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DINÂMICA DE DIFUSÃO DO PERÓXIDO DE HIDROGÊNIO ATRAVÉS DOS TECIDOS DENTÁRIOS HUMANOS

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DINÂMICA DE DIFUSÃO DO PERÓXIDO DE HIDROGÊNIO ATRAVÉS DOS TECIDOS DENTÁRIOS HUMANOS

Dissertação apresentada ao Programa de Pós-Graduação em Odontologia Integrada, da Universidade Estadual de Maringá, para obtenção do título de mestre.

Orientadora: Prof[a]. Dr[a]. Renata Corrêa Pascotto

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DINÂMICA DE DIFUSÃO DO PERÓXIDO DE HIDROGÊNIO ATRAVÉS DOS TECIDOS DENTÁRIOS HUMANOS

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“O método científico é comprovado e verdadeiro. Não é perfeito, é apenas o melhor que temos. Abandoná-lo, junto com seus protocolos céticos, é o caminho para uma idade das trevas.”

Carl Sagan

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SUMÁRIO

CONTEÚDO

1. Contextualização

A sensibilidade dentária consiste no efeito adverso mais comumente relatado na literatura após a realização do clareamento dentário de dentes vitais, (Haywood, 2000) já que tem sido relatada por 2/3 dos pacientes submetidos ao clareamento, e costuma durar de 1 a 4 dias (Sulieman, 2008).

Esta sensação dolorosa ocorre em função da permeabilidade do HP até o complexo dentino-pulpar. Uma vez que os tecidos dentários duros são altamente permeavéis a fluídos presentes na cavidade bucal ou até mesmo advindos da circulação sanguínea (Torres et al., 2007), quando aplicado na superfície externa dentária, o agente clareador leva de 5 a 15 minutos até atingir a câmara pulpar (Bowles e Ugwuneri, 1987; Cooper et al., 1992).

No esmalte, a permeação ocorre através dos espaços interprismáticos que são ocupados por matéria orgânica, já na dentina se dá pelos túbulos dentinários caracterizados como canalículos convergentes para a polpa que facilitam a permeação até o tecido pulpar (Ten Cate, 2008).

O esmalte dentário apresenta coloração branca e aspecto translúcido, possibilitando que a cor da dentina subjacente seja vista. Desta forma, a cor dentária é determinada pela dentina e modulada pela espessura, translucidez e grau de mineralização do esmalte (Sulieman, 2008). A cor final dos dentes também pode ser influenciada pelo manchamento com pigmentos de fontes diversas, bem como pelo processo de envelhecimento dentário no qual ocorre a formação de dentina reparativa resultando na diminuição da câmara pulpar.

A penetração de pigmentos e corantes através de trincas no esmalte ou diretamente na dentina exposta por desgaste do esmalte pode resultar no escurecimento dentário. Um pigmento consiste em uma substância colorida

composta de um grupamento químico cromóforo, que pode ou não se unir às substâncias orgânicas presentes na estrutura dental. Enquanto que o corante se diferencia do pigmento por possui grupos reativos cromóforos capazes de realizar ligações químicas com a matéria orgânica dentária (Baratieri et al., 2007).

A cor exibida por um objeto está diretamente relacionada ao comprimento de onda e à quantidade da luz incidente refletida ou absorvida por ele. Assim, um objeto preto absorve toda a luz incidente e resulta na ausência de luz, ou seja, ausência de cor (Torres et al., 2007). A formação de cadeias moleculares longas e complexas dentro da estrutura dentária é responsável pelo aumento do índice de absorção de luz pelo dente, resultando em seu escurecimento.

O clareamento dentário tem sido uma alternativa menos invasiva para a restauração da cor em dentes escurecidos e manchados, sendo atualmente um dos procedimentos estéticos mais procurados pelos pacientes. Existem diversas abordagens descritas na literatura com o objetivo de proporcionar o clareamento de dentes vitais (Sulieman, 2004), métodos que utilizam agentes clareadores com diferentes concentrações, com variados períodos e formas de aplicação (Joiner, 2007). Os agentes clareadores mais utilizados atualmente são o peróxido de hidrogênio (HP) e o peróxido de carbamida (CP) (Dahl et al., 2003). Quando em contato com a saliva, o CP dissocia-se em HP e úreia, sendo portanto o HP o veículo de oxigênio responsável pelo clareamento em ambos os casos (Baratieri et al., 2007).

Atualmente o clareamento caseiro supervisionado pelo dentista (HP ou CP com baixa concentração aplicado com alta frequência), tem sido

considerado o tratamento de eleição por ser uma alternativa menos agressiva que o clareamento de consultório (HP com alta concentração aplicado em curto período de tempo) (Dahl et al., 2003; Soares et al., 2010). Contudo, existem alguns casos específicos em que o clareamento caseiro não consiste na primeira opção de tratamento, como por exemplo: pacientes com múltiplas recessões gengivais, pacientes fumantes e/ou que fazem uso frequente de bebidas álcoolicas (Consolaro, 2004). Nestas situações, quando necessário o tratamento clareador, o cirugião dentista pode lançar mão da técnica de consultório com a utilização de agentes clareadores com concentração de HP entre 20-38%.

O resultado do clareamento dentário de dentes vitais é dependente da modificação da cor da dentina subjacente ao esmalte que recebeu a aplicação do agente clareador (Wiegand et al., 2005). Desta forma, o efeito clareador é consequente da permeação do peróxido através do esmalte e da dentina (Hanks et al., 1993; Kina et al. 2010). Quando em contato com os tecidos dentários, o HP tem a habilidade de gerar oxigênio ativo, radicais livres e solventes.

