (×500) did not reveal any significant morphological changes on autoclaved enamel [5, 17]; however, Amaechi et al. [6] obseved changes in surface appearance of enamel sterilized using autoclave. In the present study, morphological and compositional changes were evident after autoclaving, indicating selective removal of inorganic material on the surface and deep into the enamel. The water steam present in the sterilization process thereby may have facilitated further erosion in the enamel crystals. Besides the humid

environment, the association of steam, high temperature under pressure for a continuous and extended time could have facilitated cracking of enamel. Enamel cracked surfaces would offer more channels for the ion transfer process and may have promoted ion displacement from inner enamel [30]. In fissure enamel, a poorer prismatic arrangement, low mineral and high protein content, and therefore, more porosity have been described [15]. Removal of mineral from enamel surface associated with dissolution underneath the surface has been described for erosion process [45]. In the present study, it could be suggested that the steam interacted with protein/lipid coating of the crystals and then with the surface of the hydroxyapatite crystals themselves, which could be responsible for the observed morphological damages and decreased inorganic elements.

Unlike the other groups, GI specimens showed no alterations in any elements after sterilization. Gamma irradiation sterilization process was performed at room temperature (25 °C), providing no alteration on specimen's environment condition. Gamma irradiation has shown to produce no changes in surface microhardness or in the response to demineralization of enamel [5, 6, 8, 19]. Though, it has the undesirable effect of visible color change, which has been reported after the sterilization process with gamma irradiation [8, 19] and was also observed in the present study. No attempt was made to evaluate the color of the enamel using a color-measuring device. This alteration has been described as a dose-dependent effect of gamma irradiation, where doses up to 4.08 kGy caused color change of enamel [8, 19]. The effect of gamma irradiation on collagen protein has been described [39, 46], where due to the direct effect of ionizing radiation process, collagen could be damaged by polypeptide chain scissions, predominantly when collagen is irradiated in a dry state. Nevertheless, effect of gamma irradiation on the elemental chemical composition of enamel was observed in the present study. Besides the preservation of the surface chemical composition of enamel, GI showed higher levels of some elements when compared to the other groups.

The present findings indicated that gamma irradiation is a reliable method for enamel sterilization and once produced no changes in morphology and surface chemical composition of bovine enamel. The two main disadvantages of this method are the high cost of the equipment and the length of time required for the sterilization. The darkening effect of the enamel also must be taken into account when choosing the sterilization method. Autoclave promoted severe changes that may influence the outcomes of in vitro and in situ studies. Considering these findings, when GI and SA were used as sterilization methods, the evaluation of these data should be done carefully, as a trend in lower mineral components exists for SA sterilized specimens.

Some care should also be taken when making comparisons between GI and both EO and MI methods, once a less pronounced difference in mineral concentrations was detected. Ethylene oxide showed small alterations on both chemical composition and morphology. The complexity of sterilization process, the length of time require, and the costy must be considered. Microwave irradiation produced only minor alterations on chemical composition, corroborate the earlier positive findings [14]. Thus, microwave irradiation is emerging as an important alternative to the conventional enamel sterilization methods. This method is highly reproducible and easy to perform at low cost, and no additional technical skill is needed and is feasible and convenient.

A detailed knowledge about the effects of sterilization methods on the morphology and on the elemental chemical composition of enamel was performed and should help researchers select the sterilization method most suitable for the development of their studies, decreasing the variability and increasing the reliability of the results. The limitations of the present study include effects of sterilization procedures under the enamel surface layer, and the existence of other factors affecting enamel characteristics. Additional evaluation of sterilization methods also must be conducted to evaluate the implications of such changes on enamel specimens to be used in in vitro and in situ evaluations. Nevertheless, the present findings provide detailed knowledge about the effects of sterilization methods on the morphology and on the qualitative and quantitative elemental chemical composition of enamel and should help researchers to select the sterilization method most suitable for the development of their studies, according to each characteristic under study.

Acknowledgements We thank Dr. Peter Hammer for the XPS analysis and technical assistance. We would also like to thank ACECIL, Comércio e Esterilização a Óxido de Etileno and IPEN - Instituto de Pesquisas Energéticas e Nucleares, for their continuous support for sterilization of enamel specimens with ethylene oxide and gamma irradiation, respectively. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

Compliance with ethical standards

Conflict of interest Author Viana, PS, declares that he has no conflict of interest. Author Orlandi, MO, declares that he has no conflict of interest. Author Pavarina, AC, declares that he has no conflict of interest. Author Machado, AL, declares that he has no conflict of interest. Author Vergani, CE, declares that he has no conflict of interest.

Funding The work was supported by the State of São Paulo Research Foundation (FAPESP) within the framework of the project no. 2011/09416-0 and 2011/10358-4. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent For this type of study, formal consent is not required.

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