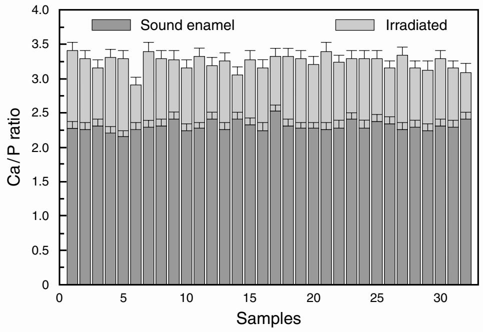
Laser Physics Letters

Laser Phys. Lett. **2**, No. 6 , 318–323 (2005) / **DOI** 10.1002/lapl.200410181

Abstract: Non-invasive methods such as X-ray Fluorescence has been applied to study of the distinct pathologies and contamination levels analysis in various biological tissues among them nails, hair, and tooth. On the other hand, several works have demonstrated that the laser could be induced chemical and morphological alterations on the enamel surface occupying an important role as co-adjuvant in the caries disease prevention. Here, we have combined X-ray fluorescence using Fundamental Parameters method aiming to evaluate the variation in the chemical contend. The Ca and P concentrations as well as the Ca/P ratio were estimated in sound human and bovine enamel surface by X-ray fluorescence (XRF). We also evaluated the effect of the lactic and the acetic acids on the sound and irradiated bovine enamel surface by Scanning Electron Microscopy (SEM) verifying the acid attack changes in the sound and irradiated bovine enamel. The laser parameters applied produced lower ablation depths, but sufficient to induce compositional changes. Our results have indicating an alteration statically significative to Ca/P ratio and also indicated an increase in the Ca/P ratio for the irradiated groups in comparison to sound groups. We also verified that the acid attack is more pronounced to sound bovine enamel surface in relation to irradiated to the bovine enamel surface indicating that the nanosecond laser can be used to reduce the demineralization process.



XRF analysis results of dental enamel surface to irradiation condition of 40 J/cm^2($\tau = 10$ ns, repetition rate 5 Hz)

Nanosecond Nd:YAG laser on dental enamel: compositional analysis by X-ray fluorescence

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Received: 27 December 2004, Accepted: 31 December 2004

Key words: dental enamel; nanosecond laser; caries prevention; X-ray fluorescence; fundamental parameters

PACS: 42.62.Be, 61.80.Ba, 68.37.Hk

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1. Introduction

The chemical changes induced in dental hard tissues irradiated by laser has been analyzed by distinct analytical methods such as Energy Dispersive X-ray spectrometry (EDS), Instrumental Neutron Activation Analysis (INAA), Absorption Atomic Spectrometry (AAS) which EDS is the more frequently utilized. In addition, non-invasive methods such as X-ray Fluorescence (XRF) has been applied to study of distinct pathologies and contamination levels in various biological tissues among them nails, hair, and tooth [1,2].

The principal advantage of the XRF resides in the fact that its provide nondestructive, semi-quantitative analysis of the major and trace elements composition determining the elements constituent of structure and sub-structure of