Os radicais livres são substâncias instáveis, reativas, que capturam elétrons de moléculas orgânicas com as quais venham a entrar em contato. Esse “roubo” de elétrons geralmente resulta na quebra de ligações químicas duplas (Dahl et al.,2003). Desta forma, o radical livre sofre um processo de redução pois se estabiliza ganhando elétrons do pigmento, enquanto que o pigmento sofre uma oxidação pois perde elétrons para o radical livre. Assim o radical consiste no agente oxidante uma vez que induz a oxidação do pigmento (Caviedes-Bucheli et al., 2008).

Os pigmentos mais saturados são macromoléculas complexas compostas de anéis aromáticos. Sob a ação dos radicais livres do HP, os anéis aromáticos dos pigmentos são convertidos em substâncias abertas mais simples com ligações insaturadas (C=C) (Plotino et al., 2008; Toledano et al., 2011). Com a ação do clareador, estas ligações químicas insaturadas são transformadas em ligações simples, saturadas, e hidrofílicas, o que permite sua eliminação da estrutura dentária por meio de difusão, completando o processo de clareamento (Baratieri et al., 2007).

A permeação do HP é dependente de sua concentração bem como do seu tempo de contato com a estrutura dentária (Hanks et al., 1993). Assim quanto maior a concentração do HP maior será sua permeação nos tecidos dentários (Sulieman 2008). Além da concentração de HP, outros fatores que aumentam a sua permeação são a sua ativação com fontes luminosas (Camargo et al., 2009), a presença de restaurações nos dentes clareados (Gokay et al., 2000) bem como a idade do paciente submetido ao tratamento (Dahl et al., 2003).

A permeação dentária ao HP foi previamente descrita por Bowles e Ugwuneri em 1987. Sua rota de permeação tem sido comparada a da Rodamina B, visto que esta substância tem solubilidade em água e pode ser dissolvida para apresentar peso molecular semelhante ao do HP. Assim como acontece com outros fluídos e até pigmentos, a entrada do HP dos tecidos dentários ocorre através dos espaços interprismáticos e dos túbulos intradentinários (Kwon et al., 2012).

Esta permeação do HP nos tecidos dentários pode promover uma irritação química no tecido pulpar, resultando no processo de inflamação pulpar

reversível, ou até mesmo irreversível em incisivos inferiores, devido à menor espessura de esmalte e dentina nestes dentes (Costa et al. 2010; Roderjan, 2012). O HP consiste em um agente químico termoinstável com alto poder oxidativo. Os radicais livres resultantes de sua dissociação podem promover reações oxidativas e assim causar danos deletérios para vários componentes celulares, incluíndo danos ao DNA celular, oxidação e fragmentação protéica, peroxidação lipídica, indução de apoptose e necrose celular (Hanks et al., 1993; Caviedes-Bucheli et al., 2008; Trindade et al., 2009).

Além dos efeitos tóxicos as células do tecido pulpar e da consequente sensibilidade pós operatória, outos efeitos adversos à clareação dentária têm sido relatados por meio de mudanças físicas e químicas nos tecidos dentários duros. Estudos recentes têm sido direcionados para a avaliação das modificações dos componentes estruturais do esmalte e da dentina após o clareamento dentário, contudo, as diferenças em suas metodologias podem ser responsáveis pela controvérsia dos resultados (Gotz et al., 2007; Severcan et al., 2008; Jiang et al., 2008).

Análises com a Espectroscopia Fotoacústica encontraram mudanças na composição e na estrutura do esmalte superficial, indicando certa destruição da hidroxiapatita após o tratamento com concentrações de 10%, 20% e 30% de HP (Bistey et al., 2007); diminuição dos compostos minerais e desnaturação protéica após aplicação de HP com concentração de 35% (Severcan et al. 2008). Corroborando com estes estudos, a Espectroscopia Raman comprovou que após o tratamento com HP o esmalte teve sua cristalinidade aumentada devido à sua desmineralização (Jiang et al., 2008; Santini et al., 2008) e indicou a oxidação do conteúdo orgânico tanto das camadas externas como internas

do esmalte (Jiang et al., 2008). Contudo, esta mesma metodologia evidenciou que o HP aplicado na forma de fitas branqueadoras nas concentrações de 13% e 16% não provocou mudanças espectrais no esmalte (Gotz et al., 2007).

De acordo com o estudo de Kawamoto e Tsujimoto em 2003, a Ressonância Magnética Nuclear demonstrou degradação e modificação estrutural de aminoácidos presentes no tecido dentinário após o clareamento dentário, e a técnica da Microscopia Eletrônica de Varredura evidenciou que tanto a dentina intertubular quanto a dentina peritubular foram dissolvidas na presença de altas concentrações de HP. As metodologias da Refletância Total Atenuada e da Espectroscopia Fotoacústica (Jiang et al., 2007) bem como a Espectroscopia Raman (Jiang et al., 2008) evidenciaram que o HP com concentração de 30% em contato direto com o tecido dentinário promoveu sua destruição pelo mecanismo de degradação protéica e reduziu a quantidade de compostos minerais deste tecido. Entretanto, estudos que avaliaram a dentina subsuperficial, simulando as técnicas de clareamento caseiro e de consultório observaram modificações insignificantes no tecido dentinário (Gotz et al., 2007; Severcan et al., 2008) e na região da junção amelodentinária (Gotz et al., 2007).

Enquanto as análises pela Microscopia Eletrônica de Varredura revelaram tecidos dentários com aspectos normais após o clareamento com diferentes concentrações de CP (10%, 20% e 35%), a Espectroscopia de Energia Dispersiva realizada nos mesmos espécimes demonstrou mudanças na composição química do esmalte e da dentina. Independentemente das concentrações e do tempo de aplicação do produto, o clareamento resultou tanto na dissolução dos compostos inorgânicos como na redução do material

orgânico dos tecidos dentários devido à redução respectiva dos minerais Ca e K (Cakir et al., 2011).

