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"CARACTERIZAÇÃO MORFOLÓGICA E ANÁLISES DO CONTEÚDO ORGÂNICO E INORGÂNICO EM LESÕES DE ESMALTE FLUORÓTICO EM TERCEIROS MOLARES HUMANOS INCLUSOS E ERUPCIONADOS EXPOSTOS A ALTOS NÍVEIS DE FLÚOR"

Tese apresentada a
Faculdade de Odontologia de
Piracicaba, da Universidade Estadual
de Campinas para obtenção do Título
de Doutor em Biologia Buco-Dental
na área de Histologia e Embriologia

Orientador: Profa. Dra. Raquel Fernanda Gerlach
Co-orientador: Prof. Dr. Frederico Barbosa de Sousa

PIRACICABA

2010

FICHA CATALOGRÁFICA ELABORADA PELA
BIBLIOTECA DA FACULDADE DE ODONTOLOGIA DE PIRACICABA
Bibliotecária: Elis Regina Alves dos Santos – CRB-8ª. / 8099

P838c

Porto, Isabel Maria.
Caracterização morfológica e análises do conteúdo orgânico e inorgânico em lesões de esmalte fluorótico em terceiros molares humanos inclusos e erupcionados expostos a altos níveis de flúor / Isabel Maria Porto. -- Piracicaba, SP: [s.n.], 2010.

Orientador: Raquel Fernanda Gerlach, Frederico Barbosa de Sousa.
Tese (Doutorado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Fluorose. 2. Microscopia de polarização. 3. Espectrometria de massa. 4. Proteínas do esmalte dentário. I. Gerlach, Raquel Fernanda. II. Sousa, Frederico Barbosa de. III. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. IV. Título.

(eras/fop)

Título em Inglês: Morphological characterization and analysis of organic and inorganic content in fluorotic enamel lesions of non-erupted and erupted third human molars exposed to high levels of fluoride

Palavras-chave em Inglês (Keywords): 1. Fluorosis. 2. Polarization microscopy. 3. Mass spectrometry. 4. Dental enamel proteins
Área de Concentração: Histologia e Embriologia.

Titulação: Doutor em Biologia Buco Dental
Banca Examinadora: Raquel Fernanda Gerlach, Luciano Bachmann, José Cesar Rosa, José Merzel, Lívia Maria Andaló Tenuta

Data da Defesa: 23-08-2010

Programa de Pós-Graduação em Biologia Buco Dental

UNIVERSIDADE ESTADUAL DE CAMPINAS
Faculdade de Odontologia de Piracicaba

A Comissão Julgadora dos trabalhos de Defesa de Tese de Doutorado, em sessão pública realizada em 23 de Agosto de 2010, considerou a candidata ISABEL MARIA PORTO aprovada.

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Dedico esta tese de doutorado aos meus pais e irmãos pelo amor incondicional e ao meu marido Ricardo por estar sempre ao meu lado, me dando apoio e força para continuar em frente, e por sempre compreender e suportar minhas ausências.

AGRADECIMENTOS

1. Primeiramente agradeço a Deus por ter iluminado e guiado meu caminho, me protegendo sempre do perigo ao longo de minhas inúmeras viagens. "A ciência humana de maneira nenhuma nega a existência de Deus. Quando considero quantas e quão maravilhosas coisas o homem compreende, pesquisa e consegue realizar, então reconheço claramente que o espírito humano é obra de Deus, e a mais notável." (Galileu Galilei).

2. Agradeço aos meus pais e familiares pela educação e amor, por ter me tornando o ser humano que sou. "Os nossos pais amam-nos porque somos seus filhos, é um fato inalterável. Nos momentos de sucesso, isso pode parecer irrelevante, mas nas ocasiões de fracasso, oferecem um consolo e uma segurança que não se encontram em qualquer outro lugar." (Bertrand Russel).

3. Agradeço ao meu marido Ricardo, por ser um anjo que Deus colocou na minha vida, por suas palavras de carinho, por seu olhar de compreensão, pelo amor despretensioso. Por nunca reclamar das minhas diversas ausências. E finalmente, muito obrigada por você existir e estar presente em minha vida. "É apenas com o coração que se pode ver direito; o essencial é invisível aos olhos." (Antoine De Saint Exupery).

4. Agradeço a minhas amigas Andrea, Carla, Élen, Giselle, Glauce, Michele, Anna e Andrezza, e também ao meu amigo César por estarem sempre ao meu lado tornando a minha existência tão alegre e divertida. "A vida é em parte o que nós fazemos dela, e em parte o que é feito pelos amigos que nós escolhemos." (Tennessee Williams).

5. Agradeço a minha orientadora Raquel por me dar oportunidades, me guiar pelo caminho do saber e por confiar sempre em meu trabalho. "Para oferecer um serviço verdadeiro você deve adicionar algo que não pode ser comprado ou calculado com dinheiro, e isso é a sinceridade e integridade." (Donald A. Adams).

6. Agradeço a todos meus colaboradores: Professor Frederico Barbosa de Sousa (Departamento de Morfologia, UFPB), Professor Jaime Aparecido Cury (Departamento de Bioquímica, FOP/UNICAMP), Professor Jose Cesar Rosa e Helen Julie Laure (Laboratório de Química de Proteínas, Hemocentro, USP), Professor Luciano Bachmann (Departamento de Física, FFCL/USP), Professora Regina Cely Barroso (Departamento de Física, UERJ) pela ajuda e dedicação para que meu trabalho fosse realizado. “A tarefa essencial do professor é despertar a alegria de trabalhar e de conhecer." (Albert Einstein).

7. Agradeço a dentista de Venâncio Aires - RS, Dra. Cláudia Souza, e ao Professor Walter Blaya Perez (Departamento de Estomatologia, UFSM) pela seleção, diagnóstico, coleta e acompanhamento dos pacientes doadores dos dentes com fluorose da cidade de Venâncio Aires - RS, sem cuja ajuda estas amostras não estariam disponíveis para esta pesquisa.

8. Agradeço à Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) por acreditar e financiar minha pesquisa. E também agradeço ao Conselho Nacional de Pesquisa (CNPq) por financiar muitos dos equipamentos e laboratórios nos quais trabalhei.

"Não existe esta coisa de homem feito por si mesmo. Somos formados por milhares de outros. Cada pessoa que alguma vez tenha feito um gesto bom por nós, ou dito uma palavra de encorajamento para nós, entrou na formação do nosso caráter e nossos pensamentos, tanto quanto do nosso sucesso."

(George Matthew Adams)

RESUMO

O esmalte é o tecido mais mineralizado do corpo. Esta característica o torna um tecido altamente conservado no período *post mortem*. Por isso ele é citado como o melhor tecido para se estudar matéria orgânica em fósseis no campo da antropologia, paleontologia e arqueologia. É possível também a determinação do sexo na ciência forense. Porém a dificuldade está em se extrair o conteúdo orgânico destes dentes, já que o esmalte maduro contém menos de 1% de proteínas. Dois estudos da presente tese descrevem um método muito eficiente em extrair proteínas do esmalte. Esta mineralização do esmalte dentário pode ser prejudicada por doenças, como a fluorose dentária por exemplo. Em nosso terceiro estudo, analisamos o perfil dos aminoácidos para saber se o flúor interfere na clivagem das amelogeninas. Neste estudo, a clivagem da amelogenina não é afetada pelo flúor em dentes humanos fluoróticos erupcionados e inclusos. O quarto estudo traz como hipótese que a distribuição espacial dos conteúdos bioquímicos nas lesões fluoróticas do esmalte se assemelha aquela observada na última onda de mineralização durante o estágio de maturação. Observou-se neste estudo que há um aumento do conteúdo orgânico e uma hipomineralização na superfície no esmalte fluorótico, tanto nos terceiros molares erupcionados quanto inclusos. Estes dados sugerem que a lesões fluoróticas em esmalte humano refletem a composição do esmalte no período tardio de maturação da amelogênese.

Palavras-chave: Proteínas, Espectrômetro de massa, Esmalte, Flúor, Mineralização.

ABSTRACT

The dental enamel is the most highly mineralized tissue in the body. Due to its characteristics, the enamel is highly inert to changes brought about by time and the environment, being a very important source of information for palaeo-, palaeanthropo-, and anthropologists. The enamel proteins are also used for sex identification in forensic science. But protein recovery from the enamel it is a challenging task. Here, we present two studies that describe procedures very effective in providing enamel samples that are adequate for protein analysis. This mineralization could be impaired by diseases like dental fluorosis, for example. In the third study, we analyzed the amino acid profile in the erupted and non-erupted fluorotic human teeth compared to control ones. In this study the cleavage of amelogenin was not affected by fluoride in the fluorotic teeth. In the fourth study we hypothesized that the composition of fluorotic lesions may resemble the enamel found during last wave of enamel mineralization. We found an increase in the organic content and a superficial hypomineralization of the fluorotic enamel, in both erupted and non-erupted human third molars. These data suggest that in both non-erupted and erupted human teeth the fluorotic lesions resembles the late maturation stage enamel, which is not mineralized until maturation is completed.

Key Words: Proteins, Mass Spectrometry, Enamel, Fluoride, Mineralization.

SUMÁRIO

INTRODUÇÃO

O esmalte é o tecido mais mineralizado do corpo. Em seu estágio final de maturação, ele apresenta mais de 95% de mineral (Smith, 1998). Esta característica o torna um tecido altamente conservado no período *post mortem*. Por isso ele é citado como o melhor tecido para se estudar matéria orgânica em fósseis no campo da antropologia, paleontologia e arqueologia (Dean, 2006; Smith & Hublin, 2008). Através do estudo de isótopos, é possível se determinar o tipo de ambiente e dieta a que nossos ancestrais foram submetidos. É possível também a determinação do sexo de uma pessoa na ciência forense. Isso porque existem duas isoformas da proteína do esmalte amelogenina: a isoforma X (AMELX) codificada pelo gene do cromossomo X e a isoforma Y (AMELOY), codificada pelo gene do cromossomo Y (Nielsen-Marsh, 2009; Pfeiffer e Brenig; 2005). Porém a dificuldade está em se extrair o conteúdo orgânico destes dentes, já que o esmalte maduro contém menos de 1% de proteínas. Estudo anterior nosso mostrou a eficácia do método de precipitação por ácido tricloroacético na extração de proteínas do esmalte (Porto et al., 2006), sendo este método até usado para extrair proteínas de dentes de fósseis de homem Neanderthal (Nielsen-Marsh et al., 2009). Porém em dois estudos da presente tese (capítulos 1 e 2) descreveremos outro método muito eficiente em extrair proteínas do esmalte maduro e muito mais conservador e de fácil manuseio.

Esta mineralização do esmalte dentário pode ser prejudicada por doenças, como a fluorose dentária por exemplo. O excesso de flúor causa lesões no esmalte conhecidas como fluorose do esmalte. Estas lesões podem variar desde manchas esbranquiçadas que comprometem a estética dos dentes até levar a perda funcional destes dentes. Um índice

para medir o grau de fluorose muito usado é o índice de Thylstrup & Fejerskov. Este índice tem uma escala de 0 a 9 que mede a severidade do grau da lesão fluorótica de acordo com a aparência clínica do esmalte (Thylstrup & Fejerskov, 1978). Quanto maior o grau, maior a severidade da lesão. Fejerskov *et al* (1977) descreveram os defeitos fluoróticos em luz polarizada e mostraram que há uma hipomineralização subsuperficial, enquanto o esmalte mais externo (aproximadamente 30 μm) é normal (Eastoe and Fejerskov, 1984; Fejerskov et al., 1977). Porém outros estudos com dentes de ratos com fluorose mostraram que não existe uma superfície hipermineralizada, há sim uma hipomineralização superficial do esmalte fluorótico que diminui em direção à junção amelodentinária (Porto et al., 2010 – anexo 1; Saiani et al., 2009 – anexo 2).