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the dental hard tissues. Another advantage is that samples often require little or no other preparation. Combined with fundamental parameter method, XRF has become a powerful and versatile technique that has found a broad range of applications in the medical and industrial fields. On the other hand, a strong supposition is associated to alterations in chemical composition of the enamel after laser irradiation and consequently an increase in the acid resistance. The laser has been appointed as an efficient auxiliary method in distinct dental procedures and its use to combat the proliferation of caries has been observed in many investigations [3–5]. Firstly, the potential of laser inhibiting the caries disease was demonstrated by Sognnaes and Stern [6,7]. In this experiment, the enamel surface acquired acid resistance after laser irradiation, which has been confirmed in new experiments [8–10] for distinct wavelengths. The nanosecond pulsed Nd:YAG laser has been utilized in few investigations [11]. To prevent dental caries, several investigations has appointed distinct suppositions about the mechanism involved to reduce demineralization rate in the enamel surface[12]. Authors has suggested that the acquired resistance of enamel after irradiation to subsurface demineralization might be due to chemical changes, such as the loss of organic matter and carbonate [5]. The de-mineralization process on the enamel surface in vitro involves a series of reactions even the development lesion [13]. Distinct methods has been applied to evaluate de-mineralization and re-mineralization process of the enamel and dentine by direct measure to mineral loss or gain of the tissues [14,15], such as microradiography, microhardness [16–18] and polarized light among others. The role of the enamel structure in the caries formation has been investigated as well as its morphology and the correlation with factors such as chemical composition [19]. In this investigation compositional analysis was carried out, which provide relative concentrations of elements in the sound and irradiated enamel surface. We also evaluate the morphological changes produced by acid attack on enamel surface to verify the loss mineral after enamel blocks laser irradiation. The lactic and acetic acids are a normal product of the fermentation of carbohydrates in the mouth. About the laser application on enamel several investigations have indicated that its acid resistant is associated with changes in the Ca/P ratio, alterations in the inorganic compounds and organic compounds, resulting in the less acid soluble surface, thus inhibiting the cariogenic process. Despite the restrict use of Nd:YAG laser due thermal effects, our previous study has showed that the temperature rise to parameters applied here is inferior to 2.5°C which justify its use without pulpal damage. The aim of this research was to use X-Ray Fluorescence to determine the relative percentage of the major elements of sound samples, and assess compositional changes that occur after nanosecond laser application. In this investigation, we have estimated the average percentage content of Ca, P, and F in human and Ca, P to bovine enamel samples and the Ca/P rate to both.

2. Materials and methods

2.1. Sample preparation

About this peculiar tissue, enamel is a protective layer of the tooth which preserve the dentine and pulp that are parts of the internal region of the tooth [20]. The enamel has chemical composition correspondent to 96 wt% inorganic basically hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, and 4 wt% organic content and water.

2.1.1. Preparation of blocks of sound enamel

Sound third molars were selected and cleaned, after removing the tooth crown horizontally. The similar procedure was applied to bovine incisors teeth. The enamel blocks of 2 mm thick were prepared using a diamond disc to select an average plain area of approximately 4 mm^2. The illustrative diagram of the sample preparation is presented in the Fig. 1.

Blocks of sound human enamel and bovine incisors enamel were analyzed semi-quantitatively by X-ray Fluorescence. All experiments were carried out in accordance with regulations of the Ethical Committee on Human and Animal Research of the Institute of Research Energetic and Nuclear (Private Communication).

Thereafter, bovine enamel blocks were immersed in 5% lactic and acetic acid to evaluate the demineralization process. The samples were separated in two groups sound and irradiated, respectively and put in individual volumes with 20 ml of lactic acid in agitation for approximately

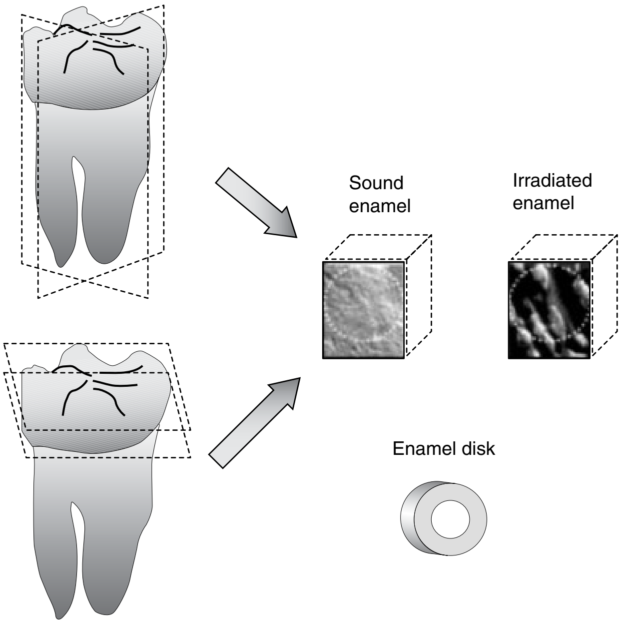

Figure 1 Illustrative diagram of the sample preparation

5 min. Only one block face was exposure to acid solution, the others block faces were recovered with the nail varnish. The acid dissolution of bovine enamel blocks was evaluated by SEM which showing the morphological changes.