Embora a permeação do HP tenha sido estudada por meio de pesquisas com câmaras pulpares de dentes extraídos (Camargo et al., 2007; Camargo et al., 2009; Camps et al., 2007), câmaras pulpares artificiais (Benetti et al., 2004; Gokay et al., 2005; Coldebella et al., 2009; Torres et al., 2010) e análise histológica após o clareamento de dentes indicados para extração (Fugaro et al., 2004; Rodriques et al., 2009), existe uma lacuna na literatura em relação a estudos que avaliem a permeação dentária do HP no proprio substrato de permeação, através do esmalte e da dentina, passando pela junção amelodentinária (Gotz et al., 2007). Além disto, grande parte dos trabalhos avalia as consequências da ação do agente clareador em contato direto com os tecidos dentários, ou seja, após aplicação no esmalte na técnica do claremanto de dentes vitais e na dentina na técnica do clareamento intracoronário. Uma vez que o HP permeia nos tecidos dentários, e age na dentina subsuperficial ao esmalte clareado, torna-se importante a investigação das modificações estruturais no tecido dentinário de dentes submetidos ao clareamento na técnica vital.

As técnicas de Espectroscopia Fotoacústica no Infravermelho com Transformada de Fourier (PAS-FTIR) Micro Espectroscopia Raman (MRS) são técnicas que possuem como vantagem a necessidade de um preparo mínimo dos espécimes, baixa complexidade da interpretação dos espectros e bandas, e caracterização química dos compostos presentes na amostra (Gotz et al., 2007; Joiner et al., 2007). Além disto, ambas as técnicas são não-destrutivas, permitindo que uma mesma amostra seja estudada antes e depois dos

procedimentos experimentais, favorecendo assim que o espécime seja seu próprio grupo controle (Santini et al., 2008). Enquanto a PAS-FTIR monitora diferentes grupos funcionais de matérias orgânicas e inorgânicas, a MRS possibilita um delineamento dos componentes minerais da amostra estudada. Desta forma, as duas técnicas se complementam e a sua associação permite uma investigação físico-química integrada.

Considerando o grande número de pacientes que procuram os consultórios odontológicos em busca de clareamento dental com resultado imediato torna-se oportuno investigar a dinâmica de difusão do HP com 25% de concentração através do esmalte e da dentina utilizando a MRS, e correlacionar estes resultados com as modificações químicas da dentina subsuperficial analisadas pela PAS-FTIR .

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HYDROGEN PEROXIDE DIFFUSION DYNAMICS IN DENTAL TISSUES

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Abstract

The aim of this study was to investigate the diffusion dynamics of 25% hydrogen peroxide (H_2O_2) through enamel-dentin layers, and to correlate it with dentin's structural alterations. Micro-Raman Spectroscopy (MRS) and Fourier Transform Infrared Photoacoustic Spectroscopy (FTIR-PAS) were used to measure the spectra of specimens before and during the bleaching procedure. H_2O_2 was applied to the outer surface of human enamel specimens for 60 minutes. MRS measurements were performed on the inner surface of enamel or on the subsurface dentin. In addition, H_2O_2 diffusion dynamics from outer enamel to dentin, passing through the dentin-enamel junction (DEJ), was obtained with Raman transverse scans. FTIR-PAS spectra were collected on the outer dentin. MRS findings revealed that H_2O_2 (O-O stretching μ-Raman band) crossed enamel, had a more marked concentration at DEJ, and accumulated in dentin. FTIR-PAS analysis showed that H_2O_2 modified dentin's organic compounds, observed by the decrease in amides I, II and III absorption band intensities. In conclusion, H_2O_2 penetration was demonstrated to be not a merely physical passage through enamel interprismatic spaces into the dentinal tubules. H_2O_2 diffusion dynamics presented a concentration gradient determined by the chemical affinity of the H_2O_2 with each specific dental tissue.

Introduction

Dental bleaching depends on the penetration of hydrogen peroxide (H_2O_2) free radicals through enamel and into dentin (Hanks *et al.*, 1993; Kina *et al.*, 2010), breaking dentin's chromogenic molecules down into smaller structures (Plotino *et al.*, 2008; Toledano *et al.*, 2011).

The literature shows that H_2O_2 applied to enamel can penetrate thouroughly into the pulp cavity (Cooper *et al.*, 1992; Benetti *et al.*, 2004). It has been suggested that H_2O_2 penetration pattern in teeth is similar to rhodamine dyes moving through enamel's interprismatic spaces and dentinal tubules (Kwon *et al.*, 2012). Previous observations have also revealed that interactions between teeth and bleaching agents involve diffusion and reaction of H_2O_2 moieties with chromogens (Camps *et al.*, 2007), so that a direct correlation between the presence of oxidative agents and the penetration potential of H_2O_2 has already been demonstrated (Sulieman *et al.*, 2004).

Although several articles have already provided information on the diffusion of bleaching agents through dental tissues (Trindade *et al.*, 2009; Kina *et al.*, 2010), further evaluation of H_2O_2 permeation through enamel, the underlying dentin-enamel junction (DEJ), and dentin tissues requires transverse cross-sectional analysis (Gotz *et al.*, 2007). Therefore, the aim of this *in vitro* study was to investigate the diffusion dynamics of 25% H_2O_2 through the enamel surface into dentin by using Micro-Raman Spectroscopy (MRS), and to correlate the results with chemical structural alterations of the subsurface dentin, measured with Fourier Transform Infrared Photoacoustic Spectroscopy (FTIR-PAS).

Materials & Methods

The study protocol was reviewed and approved by the Local Ethics Committee (0185.0.093.000-09). Eighteen sound human premolar teeth from patients ages 12-35 were used in this study.

The 35 specimens obtained (3mm x 3mm) had a naturally curved outer enamel surface with a flat surface underneath. Specimens' thickness varied from 1 to 3mm according to the study design described below. Specimen preparation and bleaching treatment followed the same protocol for both MRS and FTIR-PAS techniques.