Outra controvérsia é se o fluór causa ou não a retenção de proteínas no esmalte. Estudos mostram que o esmalte fluorótico tem um menor conteúdo mineral e maior conteúdo protéico quando comparado ao esmalte normal (Den Besten, 1986; DenBesten and Heffernan, 1989; Shinoda, 1975; Shinoda and Ogura, 1978; Triller, 1979; Wright et al., 1996; Zhou et al., 1996). Porém outros estudos não observaram aumento do conteúdo protéico em dentes com fluorose (Gerlach et al., 2000; Eastoe & Fejerskov; 1984). Porém, mudanças pós-eruptivas alteram tanto o conteúdo orgânico quanto mineral da lesão fluorótica do esmalte, portanto a descrição precisa dos componentes bioquímicos das lesões fluoróticas que nunca foram expostas à cavidade oral é ainda muito relevante. Em nosso terceiro estudo (capítulo 3), analisamos através de espectrômetro de massa o perfil dos peptídeos da amelogenina para saber se o flúor interfere na clivagem das amelogeninas. O interesse neste estudo é que estudamos dentes maduros humanos com fluorose erupcionados e também dentes com fluorose não erupcionados, portanto, que ainda não

foram expostos à cavidade oral evitando o risco de adsorção pós-eruptiva de proteínas exógenas.

Outro aspecto a se considerar são as células envolvidas na amelogênese, denominadas ameloblastos. A atividade da proteinase do esmalte conhecida como Kalicreina-4 (KLK-4) é essencial para a amelogênese normal. Uma diminuição nos níveis de expressão da KLK-4 foi detectada em células do órgão do esmalte de ratos expostos a 100 e 150 ppm de flúor (Sharma et al., 2010).

Os ameloblastos do estágio de maturação provavelmente possuem outras funções importantes além da secreção da KLK-4, tais como o controle do influxo de cálcio (Smith, 1998a). Um estudo observou que a maturação do esmalte não ocorre quando os ameloblastos do estágio de maturação são removidos cirurgicamente, e que o conteúdo mineral final do esmalte é apenas em torno de 70% (Porto et al., 2009). O mecanismo proposto para a fluorose do esmalte tem como hipótese que a toxicidade do flúor induz estresse celular nos ameloblastos do estágio de maturação que são mais sensíveis que os ameloblastos secretórios, devido ao baixo pH extracelular. Este mecanismo pode explicar o fato conhecido há tempos de que o flúor afeta predominantemente o esmalte no estágio de maturação (Sharma et al., 2010), mas isso também sugere que a aparência histopatológica das lesões fluoróticas pode se assemelhar mais a uma lesão de esmalte devido a falta de um estágio final de maturação completo. Tais defeitos foram descritos detalhadamente por Suga (1989), o qual realizou microradiografias em dentes desgastados. A onda final da mineralização é descrita movendo-se do esmalte interno em direção a superfície do esmalte (Suga, 1989). Cortes desgastados de incisivos de ratos revelou que lesões fluoróticas são também superficiais em ratos (Saiani et al., 2009), e não subsuperficiais (Angmar-Mansson

and Whitford, 1982). Tais lesões superficiais podem indicar que a fluorose do esmalte resulta de uma toxicidade direta do flúor sobre os ameloblastos do estágio de maturação, que não estão então funcionais para regular o final da maturação do esmalte. O aspecto histopatológico e a determinação do conteúdo orgânico e mineral das lesões fluoróticas com resolução espacial são, portanto importantes para a elucidação do mecanismo da fluorose.

O capítulo 4 traz o estudo que tem como hipótese que a distribuição espacial dos conteúdos bioquímicos nas lesões fluoróticas do esmalte se assemelha aquela observada na última onda de mineralização durante o estágio de maturação. Para testar esta hipótese, dados quantitativos e com resolução espacial dos conteúdos mineral, orgânico, e de água foram medidos em cortes desgastados de esmalte fluorótico humano de dentes erupcionados e inclusos em relação aos dentes controles.

CAPÍTULO 1

New techniques for the recovery of small amounts of mature enamel proteins

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Keywords: enamel, MALDI-TOF, protein extraction, enamel proteins, amelogenin.

Abstract: 198 words.

Manuscript: 2874 words.

Abstract

Currently there are no conservative techniques to obtain protein from the dental enamel, which contains 95% of mineral by weight, being the most highly mineralized tissue in the body. To recover protein, enamel is completely dissolved after being powdered. In this work we tested whether superficial enamel samples obtained by micro-etching were adequate for protein analysis by MALDI-TOF/TOF mass spectrometry and identification in protein databases. The micro-etch techniques were effective in generating adequate samples for mass-spectrometry (from 3-13.4 µm superficial enamel), being also highly conservative, since they rendered masses of enamel ranging from 0.1 to 0.4 mg. Using these techniques the separation of proteins by SDS-PAGE was not necessary, and the whole procedure was easier. Results showed successful identification of specific enamel proteins after whole crown superficial enamel etching with 11% EDTA in the case of immature porcine samples, and with 10% HCl in the case of mature human enamel. X- and Y-isoforms of amelogenin, ameloblastin, and enamelin peptides were identified. The new techniques described here allowed the successful recovery of proteins specific to the enamel, opening new avenues for the use of enamel protein information in forensics and in archeological material, where sometimes little protein is left.

1. Introduction

The dental enamel contains 95% of mineral by weight, being the most highly mineralized tissue in the body. It also contains a small amount of proteins (<1%), and most of these are specific to the enamel, such as amelogenins (1), ameloblastin (2) and enamelin (3). Amelogenins are a heterogeneous group of proteins, and a specific amelogenin sequence is derived from the X- and another from the Y-chromosome (4,5). The fact that enamel has this amazingly high amount of mineral turns it into a matrix that is highly inert to changes brought about by time and the environment (6).

Recently, enamel protein peptides have been successfully recovered from contemporary, medieval, and two Neanderthalis teeth (6). In the latter case (6), protein extraction from the tooth enamel was accomplished from ~2 mg of enamel powder from each of the Neanderthalis teeth as the starting material, and the precipitation protocol was based on the use of TCA (6), as proposed for the recovery of proteins from mature rodent enamel (7).

Although enamel protein recovery is challenging, the fact that these proteins are specific to the enamel turn their use advantageous in the case of ancient and forensic material, in which contamination with proteins from the lab (glassware, gloves, etc) or from the environment is a problem.

The recent analyses of organic matter in the enamel (8) show that the amount of organic matter decreases from the surface of the enamel toward the more inner layers (Frederico B. de Sousa, 2009, personal communication). Thus, the superficial dental enamel may be advantageous for protein analysis because it may contain more protein and is more easily accessible. Moreover, superficial etching techniques allow researchers to

obtain enamel samples without the need for using complex procedures to separate dentin from the enamel. As a matter of fact, we have been using a 20-second superficial enamel acid etching technique to collect superficial enamel samples for lead analysis (9-12), since lead and other micro-elements such as fluoride, zinc, and strontium accumulate in this outer layer of the enamel (13).

Our hypothesis in the start of this study was that we could probably obtain enough protein for protein analysis using superficial enamel etching techniques. Thus, the aim of this study was finding a method to obtain superficial enamel samples for protein analysis from mature dental enamel, and testing whether the samples were adequate for mass spectrometry and protein fingerprinting.

2. Materials and methods

2.1. Enamel samples

Porcine incisors (containing immature enamel) and human third molar (mature enamel) were used in this study. This study was approved by the Institutional Ethics Committee for Human Research (protocol number 2003.1.1329.58.2). Details are found in the Electronic Supplementary Material (ESM).

2.2. Protein Extraction Techniques

Enamel powder dissolution followed by precipitation with Trichloroacetic acid (TCA) The enamel powder was dissolved in 12% trichloroacetic acid (TCA) in a proportion of 200 µL acid/ mg enamel powder. One hour later, sodium deoxycolate was added in a final concentration of 200µg/mL, as described earlier (7). After enamel dissolution at 0°C, the solution was centrifuged at 2500 x g at 4°C for 45 min. The pellet

was ressuspended in 200µL of a 6M urea solution containing a wide range of protease inhibitors. In some samples, the centrifugation was performed using microcentrifuge filter of 30kDa-cutoff (Ultrafree-MC microcentrifuge filters, M0536, Sigma-Aldrich, USA) for 30 min, 3500 x g, at 4°C. Please refer to the ESM for all details.

b) Whole crown etching Porcine teeth (which were not fully mature) were etched using ethylenediamine tetraacetic acid (EDTA). The whole tooth crown was submersed in 2 mL of a solution of 11.2% EDTA and 1.25% sodium hydroxide, pH 7.3, for 5 min. The solution was transferred to a microcentrifuge filter of 30kDa-cutoff (Ultrafree-MC microcentrifuge filters, M0536, Sigma-Aldrich, USA) and centrifuged for 30 min, 3500 x g, at 4°C. The solution that passed through the filter and proteins below 30kDa were collected in the "flow-through". The acid and salts were removed using a microtip filled with Poros 50 R2 resin (PerSeptive Biosystems, Framingham, MA) (the proteins were retained through binding to a porous resin), eluted with 50% acetonitrile/ 0.2% formic acid, and dried in a Speed Vac (AS 290, Savant, Rochester, New York, USA) for 30 min.

The whole tooth crown of mature human third molars were submerged in 1 mL of 10% HCl containing proteinase inhibitors, for different time periods (Table 1). The samples were transferred to 30000WM filter tubes and processed as described above for the porcine teeth. Please refer to the ESM for all details.

c) Restricted area etching This etching technique was tested in both porcine (immature enamel) and human teeth (mature enamel). The procedure described by Costa de Almeida et al. (2009) (12) was followed. Briefly, a circular hole (2.0 mm diameter) was punched in an adhesive tape, which was applied to the surface of the teeth. A window of 3.14 mm^2 area was exposed, and 5 µL 11.2% EDTA/1.25% NaOH, pH 7.3, were applied

for 15 s. The extract was transferred to a low protein binding tube. This etching step was repeated twice, and the etching solutions were added to the tube. Please refer to the ESM for all details.

2.3. Determination of phosphorus

Phosphorus was determined by the colorimetric method of Fiske and Subbarow (1925) (14), as described in Costa de Almeida et al. (2007). Assuming an enamel density of 2.95 g cm^{-3} and a phosphorus content of 17.4% (15), and accepting an average presumed area of the tooth crowns used, we were able to calculate the presumed depth of the etching procedures using the different acid solutions. Details are found in the ESM.

2.4. Electrophoresis and digestion of proteins from gel bands

Protein solutions from the porcine teeth obtained by the restricted area etching procedure and protein solutions from the human teeth obtained by whole crown superficial enamel acid dissolution were employed in these analyses. Samples were then run by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) on a 15% gel with a discontinuous buffer system (16). The gel was stained with mass-spectrometry-compatible silver staining (17). The gel was photographed, and bands of different molecular weights were excised from the gel.

The procedure described by Gharahdaghi et al. (1999) (18) was employed to destain and dry the gel bands. The bands were destained with 30 mM potassium ferricyanide and 100 mM sodium thiosulfate in a 1:1 proportion and dried in a SpeedVac (AS 290, Savant) for 10 min. The gel bands were then soaked in a 200 mM solution of ammonium bicarbonate for 20 min. This solution was removed, and pure acetonitrile was added until gel bands were completely dehydrated (dehydrated gel bands had the aspect of opaque

white paper). Residual acetronitrile was removed by evaporation to dryness in the SpeedVac (AS 290, Savant) for 30 min.

The gel bands were rehydrated with a 100 mM ammonium bicarbonate solution containing 0.5 µg of trypsin (MS grade, Promega, Madison, WI), at 37°C, for 22 h. The reaction was stopped with 5 µL of formic acid. The supernatant was concentrated and desalted by means of a microtip filled with Poros 50 R2 resin, previously activated with methanol, equilibrated in 3 x 150 µL of 0.2% formic acid, followed by sample loading and further 3 x 150 µL of 0.2% formic acid for desalting. The peptides were eluted from the microtip in 30 µL of 60% methanol prepared in 5% formic acid (v/v), being then ready for mass spectrometry analysis.