2.1.2. Preparation of enamel disks

Extracted human and bovine teeth were stored in physiologic solution. The roots were cut off using a diamond disk and enamel layers were separated of dentine also using a diamond disk. Powder samples of dental enamel were pressed producing disks for posterior laser irradiation.

2.2. XRF- Instrumentation and experimental procedure

The equipment RIX 3000 X-ray spectrometer was utilized for the analysis of mineral composition of the tooth. This technique has enabled a semi-quantitative analysis, where standards were not available due to difficulty in establishing the major and trace quantity elements. The XRF analysis were carried out in two stages: first, an estimative of the majority element concentrations to sound enamel blocks was done, and second, similar procedures to determine major elements concentration after laser irradiation by wavelength dispersive X-ray fluorescence (WD-XRF). These radiations are diffracted for a crystal analyzer and seized by a detector. We have utilized a PET crystal to determine the elements Calcium (Ca) and Phosphorus (P), to the element Fluoride (F) a PAT crystal was utilized. We also selected the position crystal chamber, and the flow counter detector filled with P_{10} (mixture of argon/methane). The chemical elements were measured in the teeth using K_α line of Ca (3.69 KeV), P (2.01 KeV), and F (0.68 KeV). The spectrometer was pre-calibrated with a program based on the Fundamental Parameter (FP) method for quantitative XRF analysis which is based on a numerical procedure involving the relative values of XRF lines intensity using a spectra library of pure elements and it use the sample structure, tube spectrum and characteristics of the optical path as input for the calculation. This method for XRF quantitative analysis is based on the relative values of characteristic X-ray line intensities obtained by dividing the single intensities by the sum of the intensities of all the spectrum lines which not depending of the sample shape. Thus, the enamel blocks with an average plain area were selected.

Ca and P are major elements usually present in dental samples and frequently analyzed [1,2].

2.3. SEM examination

Scanning electron microscopy (SEM) was carried out with JEOL-5900 instrument. The samples were dehydrated in a graded sequence of aqueous ethanol (50, 70, 90, and 100% ethanol) by 20 min at each concentration.

wt%	Ca (%)	P (%)	F (%)	Ca/P ratio
Enamel	35.9	17.0	0.01	2.1
Dentine	25.9	12.6	0.02	2.06
Bone	39.4	18	0.095	2.2

Table 1 Inorganic composition of human teeth

wt%	Ca (%)	P (%)	O (%)	F (%)	Ca/P ratio
Sound	43	19	38	0.1	2.2
Irradiated	45	14	40	0.24	3.2

N = 32

Paired Student's t-test was used to identify statistical differences between sound and irradiated groups. Significant differences were found ($p < 0.05$).

Table 2 Elemental analysis by XRF of human enamel blocks (wt%) for sound and irradiated group

2.4. Irradiation conditions

A prototype of Nd:YAG (Q-switched) laser developed at the Center of Lasers and Applications - Institute of Energetic and Nuclear Research, aiming applications in the Medical Sciences that typical wavelength on 1.064 nm, 40 J/cm^2 ($\tau = 10$ ns, repetition rate 5 Hz) was used in this investigation. The samples were irradiated by scanning the total area of 4 mm^2 of the dental enamel. The sample was exposed to the beam by moving it with help of a set of step motors.

3. Results and discussion

3.1. Enamel blocks

The Table 1 show the values to Ca, P, O, and F for inorganic composition the tooth: enamel, dentine and root [21]. The results were obtained by direct determination of the Ca/P ratio. Several investigations have indicated that after the laser treatment, simultaneously to the morphological changes, a chemical re-organization occurs justifying the increase in the Ca/P ratio for the irradiated group. This chemical change is fundamental as well as acid resistant surface inhibiting the caries disease.