Specimens were individually adapted into a vinyl polysiloxane cast (Panasil R Putty, Kettenbach, USA) to guide the application of a 1mm layer of the bleaching gel, and also to ensure that H_2O_2 diffusion would occur through the enamel surface into the dentin, and not from the sides of the specimens. Each specimen was submitted to bleaching for 60min. This study was designed to simulate in-office dental bleaching and, therefore, H_2O_2 was applied to the outer enamel surface according to manufacturer's (Lase Peroxide Sensy II, DCM Equipments LTDA, Brazil) instructions. The bleaching agent used in this study was prepared using 25% H_2O_2, gelling agent, dyes, vegetable extract, amides, sequestering agent, glycol and water. Specimens' color was measured (CR 400, Konica Minolta Optics, Inc., Japan) before and after the bleaching procedure to confirm bleaching agent effectiveness.

In order to minimize dental structural composition differences, all specimens' spectra were obtained before the application of H_2O_2, and served as their own negative control group. The effect of dehydration was tested on some control samples measured every 10min, for a duration of 60min. No spectra

alteration was observed. Moreover, the spectra of the H_2O_2 agent used were also acquired.

MRS

Raman spectra were collected at room temperature in a backscattering geometry using a Bruker Senterra dispersive Raman microscope (Bruker Optik GmbH, Germany). Spectra were excited by a 785nm laser source and recorded in the 450–1800cm^{-1} spectral range. Laser power was set at 100mW and focused on the sample through a 20x objective optical microscope (0.75N.A.). Spatial resolution was 4μm, detector integration time was 3s, and each final curve resulted from 30 averaged spectra. Furthermore, in order to improve signal quality, the detector temperature was decreased to 183K. All spectra were systematically collected under the same conditions; the flatter central area of the enamel surface was selected and photographed to ensure that posterior measurements were repeated at the same place.

MRS specimens were layered with the H_2O_2 gel on the outer enamel, while MRS measurements were performed on the specimens opposing side (inner enamel side – ES, and dentin side – DS), and laterally (perpendicular to the bleached surface) from the enamel surface to dentin, passing through the DEJ - transverse scan (TS) geometry. Specimens were allocated in four groups as follows:

- ES (n=5): 1 mm enamel only specimens.
- DS2 (n=10): 2 mm enamel and dentin specimens.
- DS3 (n=10): 3 mm enamel and dentin specimens.
- TS (n=5): 3 mm enamel and dentin specimens.

For ES and DS analysis, MRS measurements were performed at 10min intervals during the bleaching procedure. For TS analysis, after the bleaching procedure was complete, specimens were dried with cotton swabs and measurements were performed every 100µm until reaching approximately 3000µm.

Spectra data were processed using OPUS® spectroscopic software (Bruker Optik GmbH, Germany). During this procedure, spectra from both untreated and treated specimens were deconvoluted, allowing H_2O_2 (O-O stretching) Raman band peak intensity to be determined.

For DS and ES groups, O-O stretching Raman band intensities were analyzed using Friedman and Wilcoxon ranks tests for the time factor (SPSS software). The results are presented as median and interquartile range. The level of significance was set at 0.05. For TS group, the band located at the same region of O-O stretching Raman band on the control spectra was subtracted from each specific treated spectrum, and a numerical mean was obtained from the five spectra resulting from the subtraction.

FTIR-PAS

FTIR-PAS spectra were recorded by a FTIR spectrometer (Varian Inc., USA), equipped with a MTEC 300 photoacoustic cell (MTEC Photoacoustics, Inc., USA). The overall spectral resolution was $8cm^{-1}$, with a moving mirror velocity of 0.64cm/s, with the final spectrum for each measure being the result of 350 scans. Spectra were obtained in the 400-$4000cm^{-1}$ spectral range. Preceding each measurement, it was necessary to purge moisture from the photoacoustic cell sample chamber and fill it with helium for approximately 5

seconds. The reference spectrum was acquired by using a carbon black reference. The depth analysis was estimated to be approximately 7.0µm (Ubaldini *et al.*, 2012).

FTIR-PAS analysis was performed in five 2mm enamel/dentin specimens on DS, using Origin software (Origin-Lab Corporation, USA) through spectra baseline correction. Peak intensity from amide I (1650cm^{-1}), amide II (1550cm^{-1}), C-H stretching (1460cm^{-1}), amide III (1230cm^{-1}), ν_3 PO_4 (1100 and 1042cm^{-1}) were normalized in relation to the peak of ν_4 PO_4 (582cm^{-1}). This peak was chosen as it presents no significant changes for phosphate peaks in Raman spectra, and because organic compounds do not make any contribution to this spectral range.

Results

Tooth color measurements taken before and after bleaching revealed effective enamel bleaching of all specimens given the increase in L* from (60±2) to (71± 2).

MRS

ES specimens (Fig. 1), presented small variations in the O-O stretching Raman band (873cm^{-1}) intensity after 10min of bleaching, from 0.004 (0.003-0.009) to 0.013 (0.009-0.018) ($p > 0.05$), remaining stable during the 60min experimental time: 0.021 (0.012-0.131) ($p > 0.05$). In DS2 and DS3 specimens, however, after 10min, H_2O_2 was detected on the subsurface dentin (Fig. 1). In those groups, the 873cm^{-1} Raman band intensity increased strongly during the bleaching treatment, showing a linear growth from 0.038 (0.033-0.052) to 0.107

(0.085-0.174) ($p < 0.05$) for DS2 (one of the measuring sequences is shown in Fig. 2), and from 0.042 (0.032-0.059) to 0.084 (0.053-0.140) ($p < 0.05$) for DS3. H_2O_2 intensities varied over time demonstrating that the penetration was more intense for DS2 than for DS3 specimens.

