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Use of Laser Fluorescence in Dental Caries Diagnosis: a Fluorescence x Biomolecular Vibrational Spectroscopic Comparative Study

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The aim of this work was to verify the existence of correlation between Raman spectroscopy readings of phosphate apatite (~960 cm^{-1}), fluoridated apatite (~575 cm^{-1}) and organic matrix (~1450 cm^{-1}) levels and Diagnodent® readings at different stages of dental caries in extracted human teeth. The mean peak value of fluorescence in the carious area was recorded and teeth were divided in enamel caries, dentin caries and sound dental structure. After fluorescence readings, Raman spectroscopy was carried out on the same sites. The results showed significant difference (ANOVA, $p<0.05$) between the fluorescence readings for enamel (16.4 ± 2.3) and dentin (57.6 ± 23.7) on carious teeth. Raman peaks of enamel and dentin revealed that ~575 and ~960 cm^{-1} peaks were more intense in enamel caries. There was significant negative correlation ($p<0.05$) between the ~575 and ~960 cm^{-1} peaks and dentin caries. It may be concluded that the higher the fluorescence detected by Diagnodent the lower the peaks of phosphate apatite and fluoridated apatite. As the early diagnosis of caries is directly related to the identification of changes in the inorganic tooth components, Raman spectroscopy was more sensitive to variations of these components than Diagnodent.

Key Words: vibrational spectroscopy, hydroxyapatite, dental caries, tooth demineralization.

Introduction

Caries is a chronic infectious multifactorial disease and the most prevalent disease in the oral cavity. It occurs due to the demineralization of tooth surfaces by organic acids (originated from the fermentation of carbohydrates by bacteria) and by organic matrix degradation (1). This process is dynamic and may be reversed at its early stages (2).

The formation of a carious lesion and its progress occurs when periods of demineralization are more frequent than those of remineralization. Thus the disease is characterized by an imbalance between the demineralization and remineralization occurring in enamel. Despite the small mineral loss of enamel is not clinically visible at very early stages, the injury exists. As demineralization progresses, a whitish area appears when the enamel continues loosing mineral (3). The mineral loss due to disease progression, causes visual changes on the tooth surface, starting in a subclinical stage (white spot) and followed by cavitation (4).

The enamel is composed of 96% inorganic material (calcium phosphate in the form of hydroxyapatite) and the remaining 4% is water and organic material. The dentin presents in its composition approximately 70% inorganic material, 18% organic material and 12% water. It is mainly composed of hydroxyapatite and its organic content is formed by approximately 90% of type I collagen. The remaining tissue is a mixture of citrate, lipids and non-collagenous proteins, including phosphor-proteins and proteoglycans (5).

Continuous laboratory studies on caries disease together with clinical research increase the knowledge on this topic resulting in changes of routines and procedures. This includes improvement of the diagnostic ability, allowing the diagnosis of subtle and early stage lesions. To be considered an ideal diagnostic method it has to be reliable, detect lesions in early stages, be able of differentiating reversible and irreversible lesions, enable its documentation, have affordable cost, be comfortable to the patient, be both fast and easy to implement, and applicable to all sites of the tooth with the same efficiency (6).

In the 1990's, good perspectives on the use of laser fluorescence for the diagnosis of caries were presented and this technology was proven able of detecting early demineralization, especially when a fluorescent dye is added to the mineralized surface (7).

The inexistence of a method able of diagnosing disease (sensitivity) and soundness (specificity) led to the development diagnostic tools, e.g. laser fluorescence (Diagnodent®; KaVo, Biberach, Germany), which has been considered a promising method (8). The principle of using a laser beam for diagnosis relies on the fact that an altered mineralized surface irradiated by a longitudinal light wave emits fluorescent radiation. Diagnodent is a diagnostic

device that has a probe that emits light directed on the mineralized surface to be examined. If this surface has some form of structural change, it will emit a fluorescent light that is captured back by the probe and the device will display values ranging from 0 to 99 (9).

Diagnodent has been tested extensively, including for detection of occlusal and smooth surface caries, comparing its results with visual inspection, histology, radiography and quantitative light-induced fluorescence (7,9).