Table 2 shows the results of analysis of Ca, P, O, and F of thirty two enamel blocks. Initially, we obtained the results for sound group and sequently to irradiated groups by XRF. The measured concentrations of Ca and P were also compared with the literature values. A table illustrative of Calcium contend values obtained for distinct experimental techniques was performed by Zaichick et al., in this table the authors obtained that Ca values vary from 29.5 ± 2.1 to 43.6 ± 0.4 [1]. Ours results have appointed for sound enamel blocks 42.6 ± 0.8. In this work, the average values obtained to sound and irradiated groups show that after irradiation an increase of 10% for Ca content and a decrease of 20% in the P contend in according to Table 2. The

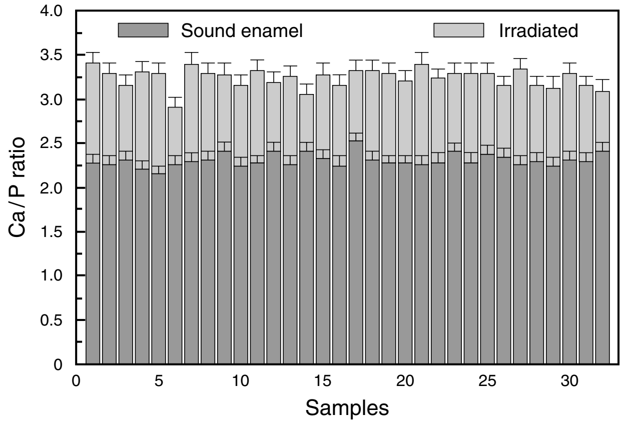

Figure 2 XRF analysis results of dental enamel surface to irradiation condition of 40 J/cm^2($\tau = 10$ ns, repetition rate 5 Hz)

graphic in the Fig. 2 shows the relative percentage of major elements concentration obtained by XRF. These data confirm that the decrease in P after irradiation has been correlated to volatile at a temperature, at least, as high as 1125°C [22], in addition to a chemical re-organization occurs justifying the increase of the Ca/P ratio for the irradiated samples.

Paired Student's *t*-test was used to identify statistical differences between sound and irradiated groups. Significant differences were found ($p < 0.05$).

3.2. Enamel disks

wt%	Ca (%)	P (%)	O (%)	F (%)	Ca/P ratio
HES	56	20	22	< 1	2.78
HEI	55	19	25	< 1	2.86
BES	51	21	28	0	2.49
BEI	52	20	27	< 1	2.59

Abbreviations. HES: Human Enamel Sound; HEI: Human Enamel Irradiated; BES: Bovine Enamel Sound; BEI: Bovine Enamel Irradiated

Table 3 Composition of bovine and human dental enamel

The dental tissues disks were studied by XRF in according with Table 3 and its performance was then compared in parallel analysis by instrumental neutron activation analysis (INAA). The relative values obtained to major elements (Ca) and minor elements (F) are similar. The results pointed out that the laser irradiated enamel with Nd:YAG have higher values of microhardness that sound surfaces, and after the irradiation, a chemical re-organization occurs. This justifies the increase of the Ca/P ratio for the irradiated samples. The concentration of calcium in enamel is higher than that of the dentin, in according to percentage of the hydroxyapatite in each tissue.

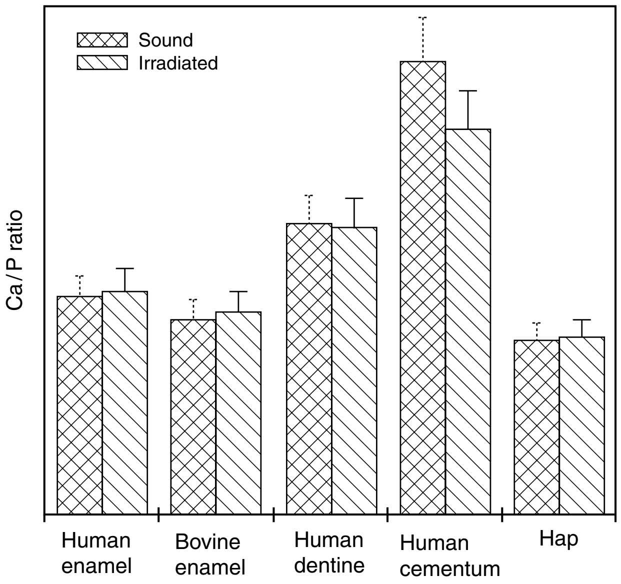

Figure 3 Ca/P ratio to human and bovine dental enamel disks and Hydroxyapatite disks (non-irradiated and irradiated groups, respectively) (Irradiation parameters: 40 Jcm^{-2}, 10 ns, 5 Hz)