2.5. Digestion of proteins

Whether dissolved enamel samples (obtained by either the biopsy technique or whole crown submersion in acid) could be directly used for MALDI-TOF analysis (without previous separation in an SDS-PAGE gel) was tested.

Prior to trypsin digestion, using reduction/alkylation of proteins, performed by ressuspending the dry protein in 20 µL of 50% acetonitrile / 100 mM ammonium bicarbonate. Next, 5 µL of 45 mM dithiothreitol (DTT) were added for 1 h at 56°C. After that, 5 µL of 100mM iodoacetamide were added for 1 h in the dark, at room temperature. The samples were diluted 5 times with 100 mM ammonium bicarbonate, 0.5 µg of trypsin were added, and trypsin hydrolysis was carried out for 22 h at 37°C. Finally, 5 µL of formic acid were added to quench the reaction. The supernatants were desalted using a microtip filled with Poros 50 R2 resin, according to the procedure described above. Details are found in the ESM.

2.6. Mass Spectrometry using matrix assisted laser desorption/ionization – time- of-flight mass spectrometer (MALDI-TOF/TOF)

Peptides were analyzed for mass precursor and ion precursor selection by data-dependent acquisition following high energy collision induced dissociation (CID), to produce product ions of each precursor ion, which were then used for amino acid sequence determination. The CID-MS/MS spectra were submitted to a database search against Swiss-Prot using the MASCOT software version 2.2.4 (Matrixscience, London, UK). Please refer to the ESM for all details.

3. Results and Discussion

Our results indicate that the superficial enamel micro etching and enamel powder dissolution techniques tested here are very effective in generating adequate samples for mass spectrometry. The acid-etching procedures employed here resulted in masses of enamel samples ranging from 0.1 to 0.4 mg (when HCl was used), based on the phosphorus content obtained in the samples.

Table 1 from the ESM shows the etching times and acid solutions tested and resulting enamel masses obtained. Results indicate that in the case of mature normal human teeth, dissolution of the enamel powder and whole crown etching with 10% HCl for 5 minutes resulted in samples with enough proteins to produce a good MS signal. Furthermore, the 5-minute etching resulted in the removal of a 13.4 μm layer of superficial enamel, corresponding to 0.23 mg of enamel. EDTA was not as effective as HCl for production of enamel samples. As expected, the longer exposures to acid will result in the removal of enamel from deeper layers, and different time periods can be employed

depending on the desired amount of enamel. Additionally, successive acid attacks can be used to remove deeper layers.

Only faint high molecular mass bands are observed in an SDS-PAGE gel when samples obtained by superficial enamel etching of mature enamel are run (not shown). Some of these bands were excised from the gel and analyzed by MS and MS/MS. While high molecular bands resulted in keratin and albumin signal, no enamel proteins were identified from such gel bands obtained from superficial mature human enamel samples separated in gels.

In the case of porcine teeth enamel samples, the SDS-PAGE gel revealed a band pattern compatible with amelogenin proteins below 27 kDa, as shown in Figure 1. The aminoacid analysis of these bands < 27 kDa confirmed the presence of amelogenin peptides in those gel bands (Figure 2A). Other contaminants like keratin and albumin were also identified in high molecular mass gel bands (bands are shown in Figure 1, and mass spectrometry graphs – MS graphs of these bands were obtained, but are not shown). Enamelin was detected in one sample from a high molecular weight band (the band is shown in Figure 1, and the MS and MS/MS of the enamelin peptide is shown in Figure 2 B and C).

MS graphs shown in Figure 3 were obtained from the direct application of purified superficial enamel samples from mature human enamel without separation of the samples in the gel. Results demonstrate that it is feasible to obtain enough protein material of < 30 kDa proteins that can be easily prepared for MS and MS/MS, rendering very good enamel protein peptide signals. When the dental crown of the immature porcine teeth was submerged in EDTA, a good signal was obtained (not shown).

Although only some MS graphs are shown, it is very interesting that the tested procedures and the described technique furnish very good MS signals by means of a procedure that is much easier than the ones described so far.

The amelogenin X-isoform was also identified in the samples obtained by superficial enamel etching (Figure 3), indicating that it may be possible to look for this peptide in such superficial enamel extracts as a means to determine sex using teeth of individuals that died a long time ago or in burned bodies, where most of the protein from other tissues is destructed. The amelogenin gene has a different sequence in the X and in the Y chromosome, and therefore amelogenin is used for sex identification using PCR (19).

We present the advantages and disadvantages of the 3 techniques tested here in Table 2 of the ESM. Whole crown etching with HCl and enamel powder dissolution and precipitation with TCA were the techniques that led to the best mature enamel samples in terms of good MS signals, thereby enabling identification of specific enamel proteins. Nonetheless, etching with EDTA also furnished immature porcine enamel samples that were adequate for MS analysis. This technique employing EDTA may find use in investigations on immature enamel. Therefore, the decision about which acid to use during enamel protein recovery will depend on the intended use.

Figure 4 summarizes the procedures used in the present work as well as the results. The superficial enamel micro-etching techniques employed here are advantageous in some aspects over the TCA dissolution/precipitation technique described in the literature (5,7): the micro-etch techniques described here provide enamel samples with smaller masses (~0.1 to 0.4 mg), which are suitable for the MS analysis of proteins. Moreover, there is no need to separate the samples by SDS-PAGE prior to mass spectrometry. Besides these

advantages (smaller amount of starting material, no need to separate proteins by electrophoresis), the sample preparation procedure is much easier than the one based on the use of powdered enamel. Another major advantage of the micro-etch techniques proposed herein is their application in the examination of past individuals and species, enabling the isolation of specific enamel proteins.

The use of the restricted area etching did not furnish samples with adequate signals for protein identification in the case of the mature human enamel under the conditions tested so far. However, this technique worked for immature enamel and may be very useful to obtain samples from small areas of interest. Successive restricted area etching will probably render enough enamel mass for adequate MS analysis. It may be desirable to obtain samples from restricted areas of the enamel in patients with enamel defects like enamel hypoplasia, enamel fluorosis, or amelogenesis imperfecta, where the enamel may later be covered with dental materials or crowns. Such samples open the possibility of characterizing the defective protein in human samples.

Due to its much higher degree of mineralization, the dental enamel is probably the best matrix to harbor better preserved proteins over very long time periods, particularly in dry environments. As pointed by Lee-Thorp and Sponheimer (2006), "enamel is denser, has a very low organic content and is more crystalline…which renders it effectively more inert and pre-fossilized" (6).

Furthermore, because the dental enamel has a set of specific proteins that are not found in other tissues (20), it offers an obvious advantage in the case of studies with such small sample amounts, since down to such small scales, contamination with proteins from the lab (glassware, gloves, etc) or from the environment where the tooth was found

(diagenesis) is always a problem. Some proteins found in the mature enamel are tightly bound to the enamel crystals (21), deserving to be further studied for their preservation state in tooth remnants as good candidates for protein analysis.

In conclusion, the micro-etch procedures described here are very conservative, removing only a very superficial layer of the enamel. They are also very effective in providing enamel samples that are adequate for protein analysis, greatly reducing the necessary starting mass of enamel. These techniques need less time to obtain samples, since the procedure is much easier than the one used currently. Since they demand less manipulation in the lab, chances of contamination with exogenous proteins (like keratin) are much less likely. Contamination is also reduced because the enamel harbors a set of enamel-specific proteins.

Acknowledgements

We gratefully acknowledge the support of the State of São Paulo Research Foundation (FAPESP) and the (Brazilian) National Research Council (CNPq).

Competing Interests Statement

The authors declare no competing interests.

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Figures

Figure 1

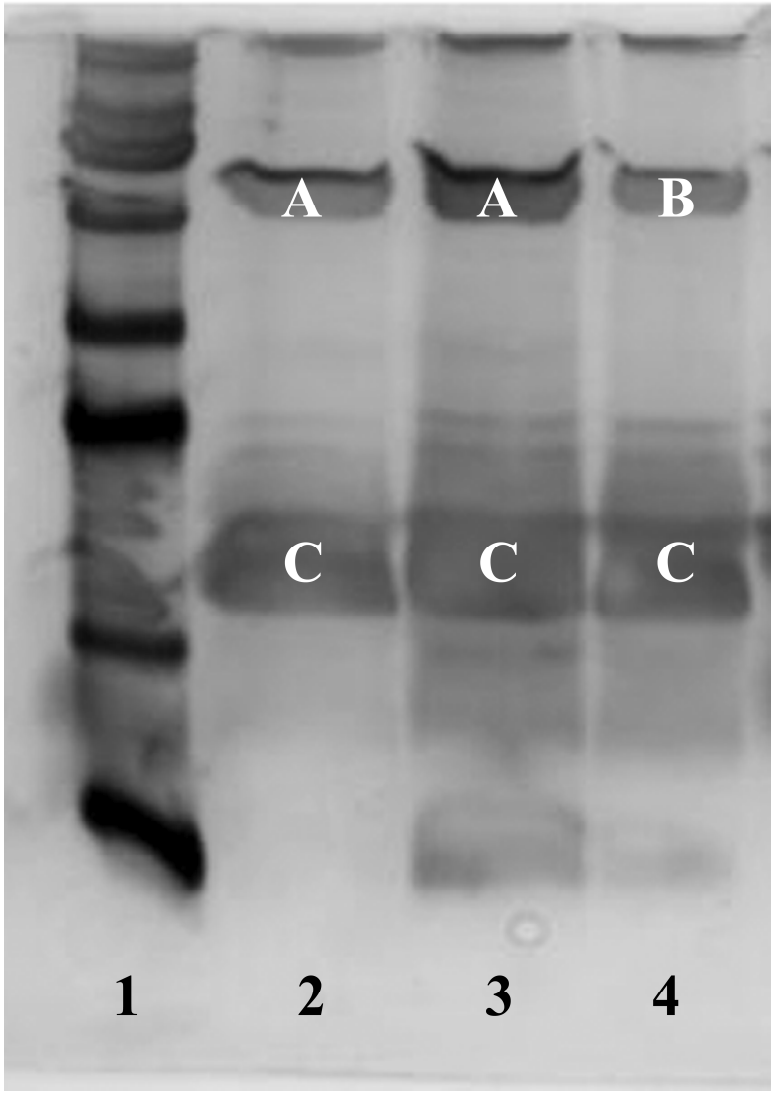

Figure 1. Silver-stained SDS-PAGE protein profile of immature porcine teeth. Lane 1: molecular weight marker. Lane 2, 3, and 4: samples from three different porcine tooth germs. Bands A: peptide fingerprinting resulted in the identification of enamelin and keratin from those bands; band B: albumin was identified in this band; band C: amelogenin was identified in this band.