Raman spectroscopy is a powerful technique for measuring light dispersion, which is used to analyze the internal structure of molecules. Raman spectroscopy is based on measurement of the wavelength and intensity of inelastically dispersed light. Raman scattering occurs at wavelengths that are displaced from the incident light by the energy of molecular vibrations. Although the mechanism of Raman scattering is different from that of infrared absorption, it provides additional information and its applications include structural and multicomponent qualitative and quantitative analyses. This technique has been used in different areas as a noninvasive diagnostic resource of biological samples, such as periimplant and bone healing, and combination with biomaterials (10-12).

The Raman spectrum of tooth shows prominent vibrational bands related its composition. Some of the main Raman bands on tissues are at ~575, ~960, ~1450 cm^{-1}. The ~1450 band is attributed to amide I and amide III stretching modes as well as to the bending and stretching modes of CH groups of lipids and proteins; the ones at ~960 and ~575 cm^{-1} are attributed to phosphate and fluoridated apatite, respectively (11,12).

The aim of this study was to correlate the results of fluorescence readings and Raman spectroscopy regarding the levels of phosphate apatite (~960 cm-1), fluoridated apatite (~575 cm-1) and organic matrix (~1450 cm-1) at different stages of dental caries in extracted human teeth.

Material and Methods

The Ethics Committee of the Dental School of the Federal University of Bahia, Brazil (Protocol #17/11) approved the present study. Twenty extracted human teeth were used. Five sound teeth (extracted due to periodontal or orthodontic reasons) and 15 teeth with carious lesions on one smooth surface (mesial, distal buccal or lingual) were collected and stored in 10% formalin. At the moment of the readings, the teeth were washed in running tap water and cleaned for removal of all traces of formalin, stains or calculi and were then stored in saline.

Fluorescence readings were taken with Diagnodent (λ=655 nm). The device was calibrated according the manufacturer's instructions and readings were made with the flat tip (point B) positioned perpendicular to the analyzed surface (6). For reading on sound dentin, a sound tooth was sectioned longitudinally with water-cooled carborundum disk to expose a smooth dentin surface.

The mean peak value of the maximum fluorescence reading was recorded and the teeth were grouped (n=5) as follows: sound enamel (0 to 10), sound dentin (0 to 10), enamel caries (11 to 20), and dentin caries (21 to 99). Reference values used where those suggested by the manufacturer (6). For all groups, five readings were taken from each sample in order to calculate the mean and standard deviation.

After the readings of laser fluorescence, the samples were analyzed using a dispersive Raman spectrometer (AndorShamrockSR303i; Andor Technology, Belfast, Northern Ireland) and a stabilized diode laser (λ = 785 nm, 500 mW; B & W TEK, Newark, DE, USA) The excitation of the sample and collection of Raman spectra was performed by a fiber optic cable (Raman Probe) positioned in contact with the samples. The band used for analysis ranged from 200 to 1800 cm^{-1}.

Detection of scattered light signal of the sample was done by a back-illuminated thinned, deep depletion (1024x128" pixels) iDUS CCD camera (Andor Technology) cooled by cooler thermocouple, reaching a working temperature of -70°C in 5 min after the start of spectrometer operation. Acquisition and storage of the spectra were performed by a microcomputer using the Andor Solis software via USB connection, controlling the exposure time of the detector, number of acquisitions *per* sample and provides storage of the spectra for further analysis and interpretation.

The exposure time to obtain the spectra was 20 s, accumulated at a single time, with power of 500 mW. This acquisition time and power does not damage the sample. The spectrometer wavelength was calibrated by the manufacturer before data collection. Verification was done by collecting the spectrum of naphthalene and comparing the position (Raman shift) of the main bands of the compound obtained in the literature, in the spectral region 500 to 1800 cm^{-1}. This is the region of interest to the Raman spectroscopy when used for biological materials analysis ("fingerprint" region). After calibration of Raman shift and the acquisition of the spectra *in vitro* they were pre-processed and stored for subsequent statistical evaluation.

The preprocessing consisted of removing the background fluorescence, which calculates a 5^{th} order polynomial, adjusted to the low-frequency spectral components (fluorescence) and its subsequent subtraction from the original data, revealing the high frequency spectral components (Raman). The spectra had their original intensities maintained. This pre-processing was carried out using an operational default protocol in the Matlab 4.0

(The Mathworks, Natick, MA, USA) software (11).