3.3. Enamel morphology subsequent acid immersion

A comparison was performed between bovine enamel surfaces treated with laser and subsequent immersion in 5% of lactic acid and acid acetic. The loss of inter-prismatic structure was more evident to bovine sound enamel in comparison to irradiated surface enamel of the two acids, however the effect of lactic acid is more strong and causes a greater loss in the organic compounds than acetic acid. This affirmation can be visualized in the scanning electron micrography presented in the Figs. 4,5,6, and 7.

Scanning electron micrography revealed changes in the surface morphology after exposure to lactic acid. The most important factor in pathogenesis of dental caries is the capacity of a large number of bacteria to produce acid specifically the lactic acid production, from dietary of carbohydrates. A 5% of lactic acid concentration applied for 5 minutes produced a clearly etched surface with demineralization visible permitting a comparison between the sound and irradiated enamel surface.

In our experiment the morphologic alteration related to mineral loss of enamel occurs after 5 min exposure at each acid. The scanning electron micrography permitted the evaluation of changes in the prismatic and inter-prismatic region.

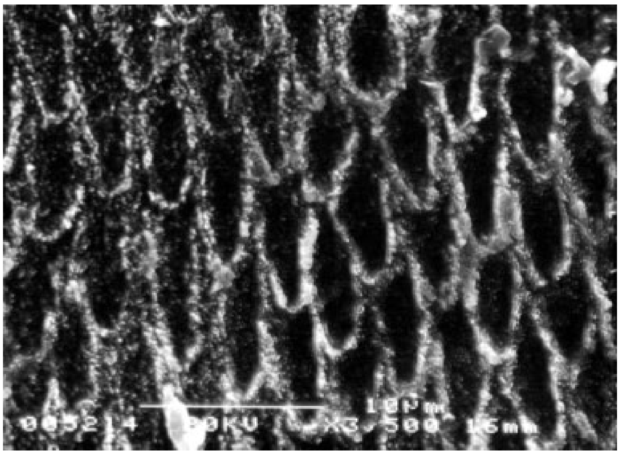

Figure 4 Scanning electron micrograph of sound bovine enamel after immersion in lactic acid. Details of the prismatic structure can be visualized in the figure. Treatment with 5% of lactic acid for 5 minutes in agitation

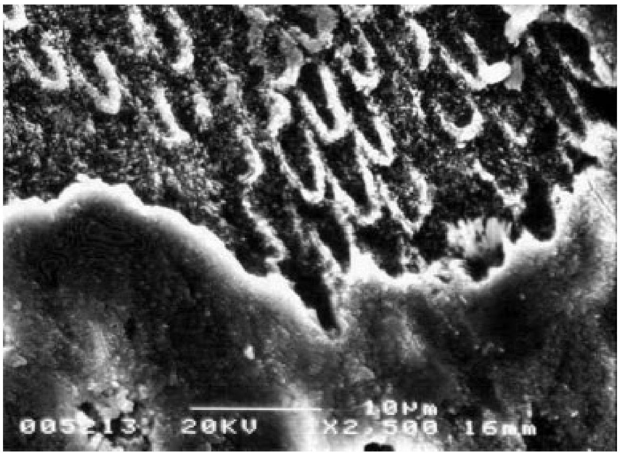

Figure 5 Scanning electron micrograph of the irradiated bovine enamel surface (40 J/cm^2($\tau = 10$ ns, repetition rate 5 Hz)) and subsequent immersion in lactic acid

The SEM image of the enamel structure sound (Fig. 4 and Fig. 6) and irradiated areas (Fig. 5 and Fig. 7) revealed changes in the prismatic and inter-prismatic region. The hard tissue structure contains inorganic (hydroxyapatite), organic (primarily collagen), and water components. The physicochemical alterations produced by laser on enamel surface to acid attack and consequently caries disease has justified the assumption that this mechanism is related to changes in Ca/P ratio, reduced carbonates, and pyrophosphate formation, as well as, reduction in water and organic contents. The changes in Ca/P rate and morphological alteration in the enamel surface is agreement with assumption previously explained.