Figure 2

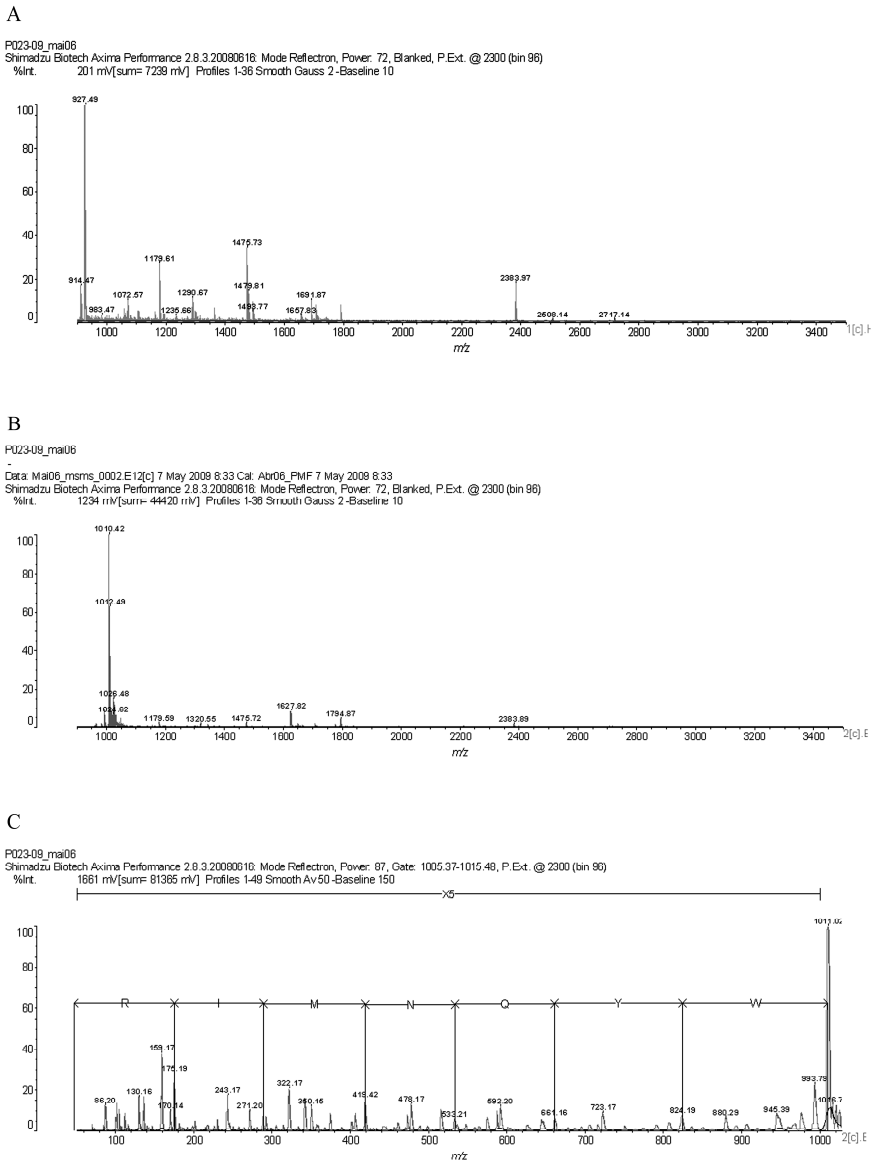

Figure 2. Porcine tooth protein identification by peptide fingerprinting. Peptides were analyzed by mass spectrometry after trypsin digestion of gel bands shown in Figure 1. A) Peptide mass fingerprint of gel band C, identified as amelogenin. B) Peptide mass fingerprint of gel band A, identified as enamelin. C) Mass spectrum of CID-MS/MS of ion m/z 1010.42 of the enamelin peptide, which allowed identification of the amino acid sequence WYQNMIR.

Figure 3

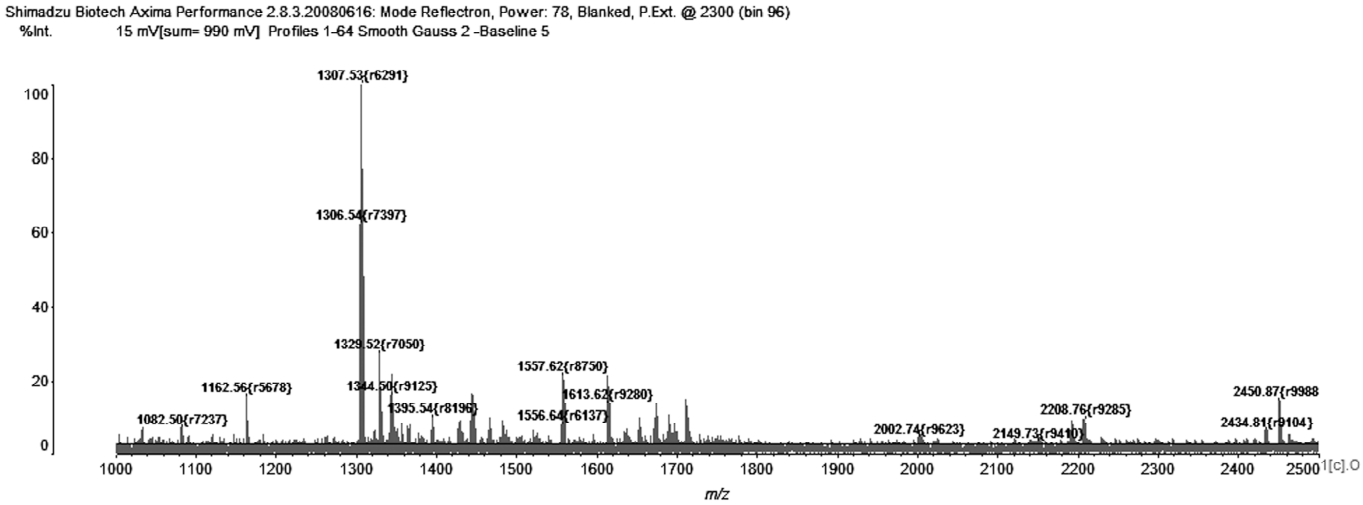

Figure 3. MS spectrum of a superficial enamel extract of mature human teeth obtained by whole crown etching in 10% HCl for 5 min. The samples were not separated by SDS-PAGE. Two Amelogenin –X isoform tryptic peptides were identified (peaks *m/z* 1308.27 and 1558.51).

Figure 4

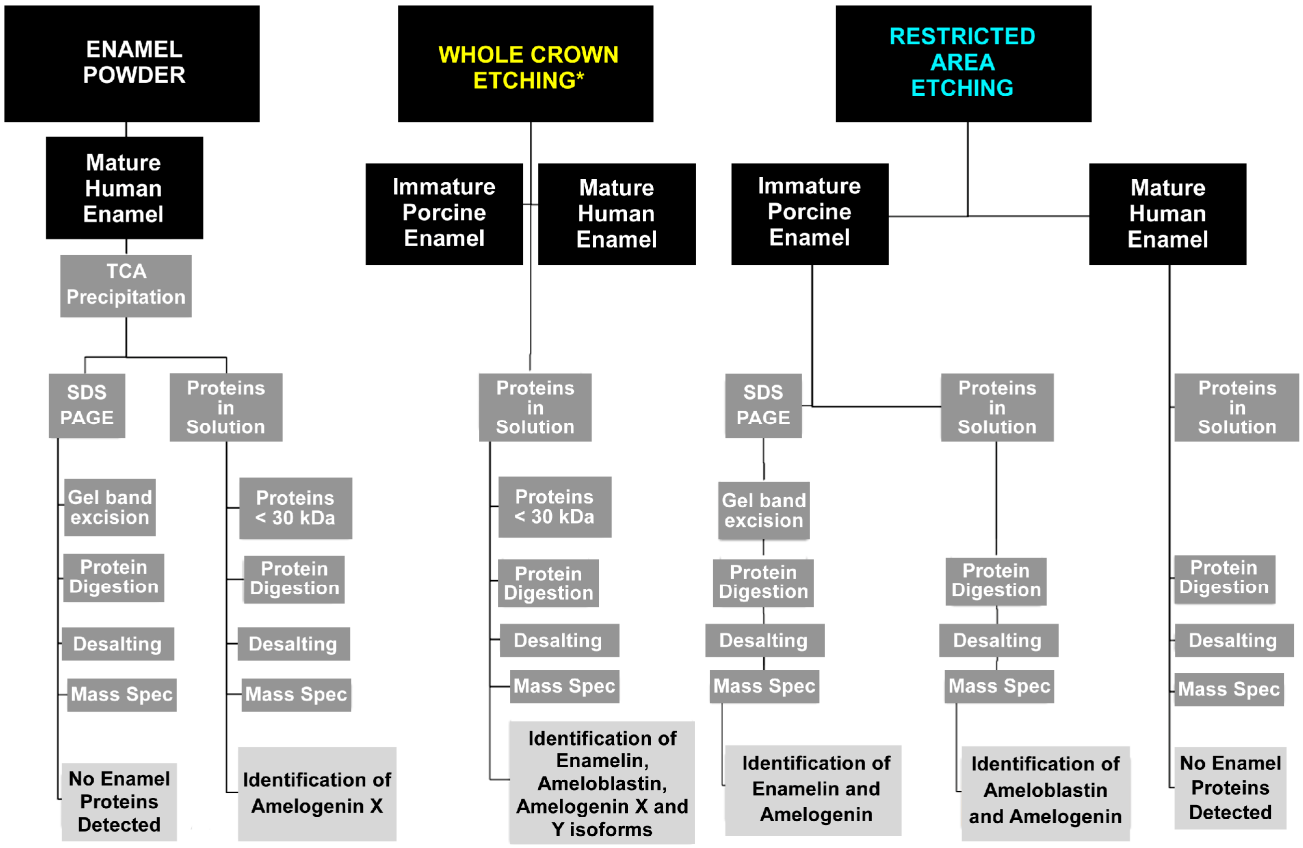

Figure 4. Schematic illustration of techniques used in this study. Left Sequence: The enamel powder is dissolved and precipitated by TCA. Middle Sequence: whole crown etching by HCl 10% for 5 min. Right Sequence: The restricted area etching (so far effective for protein recovery from immature porcine teeth). * EDTA was more effective for enamel protein extraction of porcine teeth, while HCl was more effective for human teeth.

Electronic Supplementary Material (ESM)

Material and Methods

2.1. Enamel samples

Porcine incisors were collected from two-month-old male pigs that were killed in local slaughterhouses. The incisors were washed with distilled water, to clean the tooth surface. Teeth were kept at -20°C until use. This study was approved by the Institutional Ethics Committee for Human Research (protocol number 2003.1.1329.58.2). Human third molars were extracted in the Oral Surgery Clinic of the Dental School of Ribeirao Preto, SP, Brazil. The patients were informed both verbally and in writing about the purposes of the research, and signed an informed consent document and a tooth donation term. Teeth were stored at -20°C until processing.

Porcine teeth were selected for this study because they were not fully mature, so it was expected that it would be less difficult to recover enamel specific proteins from them. The use of protease inhibitors throughout this study was essential to avoid proteolysis, and the inhibitors employed here were Phenylsulfonylfluoride, N-Ethylmaleymide, and Phenanthroline, all purchased from Sigma-Aldrich (St Louis, MO, USA) as powders, and prepared as stock solutions in methanol, being diluted in buffer solution at the appropriate concentration (2 mM for all) just prior to use.

2.2. Protein Extraction Techniques

We tested 3 techniques to obtain enamel samples: a) enamel powder dissolution followed by TCA precipitation; b) whole crown etching with different acids; and c) restricted area etching with different acids.

a) Enamel powder dissolution followed by precipitation with Trichloroacetic acid (TCA) The crowns of human third molars were powdered in a cryogenic mill Model MA 775 (Marconi, Piracicaba, SP, Brazil), using liquid nitrogen. The dentine was separated from the enamel by a classic density gradient fractionation procedure (1) using a 91% bromophorm/ 9% acetone mixture. After separation, the enamel and dentin powders were dried in a lyophilizer. Some samples were applied to glass slides and checked under a polarizing light microscope, confirming the identity of enamel and dentin, and the complete separation of the two tissues.

b) Whole crown etching Porcine teeth (which were not fully mature) were only etched using ethylenediamine tetraacetic acid (EDTA), which is the most gentle acid solution routinely used to decalcify tissues in Histology labs. This procedure resulted in adequate amounts of proteins for the identification of specific peptides from enamel protein after MALDI-TOF/TOF-MS analysis.

At first, mature human third molars were also etched with various concentrations of EDTA (different times); however, no peptide signal that resulted in enamel specific protein could be retrieved, so stronger acids were tested. Hydrochloride acid (HCl) resulted in an appropriate amount of dissolved enamel to retrieve enamel-specific proteins.

c) Restricted area etching This etching technique was tested in both porcine (immature enamel) and human teeth (mature enamel), and allowed the sampling of enamel from a specific area “restricted” by a circular hole in a tape. An important note: glycerol and other agents may be used to changes the superficial tension of the solution, resulting in acid attacks that are less invasive.