Five readings were taken from each sample at the same point where the fluorescence readings were taken in order to calculate the mean and standard deviation. The data were analyzed using the Minitab15 software (Minitab, Belo Horizonte, MG, Brazil) and significance level was set at 5%.

Results

The results of fluorescence readings are summarized in Table 1. Fluorescence readings of carious enamel and dentin showed significant differences (ANOVA, $p<0.05$) with mean values of 57.6 ± 23.7 for dentin and 16.4 ± 2.3 for enamel (Fig. 1). The readings for sound enamel and dentin were 3.6 ± 0.5 and 5.4 ± 0.5, respectively.

In the Raman spectroscopic study the ~960 cm^{-1} (phosphate apatite), ~575 cm^{-1} (fluoridated apatite) and ~1450 cm^{-1} (organic matrix) peaks were analyzed. There was significant difference ($p<0.05$) between sound enamel and dentin for all evaluated peaks (Fig. 2). The higher intensity of the peaks is related to the inorganic components (~575 cm^{-1} and ~960 cm^{-1}) in enamel.

Analysis of Raman caries peaks in enamel and dentin showed a statistically significant difference ($p<0.05$) for all peaks (Fig. 3). Raman peaks showed significant positive correlation (Pearson correlation $p<0.05$) to both enamel and dentin (Table 2).

Analyzing carious and non-carious teeth in enamel and dentin, a lower intensity of the peaks was observed on the carious ones (Fig. 4) this difference being statistically significant ($p<0.05$) only for the ~575 cm^{-1} peak in dentin.

The correlation between Diagnodent readings and Raman peaks showed a negative and significant correlation (Pearson, $p<0.05$) only between the ~575 cm^{-1} and ~960 cm^{-1} peaks in dentin caries. The ~1450 cm^{-1} Raman peak and Diagnodent readings in dentin caries showed no significant correlation. In enamel, no significant correlation was found between Diagnodent readings and Raman peaks (Table 3).

Discussion

Diagnodent emits a diode laser at 655 nm wavelength, which is absorbed by the dental tissues. Part of this light is re-emitted as fluorescence. Fluorescence increases

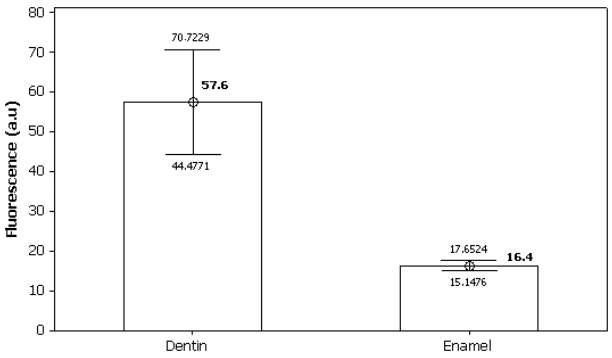

Figure 1. Mean values of Diagnodent readings in enamel and dentin.

Table 1. Summary of the results of Diagnodent fluorescence readings

Tooth	Diagnodent readings	Diagnosis
a	47/ 46/ 46/ 48/ 48	Dentin caries
b	46/ 55/ 46/ 45/50	Dentin caries
c	61/ 65/75/68/64	Dentin caries
d	99/ 99/ 99/ 98/ 99	Dentin caries
e	29/ 32/ 33/ 33/ 31	Dentin caries
f	19/ 19/ 18/ 18/ 16	Enamel caries
g	18/ 17/ 14/ 12/19	Enamel caries
h	13/ 16/ 19/ 15/ 18	Enamel caries
i	13/ 18/ 13/ 10/ 19	Enamel caries
j	12/ 14/ 13/ 16/ 14	Enamel caries
Dentin	5/ 5/ 6/ 5/ 6	Sound dentin
Enamel	4/ 4/ 3/ 4/ 3	Sound enamel

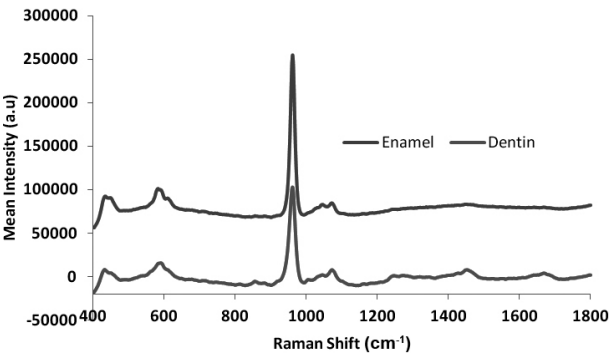

Figure 2. Raman spectra of sound enamel and dentin components.