Raman transverse scan (TS) confirmed that H_2O_2 crossed the enamel layer, presented an increased concentration at DEJ, and accumulated in the dentin (Fig. 3). This observation comes from the fact that O-O stretching Raman band (873cm^{-1}) intensity was constant through 1mm enamel and tended to rise only at the DEJ. From this point, it showed a non linear decreasing pattern until reaching the innermost surface of dentin at the final thickness of 3mm. Then, for the region after DEJ (x=0) this 873cm^{-1} band intensity variation was fitted to an exponential decay type function, $\exp(-x/x_0)$, with x being dentin thickness, and x_0 the characteristic depth where the H_2O_2 concentration decays to 1/e of the value at x=0, approximately 37%. Therefore, considering that the retention of H_2O_2 through the enamel was minimal, the fit estimated that approximately 37% of the initial H_2O_2 concentration applied on enamel was found at a depth of about 2.7mm, with 63% remaining accumulated in the dentin.

FTIR-PAS

FTIR spectra of the subsurface dentin showed significant changes after bleaching (Fig. 4). As a consequence of H_2O_2 diffusion through and reaction with dentin compounds, the relative intensity of amide I (1650cm^{-1}), amide II (1550cm^{-1}), amide III (1230cm^{-1}) and C-H stretching (1460cm^{-1}) decreased after bleaching. Moreover, this structural investigation revealed alterations through

the relative intensity increase of ν_3 PO_4 (1100cm^{-1} and 1042cm^{-1}) (Antonakosa *et al.*, 2007).

Discussion

To the best of our knowledge, this is the first report to correlate H_2O_2 ability to interact with organic dentin compounds with its penetration dynamics into dental tissues. The results not only provide new insights into the role of H_2O_2 chemical affinity during its diffusion through enamel and dentin, but also demonstrated that H_2O_2 penetrated through enamel, reaching the underlying dentin, oxidizing its organic compounds and modifying its mineral components. In fact, the data shows that after 10min, H_2O_2 reached the pulp-dentin interface (3mm), where it may cause inflammatory changes and irreversible pulp damage (Costa *et al.,* 2010).

The constant H_2O_2 Raman band intensity measured on enamel's inner surface (ES) over the 60min bleaching period may be attributed to the fact that H_2O_2 hardly interacted with the components of enamel tissue due to the low presence of organic compounds in enamel's interprismatic spaces. On the other hand, the marked increase of the Raman band in subsurface dentin at 2 and 3mm (DS2 and DS3) provides evidence that H_2O_2 diffused through enamel and reacted with dentin compounds. While the highest concentration of H_2O_2 in DS2 specimens was found in the outer dentin, in DS3 specimens it was found to be further inward. This difference may be explained by the fact that the most organic region of the dentin layer is near the DEJ (Xu *et al.,* 2009).

Transverse scan spectra from TS specimens (Fig. 3) confirmed the results found for ES, DS2 and DS3. In agreement with ES data, TS analysis showed that H_2O_2 rapidly crossed the enamel layer. This suggests that during

its penetration along the interprismatic spaces, H_2O_2 probably did not attach itself to enamel compounds. On the other hand, the diffusion of H_2O_2 through dentinal tubules concentrated at DEJ, accumulated 2mm into the dentin (63%), and decreased at the thickness of 3mm (37%). Although changes in the subsurface dentin due to bleaching procedures is a controversial issue (Gokay *et al.*, 2004; Kawamoto *et al.*, 2004; Gotz *et al.*,2007), the spectroscopic findings of this study demonstrated that H_2O_2 interacted with dentin tissues, presenting a concentration gradient at DEJ during its diffusion dynamics.

The strong oxidizing ability of H_2O_2 has been previously described as being responsible for the reduction of organic components in dentin when the bleaching gel is applied directly on dentin specimens (Cakir *et al.*, 2011; Chng *et al.*, 2005; Jiang *et al.*, 2007; Rotiesten, 1992). Nevertheless, little information is available concerning the chemical modifications that occur in subsurface dentin during the diffusion of H_2O_2 through this tissue (Severcan *et al.*, 2008).

FTIR-PAS chemical analysis (Fig. 4) revealed the relative reduction of amide I, II and III and C-H stretching optical bands. Spectral changes in this region showed collagen denaturation (Spencer *et al.*, 2001; Wang and Yao, 2010), indicating that an oxidation reaction occurred as a consequence of H_2O_2 interaction with dentin, causing modifications on its protein secondary structure (Kawamoto and Tsujimoto, 2004). Other authors have found that the relative increase in mineral optical bands spectra could denote changes in dentin crystallinity, due to the structural rearrangement of dentin amorphous compounds (Jiang *et al.,* 2007). However, this modification on the mineral matrix could not be detected with Raman spectroscopy.

The variance on the 1042cm^{-1} optical band intensity could have been affected by two distinct mechanisms. First, protein oxidation could have decreased this band intensity, since amino acids in dentin present optical absorption at that wavelength (Kawamoto and Tsujimoto, 2004). Moreover, the reaction between H_2O_2 and dentin's inorganic compounds could result in the formation of acid by-products such as hydrogen phosphate, increasing band intensity (Santini *et al.*, 2008). Although both phenomena may occur simultaneously, the bleaching procedure performed led to increased 1042cm^{-1} intensity, suggesting that by-products were formed.

Dentinal tubule diameter and density influence dentin permeability (Chng *et al.*, 2002, Hairul Nizam *et al.*, 2005; Zimmerman *et al.*, 2010). In the present study H_2O_2 was applied on the outer surface of enamel and, therefore, during its course of penetration, the bleaching agent had to diffuse from small to large tubule diameters (Xu *et al.,* 2009). Thus, H_2O_2 accumulation on the outer dentin layer could have been the result of both H_2O_2 chemical affinity with dentin's organic compounds as well as the reduced diameter of dentinal tubules in that region.

MRS and PAS-FTIR techniques permit nondestructive ultrastructure analysis of natural, non-fixed specimens (Gotz *et al.*, 2007; Joiner *et al.*, 2007). Thus, the specimens could be studied before and after the experimental procedures, allowing each sample to serve as its own control (Bistey *et al.*, 2007). Furthermore, the association of both techniques allowed for an integrated physical-chemical evaluation.