Briefly, a circular hole (2.0 mm diameter) was punched in an adhesive tape, which was applied to the surface of the teeth. A window of 3.14 mm^2 area was exposed, and 5 µL 11.2% EDTA/1.25% NaOH, pH 7.3, were applied for 15 s. The extract was transferred to a low protein binding tube. This etching step was repeated twice, and the etching solutions were added to the tube. At the end, 5 µL of distilled water were applied to the tape window for 5 s and added to the same tube, which contained approximately 20 µL water.

We usually use glycerol (70%, v/v) to obtain clinical samples from deciduous teeth *in vivo* (2-5). However, in this study glycerol was not employed in the etching solutions because it interferes in the drying of the MALDI/TOF matrix, and should therefore be avoided in samples intended for mass spectrometry.

2.3. Determination of phosphorus

For the human samples obtained by whole crown etching, the amount of phosphorus was determined, so as to calculate the "approximate" mass of enamel obtained by the different acids employed here (12% TCA, 11.2% EDTA, and 10% HCl). We use the term "approximate" because the precise superficial area that was etched cannot be directly measured, and therefore the depth cannot be considered a precise value, although the expected error should not exceed ±10%. We decided to check the mass of enamel by this chemical procedure because most of the samples did not furnish good signals (except those obtained by etching in concentrated HCl).

To be able to quantify the sample masses obtained by the whole crown and the restricted area etching techniques (since they dissolve the enamel, and therefore the initial mass cannot be weighed), we determined phosphorus in the samples to calculate the mass of enamel obtained.

2.4. Electrophoresis and digestion of proteins from gel bands

Twenty μL of sodium dodecyl sulfate (SDS)-reducing buffer (0.06M Tris-HCl, pH6.8, 2% SDS, 10% glycerol, 0.025% Bromophenol Blue, 5% 2-mercaptoethanol) were added to the samples, which were heated to 95°C for 4 min.

A protein standard solution was used (Page Ruler ^{TM} Plus Prestained Protein Lader, Fermentas Life Sciences, Glen Burnie, Maryland, USA).

2.5. Digestion of proteins

Initially, samples were heated for 5 min at 100°C. Then, 60 μL of 100 mM ammonium bicarbonate and 0.5 μg trypsin were added, and the mixture was incubated for 22 h at 37°C. When no peptide signal was obtained, samples were processed by another procedure prior to trypsin digestion, using reduction/alkylation of proteins,

2.6. Mass Spectrometry using matrix assisted laser desorption/ionization – time- of-flight mass spectrometer (MALDI-TOF/TOF)

The samples were redissolved in a CHCA-5mg/mL (alfa-cyano- 4-hydroxicinnamic acid, Sigma, USA) matrix with 50% acetonitrile/ 0.1% TFA (trifluoroacetic acid), crystallized in MALDI target, and mass spectrometry was accomplished in MALDI-TOF/TOF Axima Performance (Kratos-Shimadzu Biotech, Manchester, UK). The mass spectrometer was calibrated with external calibration using synthetic peptides (ProteoMass Peptide and Protein Maldi-MS Calibration Kit, Sigma-Aldrich, St.Louis, MO). The mass accuracy was less than 50 ppm.

Results

We present the advantages and disadvantages of the 3 techniques tested here in Table 2 of the ESM.

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Table 1. Resultant etching depth (µm) after whole crown submersion

Acid	Time	Depth of acid etching (µm)	Efficiency of enamel etching
11.2% EDTA	15 min	0.02 ±0.009	--
11.2% EDTA	20 min	0.03±0.01	--
11.2% EDTA	30 min	0.036±0.02	--
11.2% EDTA	4 h	0.778±0.014	--
11.2% EDTA	16 h	2.33±0.9	+
10% HCl	1 min	3.6±0.08	++
10% HCl	2 min	7.4±1.1	+++
10% HCl	5 min	13.4±2.3	++++
12% TCA	5 min	2.55± 0.4	+
12% TCA	15 min	3.07±0.1	++
12% TCA	30 min	3.44±0.1	++

(n=4 for each group) using different acid etching procedures on erupted human teeth.

Table 2. Advantages e disadvantages of the different protein extraction procedures

Technique	Advantages	Disadvantages	Enamel Proteins identified
Enamel powder dissolution and protein precipitation with TCA	Larger amount of enamel proteins can be obtained (theoretically) since larger amounts of starting material is available	Larger volumes of starting material are difficult to handle during the cleansing and concentration steps (ZipTip).Information of different layers is lost	Amelogenin X isoform
Whole crown submersion in acid	Easy and low cost technique.Can be used to study enamel proteins in ancient teeth as well as lesions that affect superficial enamel, as enamel fluorosis	Does not reach deeper enamel proteins. However, if successive crown submersions are made, different layers of enamel will be reached.	Amelogenin, amelogenin X isoform, amelogenin Y isoform, enamelin and ameloblastin
Restricted area acid etching	Less invasive. Can be used in vivo to collect samples from the teeth of patients, may be useful in the characterization restricted area enamel defects.	Needs more technical skill when done in vivo. Does not reach deeper enamel proteins, but may reach depper layers if successive samples are obtained from the same site	Amelogenin, enamelin and ameloblastin.

CAPÍTULO 2

Recovery of immature and mature enamel proteins and protein identification by MALDI TOF/TOF MS

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Running title: Protein recovery from dental enamel

Abstract

The dental enamel is the most highly mineralized tissue in the body, with less than 1% protein when mature. Protein recovery from the enamel represents a promising possibility of gaining information about the past, but it is a challenging task. In this work we tested a whole crown micro-etch technique and a restricted area micro-etch technique to obtain superficial enamel samples from mature and immature enamel. These etching techniques were compared with the conventional enamel powder method used for protein extraction. Samples were analyzed by MALDI-TOF/TOF mass spectrometry, and the resulting spectra were searched against the Swiss-Prot protein database using the Mascot software for protein identification. The mass of enamel obtained by the restricted area micro-etch technique was also calculated, on the basis of the phosphorus content. The acid-etching procedures employed here are highly conservative, since they render masses of enamel samples ranging from 0.1 to 0.4 mg, and may be carried out superficially or at different depths within the enamel. Using our protocol, the separation of proteins in gel is not necessary. Results showed successful identification of specific enamel proteins after whole crown superficial enamel etching with 11% EDTA in the case of immature porcine samples, and with 10% HCl and 12% TCA precipitation in the case of mature human enamel. Among the recovered proteolytic fragments, it was possible to identify the X-isoforms of amelogenin, ameloblastin, and enamelin peptides. In conclusion, the techniques proposed here allowed the successful recovery of proteins specific to the dental enamel

from samples obtained in a very conservative manner, which may also be important in forensic and/or archeological science.

Keywords: enamel, MALDI-TOF, protein extraction, enamel proteins, amelogenin.

1. Introduction

The mature dental mature enamel is the most mineralized tissue in mammals, with 95% of mineral (weight %), less than 1% organic matter, and an even smaller amount of proteins, most of which are specific to the dental enamel (Smith, 1998). For comparison, bone is a mineral tissue that contains 70% of mineral (by weight) and around 28% of collagen type I (Nanci, 2008), the most abundant protein in vertebrates (Alberts et al., 2002).

Due to its characteristics, the enamel is highly inert to changes brought about by time and the environment, being a very important source of information for palaeo-, palaeanthropo-, and anthropologists. Indeed, these professionals use the morphological aspects of enamel to infer data on type of food ingested. Additionally, its histological aspects can provide useful information on many different physiological aspects of past species (Dean, 2006; Smith and Hublin, 2008). As recently stated, much of what is known about (human and primate) evolution derives from dental remains (Smith, 2008).

Proteins are the building blocks and the machinery of the organisms, and due to their different levels of expression, alternative splicing, and many possible post-translational modifications (Alberts et al., 2002), they are not directly related to the information conveyed in the DNA. Therefore, the search for information on proteins from past species is extremely important.

In this regard, an amazing achievement has been the publication of mass spectrometry data from bone extracts of an 80 million-year dinosaur (*Brachylophosaurus Canadensis*) (Schweitzer et al., 2009), describing the identification of eight total collagen peptide sequences, amounting to 149 amino acids of the collagen sequence. These collagen peptides were shown to contain OH-proline amino acids (post-translationally modified amino acid). The hypothesis that endogenous proteins can persist across geological time is still controversial, but data support the likelihood that protein remnants are preserved in deep burial in sandstone (Schweitzer et al., 2007). There are also theories that point to the importance of the "packaging" of mineral-bound biopolymers in bones for the long-term survival of proteins (Collins et al., 2000). A case in point is osteocalcin, a small protein that is strongly bound to collagen and mineral (Collins et al., 2000). The complete sequence of the protein osteocalcin (5.5 kDa) obtained by using small amounts (20 mg) of two bison bones older than 55 k-years has been reported (Nielsen-Marsh et al., 2002). In 2005, osteocalcin protein sequences were also reported from Neanderthalis dating back to ~75,000 years, and samples were also obtained from bone (Nielsen-Marsh et al., 2005).

Our hypothesis in the start of this study was that we could probably obtain enough protein from the superficial enamel by using superficial enamel etching with acidic solutions to recover enough protein for mass spectrometry and protein fingerprinting. In this way, we hoped that adequate signals for specific enamel proteins would be detected, which should provide reliable protein identification.

This study aimed at finding a method to obtain better quality samples for protein analysis from mature dental enamel. After the extraction, porcine and human enamel protein samples were analyzed using matrix-assisted laser desorption/ionization time-of-

flight mass spectrometry (MALDI-TOF/TOF MS). Results indicated that the micro-etch techniques tested here resulted in samples that were adequate for mass spectrometry and peptide fingerprinting, with successful identification of specific enamel proteins after whole crown superficial enamel etching.

2. Material and methods

2.1. Enamel samples

Porcine incisors were collected from two-month-old male pigs that were killed in local slaughterhouses for commercial purposes. Teeth were kept at -20°C until use. The incisors were washed with distilled water, to clean the tooth surface.

This study was approved by the Institutional Ethics Committee for Human Research (protocol number 2003.1.1329.58.2). Third molars were extracted in the Oral Surgery Clinic of the Dental School of Ribeirao Preto, SP, Brazil. The patients were informed both verbally and in writing about the purposes of the research, and signed an informed consent document and a tooth donation term. Teeth were stored at -20°C until processing.

Porcine teeth were selected for this study because they were not fully mature, so it was expected that it would be less difficult to recover enamel specific proteins from them. The use of protease inhibitors throughout this study was essential to avoid proteolysis, and the inhibitors employed here were Phenylsulfonylfluoride, N-Ethylmaleymide, and Phenanthroline, all purchased from Sigma-Aldrich (St Louis, MO, USA) as powders, and prepared as stock solutions in methanol, being diluted in buffer solution at the appropriate concentration (2 mM for all) just prior to use.

2.2. Protein Extraction Techniques

Enamel powder dissolution followed by precipitation with Trichloroacetic acid (TCA) The crowns of human third molars were powdered in a cryogenic mill Model MA 775 (Marconi, Piracicaba, SP, Brazil), using liquid nitrogen. The dentine was separated from the enamel by a classic density gradient fractionation procedure (Mainly and Hodge, 1939) using a 91% bromophorm/ 9% acetone mixture. After separation, the enamel and dentin powders were dried in a lyophilizer. Some samples were applied to glass slides and checked under a polarizing light microscope, confirming the identity of enamel and dentin, and the complete separation of the two tissues.