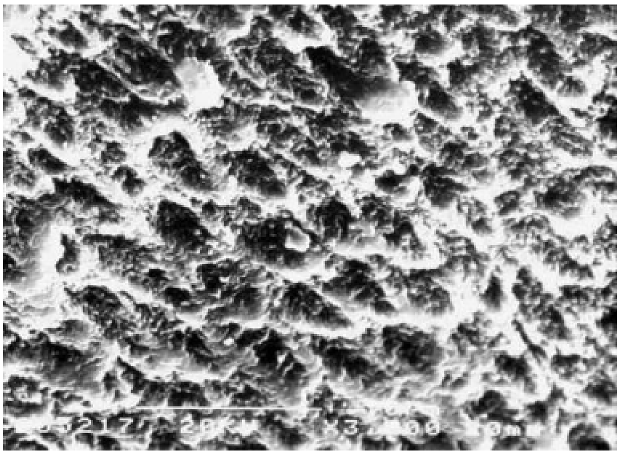

Figure 6 Scanning electron micrograph of sound bovine enamel after immersion in acetic acid. Treatment with 5% of acetic acid for 5 minutes in agitation

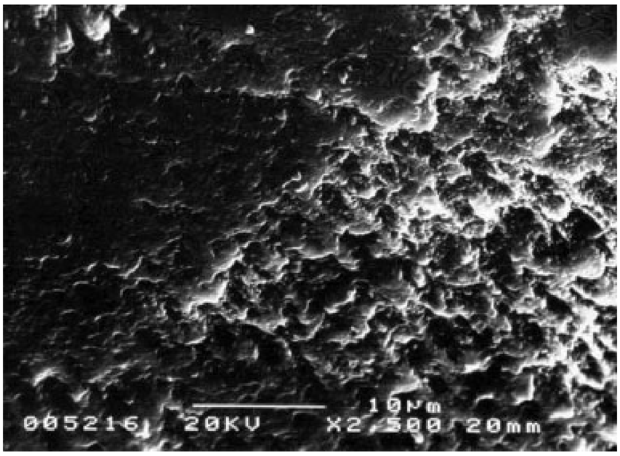

Figure 7 Scanning electron micrograph of the irradiated bovine enamel surface (40 J/cm^2($\tau = 10$ ns, repetition rate 5 Hz)) and subsequent immersion in acetic acid

4. Conclusion

The XRF analysis of human and bovine enamel samples shows reliable differences in the element concentrations of major elements after nanosecond laser irradiation, consequently the Ca/P rate has been also modified. An optimized procedure has been applied in our group for determination of the Ca/P rate in healthy and irradiated dental tissues(to distinct wavelength and irradiation parameters). These results show the large potential of combining XRF using the FP methods to analyze alterations induced by laser. The results obtained by XRF and acid attack demonstrate, first, that these techniques are suitable for determining the Ca/P rate, and second, that the acid attack permits identify the acid resistance induced by this laser on the enamel surface, as by SEM of enamel surfaces immersed in acid lactic and acetic. Simultaneously, we have compared percentage contend and Vickers microhardness for dental enamel surfaces [2]. The results obtained are consistent with other studies indicating a greater Ca/P rate in irradiated group in relation to the sound group, and an increase in the microhardness values after irradiation of enamel surface. Our results suggest that nanosecond pulsed Nd:YAG laser can be used, to conditions employed here, in enamel surfaces for acid attack prevention. Studies has suggested that laser can be used to prevent enamel decalcification by altering the crystalline structure of enamel [23]. However, new experiments are still necessary to evaluate this complex mechanism.

Acknowledgements This work was supported by the State of São Paulo Research Foundation-FAPESP, Process 00/07378-9.

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