Although the findings of the present study may have clinical relevance, some differences exist in comparison to actual clinical conditions. First of all, the

use of extracted teeth probably allowed a quicker penetration of the bleaching agent towards the pulp, as they were devoid of the dentinal fluid produced by intrapulpal pressure (Sulieman *et al.*, 2004; Zimmerman *et al.*, 2010). Secondly, the absence of pulp tissues may also have changed the absorption patterns of bleaching by-products (Kina *et al.*, 2010).

Therefore, considering the oxidizing action of H_2O_2 on organic dentin components observed in this study, it is possible to conclude that H_2O_2 diffusion dynamics seem to be determined by the chemical affinity of the bleaching agent with the organic portion of each specific dental tissue. Taking into account that organic enamel compounds correspond to just 2% of its total volume, in all probability H_2O_2 passed through this tissue with minimal interaction with its organic matrix, presenting a homogeneous distribution. H_2O_2 interaction with dentin, on the other hand, which consists of 38% organic compounds, demonstrated different patterns according to dentin's depth. H_2O_2 penetrated the inner dentin, decreasing its diffusion gradient until reaching a final dentin thickness of 3mm, in such a way that approximately 63% of H_2O_2 became attached to dentin tissue molecules, reacting with dentin's organic components, which may result in undesirable clinical side effects.

Acknowledgments

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Figure legends

1) Intensity change of the O-O stretching band (873cm^{-1}) as a function of time for ES, DS2 and DS3 specimens. O-O stretching band showed no increase for ES, but rose for DS2 and DS3. H_2O_2 penetration was more intense for DS2 than for DS3 specimens.

2) Raman spectra of dentin with and without treatment monitored over 60 minutes (DS2). Raman spectrum shift at around 873cm^{-1} is attributed to the stretching O-O. Raman band growth started at 10min for the treated group and continued rising for up to 60min.

3) Relative intensity of the O-O stretching band as a function of specimen's depth. Lateral analysis demonstrating that H_2O_2 crossed the enamel layer, had increased concentration at DEJ, and accumulated in the dentin.

4) FTIR-PAS spectra as a function of H_2O_2 treatment time. The inset shows the variation of the optical bands intensities at P(1): 1650cm^{-1} (amide I); P(2): 1550cm^{-1} (amide II); P(3): 1460cm^{-1} (C-H stretching); P(4) 1230cm^{-1} (amide III); P(5): 1100cm^{-1} (ν_3 PO_4) and P(6):1042cm^{-1} (ν_3 PO_4). The reference peak was centered at 582cm^{-1}. Reduction of dentin compounds over bleaching treatment time is observed.

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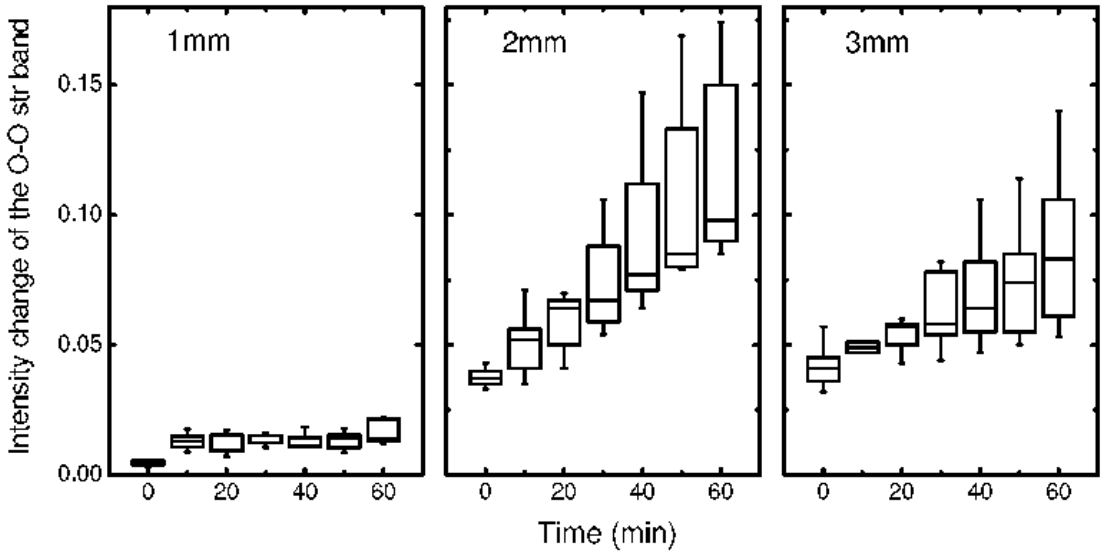
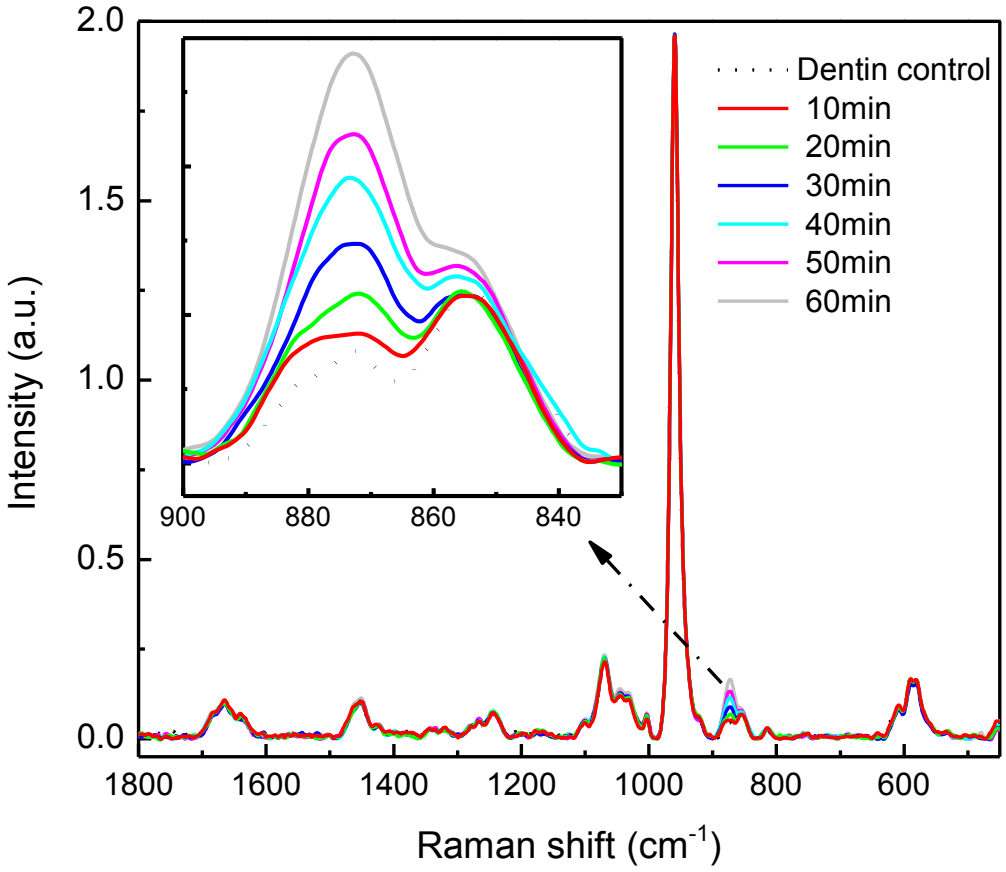
Figures