The enamel powder was dissolved in 12% trichloroacetic acid (TCA) in a proportion of 200 µL acid/ mg enamel powder. One hour later, sodium deoxycolate was added in a final concentration of 200µg/mL, as described earlier (Porto et al., 2006). After enamel dissolution at 0°C, the solution was centrifuged at 2500 x g at 4°C for 45 min. The pellet was ressuspended in 200µL of a 6M urea solution containing a wide range of protease inhibitors, as described before. In some samples, the centrifugation was performed using microcentrifuge filter of 30kDa-cutoff (Ultrafree-MC microcentrifuge filters, M0536, Sigma-Aldrich, USA) for 30 min, 3500 x g, at 4°C.

Whole crown etching Porcine teeth (which were not fully mature) were only etched using ethylenediamine tetraacetic acid (EDTA), which is the most gentle acid solution routinely used to decalcify tissues in Histology labs. This procedure resulted in adequate amounts of proteins for the identification of specific peptides from enamel protein after MALDI-TOF/TOF-MS analysis. The whole tooth crown was submersed in 2 mL of a solution of 11.2% EDTA and 1.25% sodium hydroxide, pH 7.3, for 5 min. The solution was transferred to a microcentrifuge filter of 30kDa-cutoff (Ultrafree-MC microcentrifuge

filters, M0536, Sigma-Aldrich, USA) and centrifuged for 30 min, 3500 x g, at 4°C. The solution that passed through the filter and proteins below 30kDa were collected in the "flow-through". The acid and salts were removed using a microtip filled with Poros 50 R2 resin (PerSeptive Biosystems, Framingham, MA) (the proteins were retained through binding to a porous resin), eluted with 50% acetonitrile/ 0.2% formic acid, and dried in a Speed Vac (AS 290, Savant, Rochester, New York, USA) for 30 min.

At first, mature human third molars were also etched with various concentrations of EDTA (different times); however, no peptide signal that resulted in enamel specific protein could be retrieved, so stronger acids were tested. Hydrochloride acid (HCl) resulted in an appropriate amount of dissolved enamel to retrieve enamel-specific proteins. The whole tooth crown was submerged in 1 mL of 10% HCl containing proteinase inhibitors for 5 min. The samples were transferred to 30000WM filter tubes and processed as described above for the porcine teeth.

Restricted area etching This etching technique was tested in both porcine (immature enamel) and human teeth (mature enamel), and allowed the sampling of enamel from a specific area "restricted" by a circular hole in a tape. The procedure described by Costa de Almeida et al. (2009) was followed. Briefly, a circular hole (2.0 mm diameter) was punched in an adhesive tape, which was applied to the surface of the teeth. A window of 3.14 mm^2 area was exposed, and 5 µL 11.2% EDTA/1.25% NaOH, pH 7.3, were applied for 15 s. The extract was transferred to a low protein binding tube. This etching step was repeated twice, and the etching solutions were added to the tube. At the end, 5 µL of distilled water were applied to the tape window for 5 s and added to the same tube, which contained approximately 20 µL water.

2.3. Determination of phosphorus

For the human samples obtained by whole crown etching, the amount of phosphorus was determined, so as to calculate the "approximate" mass of enamel obtained by the different acids employed here (12% TCA, 11.2% EDTA, and 10% HCl). We use the term "approximate" because the precise superficial area that was etched cannot be directly measured, and therefore the depth cannot be considered a precise value, although the expected error should not exceed ±10%. We decided to check the mass of enamel by this chemical procedure because most of the samples did not furnish good signals (except those obtained by etching in concentrated HCl). Phosphorus was determined by the colorimetric method of Fiske and Subbarow (1925), as described in Costa de Almeida et al. (2007). Assuming an enamel density of 2.95 g cm^{-3} and a phosphorus content of 17.4% (Koo and Cury, 1998), and accepting an average presumed area of the tooth crowns used, we were able to calculate the presumed depth of the etching procedures using the different acid solutions.

2.4. Electrophoresis and digestion of proteins from gel bands

Protein solutions from the porcine teeth obtained by the restricted area etching procedure and protein solutions from the human teeth obtained by whole crown superficial enamel acid dissolution were employed in these analyses. Twenty μL of sodium dodecyl sulfate (SDS)-reducing buffer (0.06M Tris-HCl, pH6.8, 2% SDS, 10% glycerol, 0.025% Bromophenol Blue, 5% 2-mercaptoethanol) were added to the samples, which were heated to 95°C for 4 min. Samples were then run by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) on a 15% gel with a discontinuous buffer system (Laemmli, 1970). The gel was stained with mass-spectrometry-compatible silver staining (Simpson, 2003). A protein

standard solution was used (Page Ruler ^{TM} Plus Prestained Protein Lader, Fermentas Life Sciences, Glen Burnie, Maryland, USA). The gel was photographed, and bands of different molecular weights were excised from the gel.

The procedure described by Gharahdaghi et al. (1999) was employed to destain and dry the gel bands. The bands were destained with 30 mM potassium ferricyanide and 100 mM sodium thiosulfate in a 1:1 proportion and dried in a SpeedVac (AS 290, Savant) for 10 min. The gel bands were then soaked in a 200 mM solution of ammonium bicarbonate for 20 min. This solution was removed, and pure acetonitrile was added until gel bands were completely dehydrated (dehydrated gel bands had the aspect of opaque white paper). Residual acetronitrile was removed by evaporation to dryness in the SpeedVac (AS 290, Savant) for 30 min.

The gel bands were rehydrated with a 100 mM ammonium bicarbonate solution containing 0.5 µg of trypsin (MS grade, Promega, Madison, WI), at 37°C, for 22 h. The reaction was stopped with 5 µL of formic acid. The supernatant was concentrated and desalted by means of a microtip filled with Poros 50 R2 resin, previously activated with methanol, equilibrated in 3 x 150 µL of 0.2% formic acid, followed by sample loading and further 3 x 150 µL of 0.2% formic acid for desalting. The peptides were eluted from the microtip in 30 µL of 60% methanol prepared in 5% formic acid (v/v), being then ready for mass spectrometry analysis.

2.5. Digestion of proteins

Whether dissolved enamel samples (obtained by either the biopsy technique or whole crown submersion in acid) could be directly used for MALDI-TOF analysis (without previous separation in an SDS-PAGE gel) was tested.

Initially, samples were heated for 5 min at 100°C. Then, 60 μL of 100 mM ammonium bicarbonate and 0.5 μg trypsin were added, and the mixture was incubated for 22 h at 37°C. When no peptide signal was obtained, samples were processed by another procedure prior to trypsin digestion, using reduction/alkylation of proteins, performed by ressuspending the dry protein in 20 μL of 50% acetonitrile / 100 mM ammonium bicarbonate. Next, 5 μL of 45 mM dithiothreitol (DTT) were added for 1 h at 56°C. After that, 5 μL of 100mM iodoacetamide were added for 1 h in the dark, at room temperature. The samples were diluted 5 times with 100 mM ammonium bicarbonate, 0.5 μg of trypsin were added, and trypsin hydrolysis was carried out for 22 h at 37°C. Finally, 5 μL of formic acid were added to quench the reaction. The supernatants were desalted using a microtip filled with Poros 50 R2 resin, according to the procedure described above.

Mass Spectrometry using matrix assisted laser desorption/ionization – time- of-flight mass spectrometer (MALDI-TOF/TOF)

The samples were redissolved in a CHCA-5mg/mL (alfa-cyano- 4-hydroxicinnamic acid, Sigma, USA) matrix with 50% acetonitrile/ 0.1% TFA (trifluoroacetic acid), crystallized in MALDI target, and mass spectrometry was accomplished in MALDI-TOF/TOF Axima Performance (Kratos-Shimadzu Biotech, Manchester, UK). The mass spectrometer was calibrated with external calibration using synthetic peptides (ProteoMass Peptide and Protein Maldi-MS Calibration Kit, Sigma-Aldrich, St.Louis, MO). The mass accuracy was less than 50 ppm. Peptides were analyzed for mass precursor and ion precursor selection by data-dependent acquisition following high energy collision induced dissociation (CID), to produce product ions of each precursor ion, which were then used for amino acid sequence determination. The CID-MS/MS spectra were submitted to a database

search against Swiss-Prot using the MASCOT software version 2.2.4 (Matrixscience, London, UK).

3. Results

Our results indicate that the superficial enamel micro etching and enamel powder dissolution techniques tested here are very effective in generating adequate samples for mass spectrometry. The acid-etching procedures employed here resulted in masses of enamel samples ranging from 0.1 to 0.4 mg (when HCl was used), based on the phosphorus content obtained in the samples.

Figure 1 shows one representative SDS-PAGE gel in which human mature superficial enamel samples, extracted by whole crown etching with EDTA, were run and stained with silver nitrate, which only rendered faint bands at high molecular weights (as expected for mature enamel). Some of these bands were excised from the gel and analyzed by MS and MS/MS. While high molecular bands resulted in keratin and albumin signal, no enamel proteins were identified from such gel bands obtained from superficial mature human enamel samples separated in gels.

In the case of porcine teeth enamel samples, the SDS-PAGE gel revealed a band pattern compatible with amelogenin proteins below 27 kDa (not shown). The aminoacid analysis of these bands < 27 kDa confirmed the presence of amelogenin peptides in those gel bands (not shown). Other contaminants like keratin and albumin were also identified in high molecular mass gel bands (not shown). Enamelin was detected in one sample from a high molecular weight band (not shown).

MS graphs shown in Figures 2 to 4 were obtained from the direct application of purified superficial enamel samples from immature porcine and mature human enamel without separation of the samples in the gel. Results demonstrate that it is feasible to obtain enough protein material of < 30 kDa proteins that can be easily prepared for MS and MS/MS, rendering very good enamel protein peptide signals (Figures 2 to 4).

When the dental crown of the immature porcine teeth was submerged in EDTA, a good signal was obtained, indicating that more ions and more proteins were identified. The search for peptides generated by trypsin digestion resulted in the identification of two peptides of enamelin and one peptide of a basic proline-rich protein (not shown). The search for all peptides resulted in the identification of four peptides of ameloblastin and two peptides of enamelin (Figure 3).

Although only some MS graphs are shown, it is very interesting that the tested procedures and the described technique furnish very good MS signals by means of a procedure that is much easier than the ones described so far. Even the peptide specific to the amelogenin X-isoform was obtained (not shown), indicating that it may be possible to look for this peptide in such superficial enamel extracts as a means to determine sex using teeth of individuals that died a long time ago.

4. Discussion

Whole crown etching with HCl and enamel powder dissolution and precipitation with TCA were the techniques that led to the best mature enamel samples in terms of good MS signals, thereby enabling identification of specific enamel proteins. Nonetheless, etching with EDTA also furnished immature porcine enamel samples that were adequate

for MS analysis. This technique employing EDTA may find use in investigations on immature enamel. Therefore, the decision about which acid to use during enamel protein recovery will depend on the intended use.

Glycerol and other agents may be used to restrict the acid attack, since glycerol changes the superficial tension of the solution. We usually use glycerol (70%, v/v) to obtain clinical samples from deciduous teeth *in vivo* (Gomes et al., 2004; Costa de Almeida et al., 2007; de Almeida et al., 2008; Costa de Almeida et al., 2009). However, in this study glycerol was not employed in the etching solutions because it interferes in the drying of the MALDI/TOF matrix, and should therefore be avoided in samples intended for mass spectrometry.

Studies on the enamel of continuously growing teeth may also benefit from the possible recovery of proteins from restricted areas of the enamel, both of contemporary (Porto et al., 2009) or past species (Fraser et al., 2008). In this regard, it is interesting that the ratios of carbon isotopes, formed at different times, clearly preserves seasonal changes in diet and local environmental conditions, and such restricted area techniques may facilitate sampling.

Enamel also contains some proteins that remain after maturation (for instance, tuftelin and enamelin fragments, please find details in Bartlett and Simmer, 1999). Some enamel proteins are tightly bound to the enamel crystals (Aoba et al., 1987), deserving to be further studied for their preservation state for protein analysis in tooth remnants. This is particularly relevant because of the amazing protein data obtained from osteocalcin (Nielsen-Marsh et al., 2002).