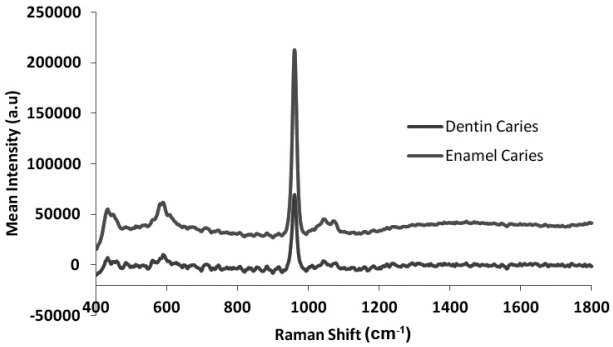

Figure 3. Raman spectra of caries in enamel and dentin.

with the progression of caries (7,13). In the present study, significant differences were observed between the Diagnodent readings for caries in enamel and dentin, with higher values of fluorescence related to dentin. This shows that more advanced processes of caries as in dentin have higher fluorescence.

Caries begins on tooth enamel, which has hydroxyapatite (HA) as its main component. When a solution containing fluoride comes in contact with HA, OH^- ions may be replaced by F- ions resulting in the formation of fluoridated apatite. This compound is found both in enamel and dentin (14,15).

Raman spectroscopy shows the chemical structure of the tissues. In this study were observed enamel and dentin peaks related to the inorganic components ~960 cm^{-1} (phosphate apatite) and ~575 cm^{-1} (16) (attributed to fluoridated apatite), both with greater intensity in enamel, confirming its higher mineral content. The ~1450 cm^{-1} peak related to the organic content was observed in higher intensity in dentin.

Raman spectroscopy of carious and non-carious teeth revealed reduction of the intensity of peaks from inorganic components, but were not statistically significant. Unlike a previous study (17) that reported changes in the Raman peak of phosphate between sound and carious enamel, in this study was observed a significant reduction only for the ~575 cm^{-1} peak in dentin caries.

In the correlation of Diagnodent readings with Raman peaks was observed a negative and significant correlation only between the ~575 cm^{-1} and ~960 cm^{-1} peaks and dentin caries. The lower the intensity of these inorganic components the higher the value obtained in Diagnodent readings in dentin. This finding reinforces the observation of another study (18), which concluded that the depth of dental caries has greater influence than the mineral loss in Diagnodent readings. The increased values in the Diagnodent readings were probably due to a higher fluorescence, which might be attributed to bacterial metabolic activity (9) and also to the amount of organic matrix (13,18) rather than from disintegration of crystals.

There was no significant correlation between Diagnodent readings in enamel and Raman peaks, proving that Diagnodent does not measure accurately small changes in mineral content. These findings corroborate a previous study (19) in which Diagnodent showed the lowest

Table 2. Correlation between Raman peaks obtained in enamel and dentin

Correlated Peaks	r*	p value
Enamel ~575 X ~960	0.934	>0.001
Enamel ~575 X ~1450	0.746	0.001
Enamel ~960 X ~1450	0.671	0.006
Dentin ~960 X ~1450	0.785	0.001
Dentin ~960 X ~575	0.863	>0.001
Dentin ~575 X ~1450	0.663	0.007

*Pearson's correlation.

Table 3. Correlation between Raman peaks and Diagnodent® readings in carious teeth

Correlated peaks	r*	p value
Dentin ~575; Diagnodent Dentin	- 0.645	0.009**
Dentin ~960; Diagnodent Dentin	-0.807	>0.001**
Dentin ~1450; Diagnodent Dentin	-0.370	0.175
Enamel ~575; Diagnodent Enamel	0.232	0.389
Enamel ~960; Diagnodent Enamel	0.234	0.402
Enamel ~1450; Diagnodent Enamel	0.140	0.620

*Pearson's correlation; **Statistically significant.