Figure 1



1) Intensity change of the O-O stretching band (873cm^{-1}) as a function of time for ES, DS2 and DS3 specimens. O-O stretching band showed no increase for ES, but rose for DS2 and DS3. H_2O_2 penetration was more intense for DS2 than for DS3 specimens.

Figure 2



2) Raman spectra of dentin with and without treatment monitored over 60 minutes (DS2). Raman spectrum shift at around 873cm^{-1} is attributed to the stretching O-O. Raman band growth started at 10min for the treated group and continued rising for up to 60min.

Figure 3

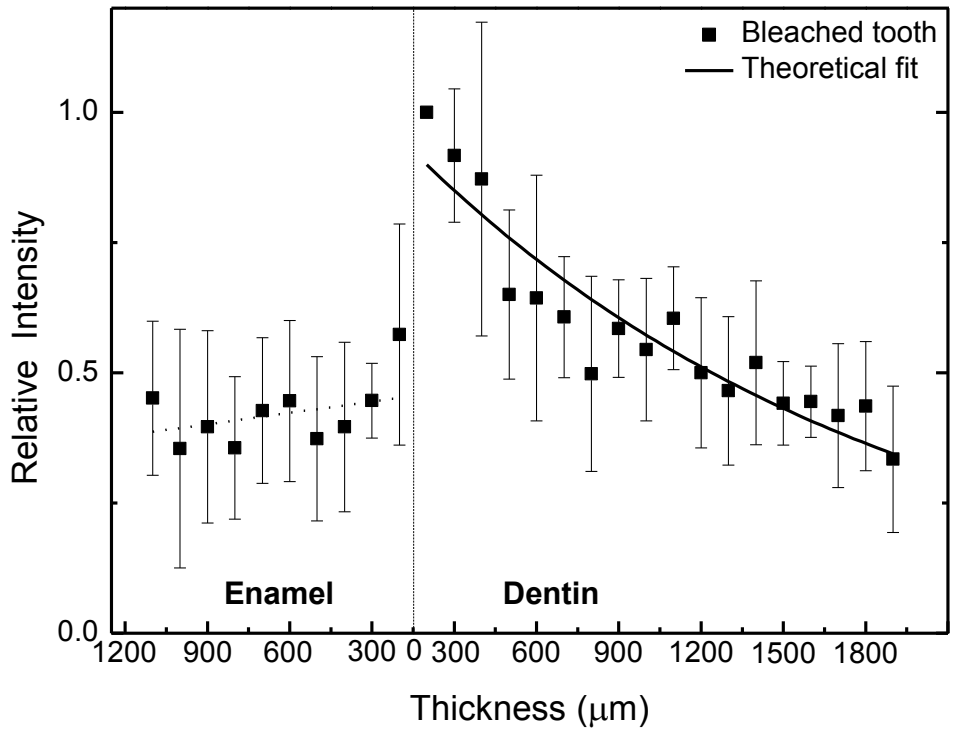


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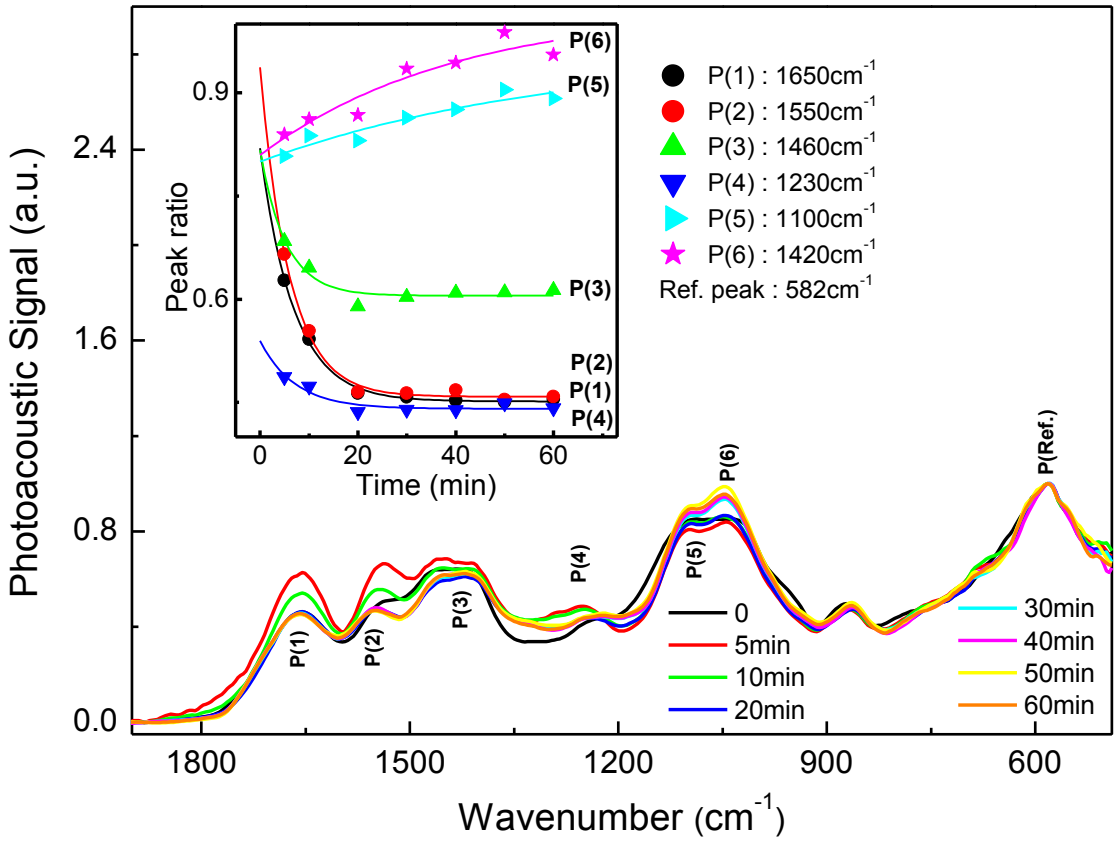
Figure 4



4) FTIR-PAS spectra as a function of H_2O_2 treatment time. The inset shows the variation of the optical bands intensities at P(1): 1650cm^{-1} (amide I); P(2): 1550cm^{-1} (amide II); P(3): 1460cm^{-1} (C-H stretching); P(4) 1230cm^{-1} (amide III); P(5): 1100cm^{-1} (ν_3 PO_4) and P(6):1042cm^{-1} (ν_3 PO_4). The reference peak was centered at 582cm^{-1}. Reduction of dentin compounds over bleaching treatment time is observed.

1. ANEXOS

ANEXO A (comitê)

Universidade Estadual de Maringá
Pró-Reitoria de Pesquisa e Pós-Graduação
Comitê Permanente de Ética em Pesquisa Envolvendo Seres Humanos
Registrado na CONEP em 10/02/1998

CAAE Nº. 0185.0.093.000-09 **PARECER Nº. 506/2009**

Pesquisador (a) Responsável: RENATA CORRÊA PASCOTTO

Centro/Departamento: CCS/Departamento de Odontologia

Título do projeto: Análise de permeabilidade dos agentes clareadores através dos tecidos dentários por meio da técnica Espectroscopia Fotoacústica no infravermelho via transformada de Fourier.

Considerações:

Atendendo ao parecer 368/2009 de 31/7/09, a pesquisadora reencaminha o TCLE reformulado nos termos da Resolução 196/96, incluindo modelo formulado para participantes menores de 18 anos, em que será necessária a autorização de um responsável. Quanto às autorizações das instituições/departamentos relacionados no projeto como "participantes" na execução de etapas do estudo (departamentos de Física e Química da UEM e de odontologia da UNOPAR), a mesma esclarece que a pesquisa não utilizará as dependências do laboratório de Química, e encaminha termo de autorização para a utilização