Another obvious interesting feature of the enamel is the fact that it is produced over many years; therefore, isotopic signatures found in the enamel formed at different times have the potential to unravel information about some of the components of the diet since birth up to the age of ~17 years (in contemporary humans).

An amazing possibility in the study of past species is the use of direct data from tissue samples derived from stable isotope mass spectrometry, which can be accomplished using enamel (Sponheimer and Lee-Thorp, 1999; Lee-Thorp et al., 2007). The C and O isotopic signatures found in the dental enamel may be complimentary to the ones found in bone, aiding determination of important aspects of the diets of animals and hominids (Lee-Thorp and Sponheimer, 2006). While such isotopic signatures of enamel apatites can be reliably followed in hominid fossils aged 2-3 million, bone apatite isotopes can be only reliably followed up in 200,000-year fossils (Lee-Thorp and Sponheimer, 2006). While stable ratios of C isotopes render information on the consumed vegetation (Sponheimer and Lee-Thorp, 1999), stable ratios of O isotopes in mammalian tissues inform on environmental conditions (Smith et al., 2010). Stable N isotopes furnish information on the proteins in the diet (Smith et al., 2010) and may shed light on aspects like breastfeeding. So far, only C and O isotopes have been determined in enamel remains (Wright and Schwarcz, 1999), with all the information on N isotopes having been derived from bone and dentin (Wright and Schwarcz, 1999; Smith et al., 2010). Therefore, the ability to recover protein from the enamel could enhance the quality of the data on protein in the diet, since (theoretically) the enamel can harbor preserved protein for a much longer time.

The possible recovery of well preserved protein fragments from the enamel may open new perspectives for the use of a tissue that contains preserved chemical and time

information in its very structure. Protein has not yet been adequately searched for in the enamel.

The new techniques described in our study may also be important for sex determination in forensic science because different isoforms of amelogenin are encoded by the X and Y chromosomes, which is the basis for its large use in sex determination by PCR (Steinlechner et al., 2002).

The prospect of obtaining enamel samples from different layers of the enamel may also facilitate analysis of microelements in archeological remains. Ratios between strontium isotopes have already been determined in enamel samples (Tykot, 2004).

5. Conclusion

In conclusion, the micro-etch procedures described here are very conservative, removing a very superficial layer of the enamel only. They are also very effective in providing enamel samples that are adequate for protein analysis, greatly reducing the necessary starting mass of enamel. These techniques described in our study may also be important in forensic and/or archeological science.

Acknowledgements

We gratefully acknowledge the support of the State of São Paulo Research Foundation (FAPESP) and the (Brazilian) National Research Council (CNPq).

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Figures

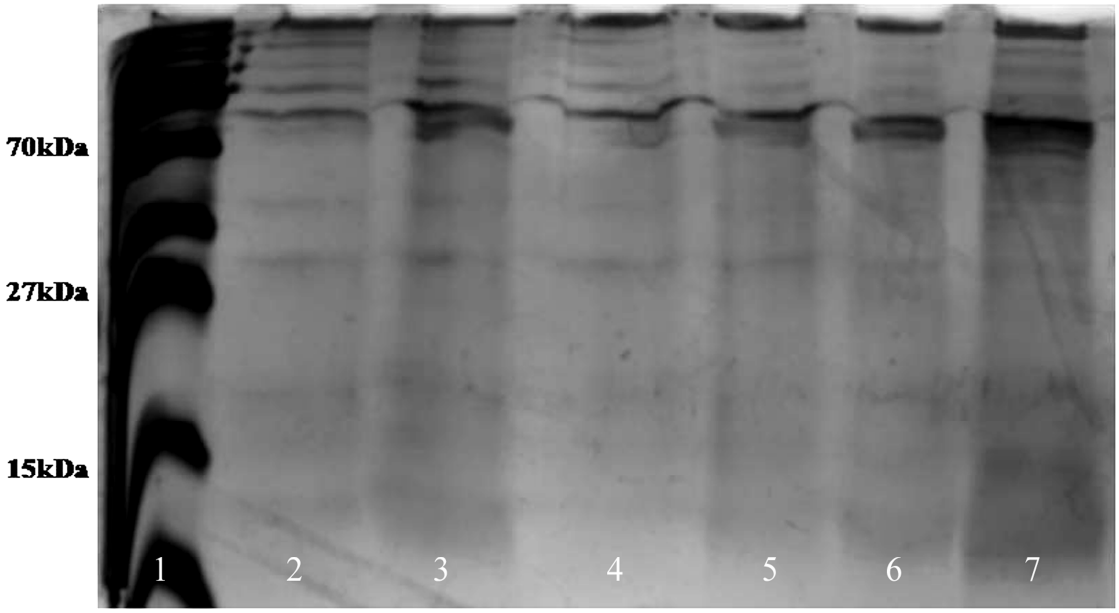


Figure 1. Silver-stained SDS-PAGE protein profile of human mature teeth. Lane 1: molecular weight marker. Lane 2-7: proteins extracted from human teeth (with mature enamel) by whole crown etching with 11.2% EDTA for 5 min.

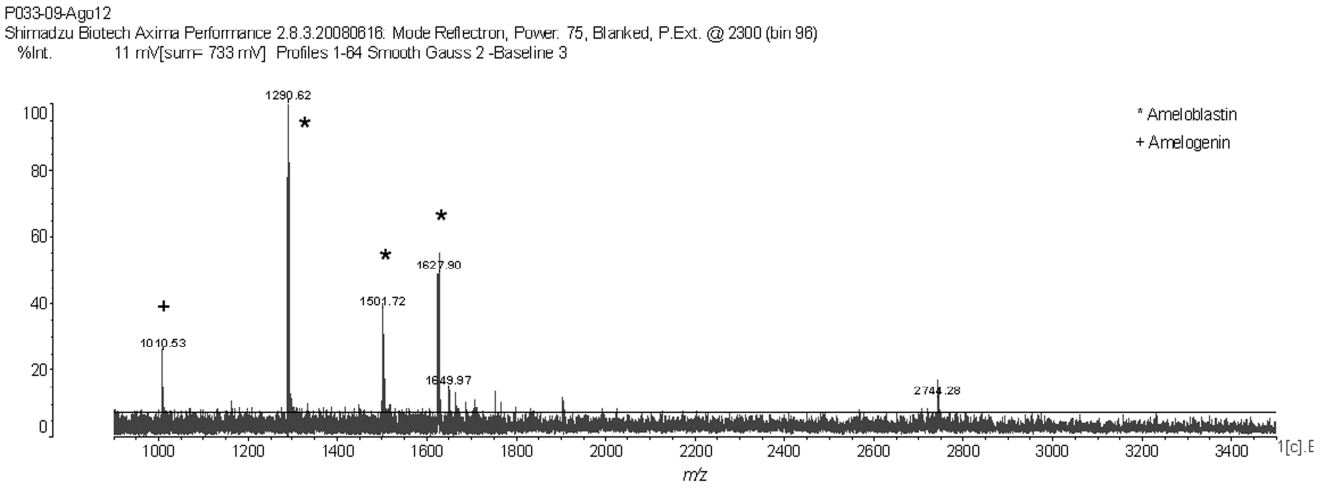
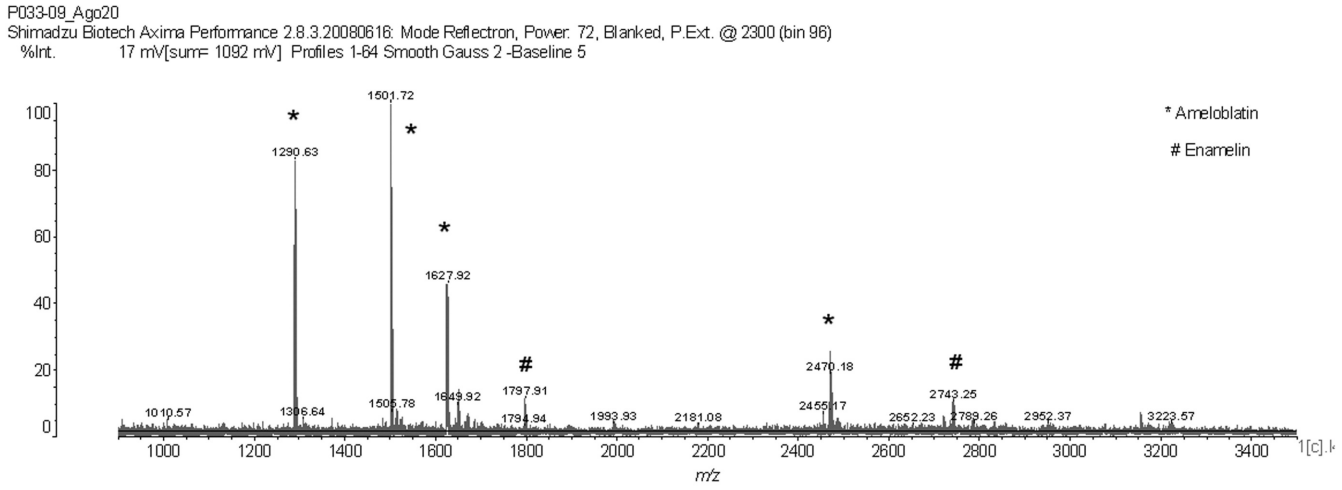


Figure 2. MS spectrum resulted in the identification of ameloblastin (*) and amelogenin (+) peptides in a porcine enamel sample obtained by the restricted area etching procedure directly submitted to mass spectrometry (without protein separation in an SDS-PAGE gel).

A



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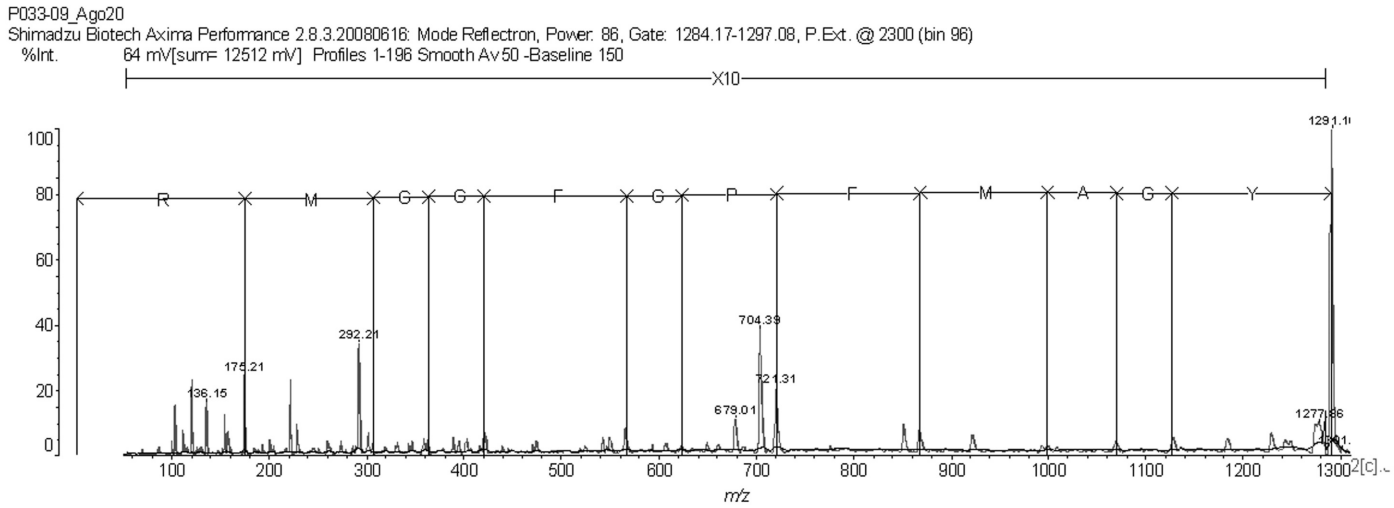

Figure 3. MS analysis of superficial enamel samples obtained from immature porcine teeth by whole crown etching with 11.2% EDTA for 5 min. A) Ameloblastin (*) and enamelin (#) were identified by mass spectrometry. B) Mass spectrum of CID-MS/MS of m/z 1290.63 of the ameloblastin peptide, which allowed the deduction of the amino acid sequence YGAMFGGMR.