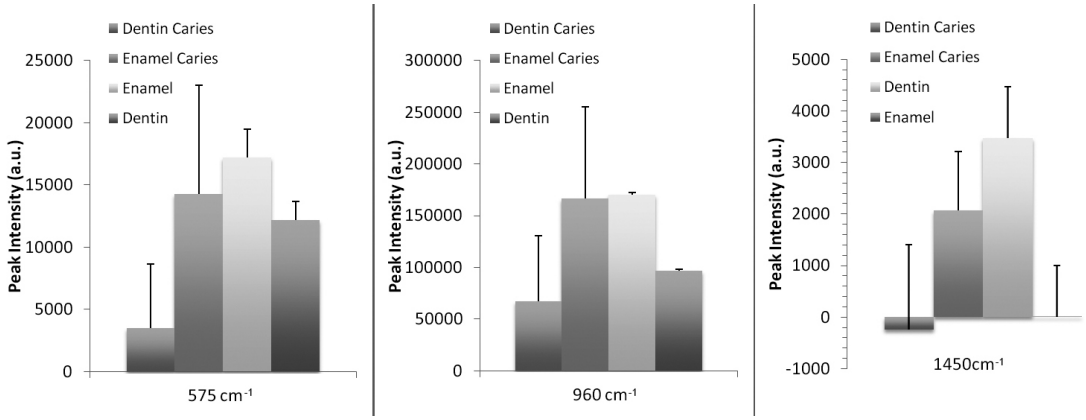

Figure 4. Mean values of Raman readings in sound and carious enamel and dentin.

sensitivity (0.66-0.75) when compared with all other studied detection methods. According to the authors (19), less sensitivity could be explained by the Diagnodent pen device, since the study refers mainly to enamel lesions and not to dentinal ones. In general, all studies agree that Diagnodent performs well when used for dentinal lesions (9).

Within the limitations of this *in vitro* study, these results suggest that Diagnodent readings reflect changes in organic matter instead of inorganic contents. In a previous report (18) the correlation between mineral loss in enamel, measured by microradiography, and Diagnodent readings was only 0.65.

Another study (20) revealed differences between sound enamel and initial caries at the peak of HA, disagreeing with the findings of this study in that the ~960 cm^{-1} peak in enamel caries and sound enamel had no significant difference.

Since the early diagnosis of caries is directly related to the identification of changes in inorganic components of the tooth, Raman spectroscopy was more sensitive to variations of these components when compared with Diagnodent.

Resumo

O objetivo desse estudo foi verificar por meio da espectroscopia Raman, a existência de correlação entre os níveis de apatita fosfatada (~960 cm^{-1}), apatita fluoretada (~575 cm^{-1}) e matriz orgânica (~1450 cm^{-1}) e as leituras do Diagnodent® em diferentes estágios de cárie dental em dentes humanos extraídos. O valor médio do pico de fluorescência na área da cárie foi anotado e os dentes divididos em cárie de esmalte, dentina e dente hígido. Após as leituras de fluorescência, foi realizada a espectroscopia Raman nos mesmos sítios. Os resultados mostraram diferença significante (ANOVA $p<0,05$) entre as leituras de fluorescência para esmalte ($16,4 \pm 2,3$) e dentina ($57,6 \pm 23,7$) nos dentes cariados. Os picos Raman para esmalte e dentina evidenciaram que os picos ~575 e ~960 cm^{-1} foram mais intensos em cárie de esmalte. Houve correlação negativa e significante ($p<0,05$) entre os picos ~575 e ~960 cm^{-1} e cárie de dentina. Pode-se concluir que quanto maior a fluorescência detectada pelo Diagnodent menor o pico da apatita fosfatada e fluoretada. O diagnóstico precoce da cárie está diretamente relacionado com a identificação de mudanças nos componentes inorgânicos do dente, assim a espectroscopia Raman foi mais sensível para variações desses componentes quando comparada ao Diagnodent.

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