de equipamento do Departamento de Física, bem como um protocolo de intenções assinados pelo Chefe do Departamento de Odontologia da UEM e o Coordenador do Programa de Pós-graduação *stricto sensu* em Odontologia da UNOPAR, em que se propõem apoio mútuo na elaboração e desenvolvimento de pesquisa de iniciação científica, trabalhos de conclusão de curso, dissertação e teses.

Diante do exposto, somos de parecer favorável à aprovação do referido projeto.

Situação: **APROVADO**

CONEP: (X) para registro ( ) para análise e parecer Data: **9/10/2009**

O pesquisador deverá apresentar Relatório Final para este Comitê em: 31/8/2010.

O protocolo foi apreciado de acordo com a Resolução nº. 196/96 e complementares do CNS/MS, na 183ª reunião do COPEP em 9/10/2009.

PROFª.DRª. Ieda Harumi Higarashi
Presidente do COPEP

Em suas comunicações com esse Comitê cite o número de registro do seu CAAE.
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ANEXO B

The *Journal of Dental Research* (*JDR*) is a peer-reviewed scientific journal dedicated to thedissemination of new knowledge and information on all science relevant to dentistry and to theoral cavity and associated structures in health and disease. The *Journal of Dental Research*'sprimary readership consists of oral, dental and craniofacial researchers, clinical scientists, hardtissuescientists, dentists, dental educators, and oral and dental policy-makers. The *Journal* ispublished monthly, allowing for frequent dissemination of its leading content. The *Journal ofDental Research* also offers OnlineFirst, by which forthcoming articles are published onlinebefore they are scheduled to appear in print.Authors of all types of articles should be aware of the following guidelines when submitting toJDR.

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Discovery!: Essays that explore seminal events and creative advances in thedevelopment of dental research are considered for the "Discovery!" section of thejournal. Manuscripts submitted for "Discovery!" have a limit of 2,500 words and a totalof 2 figures or tables. Manuscripts are to be submitted by invitation only. Questionsregarding "Discovery!" should be directed to Dr. Marty Taubman, atmtaubman@forsyth.org.

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