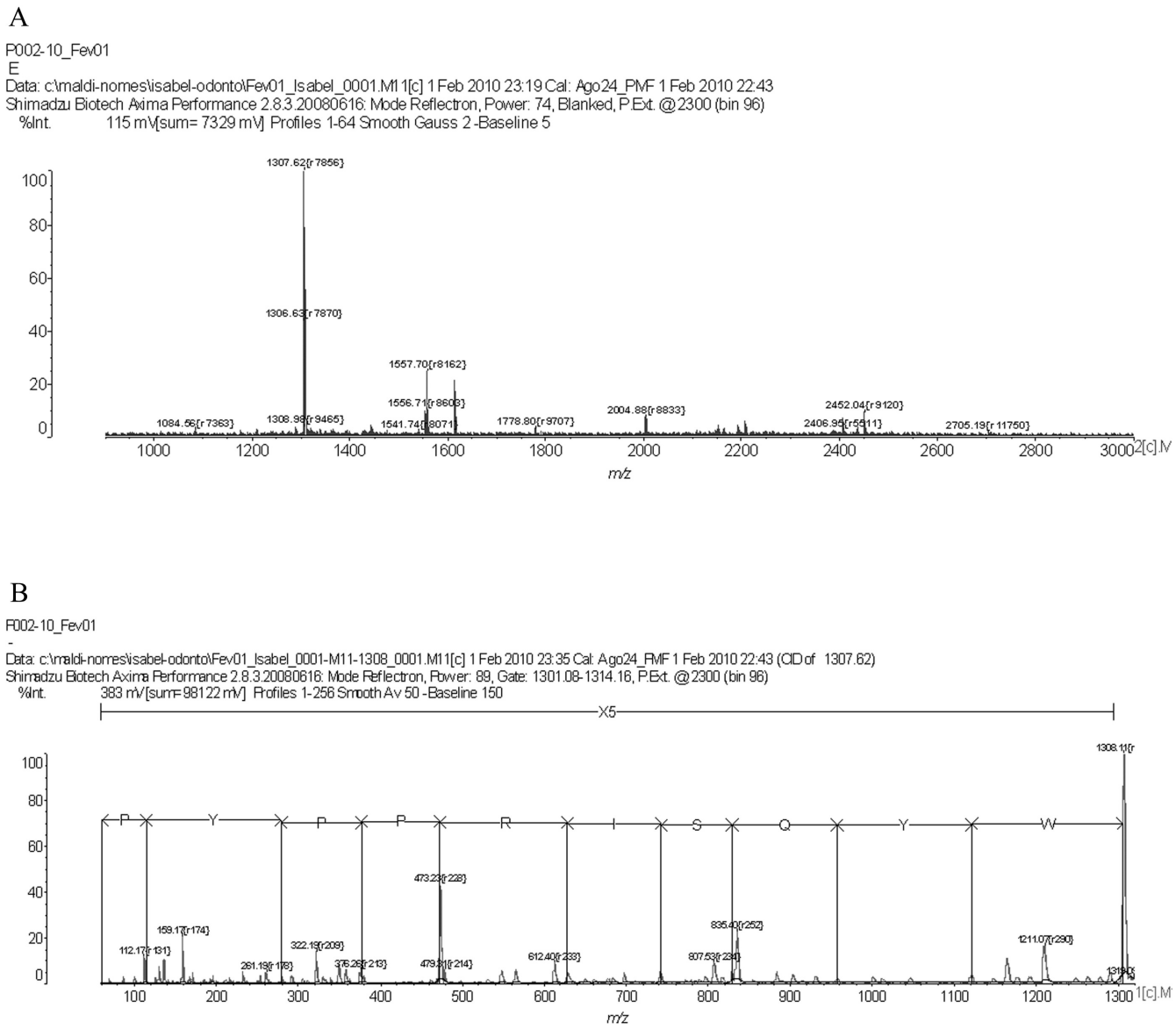

Figure 4. MS analysis of enamel extracted from mature human teeth. A) MS spectrum of enamel powder of mature human teeth obtained by TCA precipitation. The samples were not separated by SDS-PAGE, but directly prepared for MS. Three Amelogenin –X isoforms tryptic peptides were identified. B) Mass spectrum of CID-MS/MS of ion m/z 1307.62 of the amelogenin peptide, which allowed deduction of the amino acid sequence of an amelogenin –X isoform peptide with the amino acid sequence WYYQSIRPPYP.

CAPÍTULO 3

Amino acids profile in erupted and non-erupted human fluorotic mature teeth analyzed by MALDI TOF/TOF MS

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Running title: Amino acids profile in human fluorotic teeth

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Abstract

Excess of fluoride causes a disease called fluorosis that affects and cause a hypomineralization of the enamel. Some studies showed that fluoride alters the removal of enamel proteins, but other studies did not demonstrated any alteration in protein content or in enamel proteinases between fluorotic and control enamel. The aim of this study is analyze the amino acid profile in erupted and non-erupted fluorotic human teeth compared to control ones, using a superficial etching technique to extract only proteins of enamel surface where the retention of proteins in fluorosis is higher. No differences were found in protein profile between fluorotic and control teeth. In this study the cleavage of amelogenin was not affected by fluoride in fluorotic teeth. Possibly, the hydrolysis of amelogenin is not affected, but maybe the peptides originated by this cleavage cannot leave the enamel.

Introduction

Excess of fluoride causes a disease called fluorosis that affects and cause a hypomineralization of the enamel (Saiani et al., 2009; Yanagisawa et al., 1989). Some studies showed that fluoride alters the removal of enamel proteins, either by modification of proteins or by decreasing the secretion and activity of proteinases (Den Besten, 1986; DenBesten and Heffernan, 1989; Wright et al., 1996) The retention of these proteins occurs in superficial fluorotic enamel (Porto et al., 2010a; Saiani et al., 2009). But other studies did not demonstrated any alteration in protein content or in enamel proteinases between fluorotic and control enamel (Gerlach et al., 2000). Eastoe and Fejerskov (1984) (Eastoe and Fejerskov, 1984) showed that human fluorotic enamel showed an increased proportion of proline rich protein like amelogenin than control enamel, while Wright et al (1996) (Wright et al., 1996) showed that amino acids profile were similar for fluorosed or normal enamel. Furthermore, these two studies used erupted teeth for enamel protein analysis, and therefore the presence of salivary proteins cannot be ruled out. Since the proposed mechanism for enamel fluorosis involves the inhibition of the activity of enamel proteinases, the information on the peptides present in unerupted fluorotic enamel is very relevant, since uncut peptides might be a proof that proteolysis did not occur as in control enamel. So far, no study has compared the peptide sequence of the polypeptides found in the fluorotic enamel. Thus, the aim of this study is analyze the amino acid profile in erupted and non-erupted fluorotic human teeth compared to control ones, using a superficial etching technique to extract only proteins of enamel surface where the retention of proteins in fluorosis is higher.

Material and Methods:

Enamel samples

This study was approved by the Ethics Committee for Human Research (protocol number 2003.1.1329.58.2). The fluorotic third molars were extracted in the city of Venâncio Aires, State of Rio Grande do Sul, Brazil, in a district where severe fluorosis (water containing approximately 8 ppm of fluoride) (Marimon et al., 2007b) and the control teeth were extracted in the city of Ribeirão Preto, State of São Paulo, Brazil, at the Oral Surgery Clinics of the Dental School of Ribeirão Preto – University of Sao Paulo (Ribeirao Preto, SP, Brazil). The patients were informed both verbally and in writing about the purposes of the research, and signed an informed consent document and a tooth donation term. Teeth were stored at -20°C until processing.

Protein extraction and analysis in Mass Spectrometry using matrix assisted laser desorption/ionization – time- of- flight mass spectrometer (MALDI/TOF)

The whole tooth crown was submerged in 1 mL of 10% hydrochloride acid (HCl) containing proteinase inhibitors for 5 min. The solution was transferred to a microcentrifuge filter of 30kDa-cutoff (Ultrafree-MC microcentrifuge filters, M0536, Sigma-Aldrich, USA) and centrifuged for 30 min, 3500 x g at 4°C. The solution that passed through the filter, and proteins below 30kDa were collected in the "flow-through The acid and salts were removed using microtip filled with Porous 50 R2 resin (PerSeptive Biosystems, Framingham, MA), eluted with 50% acetonitrile/0.2% formic acid and dried in a Speed Vac (AS 290, Savant, Rochester, New York, USA) for 30 min. The reduction/alkylation was performed and samples were diluted 5X with 100 mM ammonium bicarbonate and 0.5 μg of trypsin were added and left for 22 h at 37°C. The supernatants

were desalted using a micro-tip filled with Porous 50 R2 resin, and dried for 10 min under vacuum. The samples were then inserted in the mass spectrometry (MS) and tandem mass spectrometry (MS/MS) analysis and results were analyzed in the Mascot program using Swiss-Prot data bank.

Results

No differences were found in protein profile between fluorotic and control teeth.

In non-erupted fluorotic teeth two peptides of amelogenin –X isoform were observed in MS analysis (Fig. 1B), which amino acid sequences are K.WYQSIRPPYP.S and A.MPLPPHPGHPGYINF.S, when MS/MS analysis was done. These peptides are the same of non-erupted control teeth (Fig. 1A).

In erupted teeth, fluorotic group showed one peptide of amelogenin –X isoform in MS analysis (Fig. 2B), which had amino acid sequence K.WYQSIRPPYP.S, the same peptide and sequence observed in the control group (Fig. 2A)

Discussion:

Mature enamel contains less than 1% of protein (Smith, 1998b). This factor makes the protein extraction and characterization a challenge. We have been studied techniques to extract enamel proteins for a better characterization of these proteins. In a previous study, we developed a method to extract proteins from mature enamel highly efficient and very easy (Porto, I.M., personal communication). The fluorotic lesions cause aesthetic and morphological problems and teeth and the retention of proteins in fluorotic enamel is still controversial. In others studies we demonstrated that there is the retention of enamel protein

in the surface of the fluorotic enamel in rat teeth and human teeth, both erupted and non-erupted teeth. Here in this study, we used a superficial etching technique to extract protein from enamel surface developed by us (Porto, I.M., personal communication), but we not observed any differences in the peptide sequences. Previous study (Nagano et al., 2009) demonstrated that the peptide sequence A.MPLPPHPGHPGYINF.S is derived by kallikrein-4 (KLK-4) cleavage of tyrosine-rich amelogenin polypeptide (TRAP) and leucine-rich amelogenin protein (LRAP) in porcine enamel. The other peptide sequence that we achieved K.WYQSIRPPYP.S is alike the other sequence (K.WYQNMIR.H) showed by Nagano et al. (2009) (Nagano et al., 2009) resulted of TRAP and LRAP cleavage by KLK-4. Due the low content of protein in mature enamel, probably these two sequences that we observed should be the more abundant in mature enamel both as control as fluorotic teeth.

Despite our previous studies showed that there is a higher content of protein in superficial fluorotic enamel when compared to control enamel (Porto et al., 2010; Saiani et al., 2009), in this study the cleavage of amelogenin was not affected by fluoride in fluorotic teeth. Possibly, the hydrolysis of amelogenin is not affected, but maybe the peptides originated by this cleavage cannot leave the enamel.

Acknowledgements

The authors state that there is no possible conflict of interest such as patent, ownership, stock ownership, consultancies or speaker's fee. This study was supported by The State of Sao Paulo Research Foundation (FAPESP) and the (Brazilian) National Research Council (CNPq).

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Figures

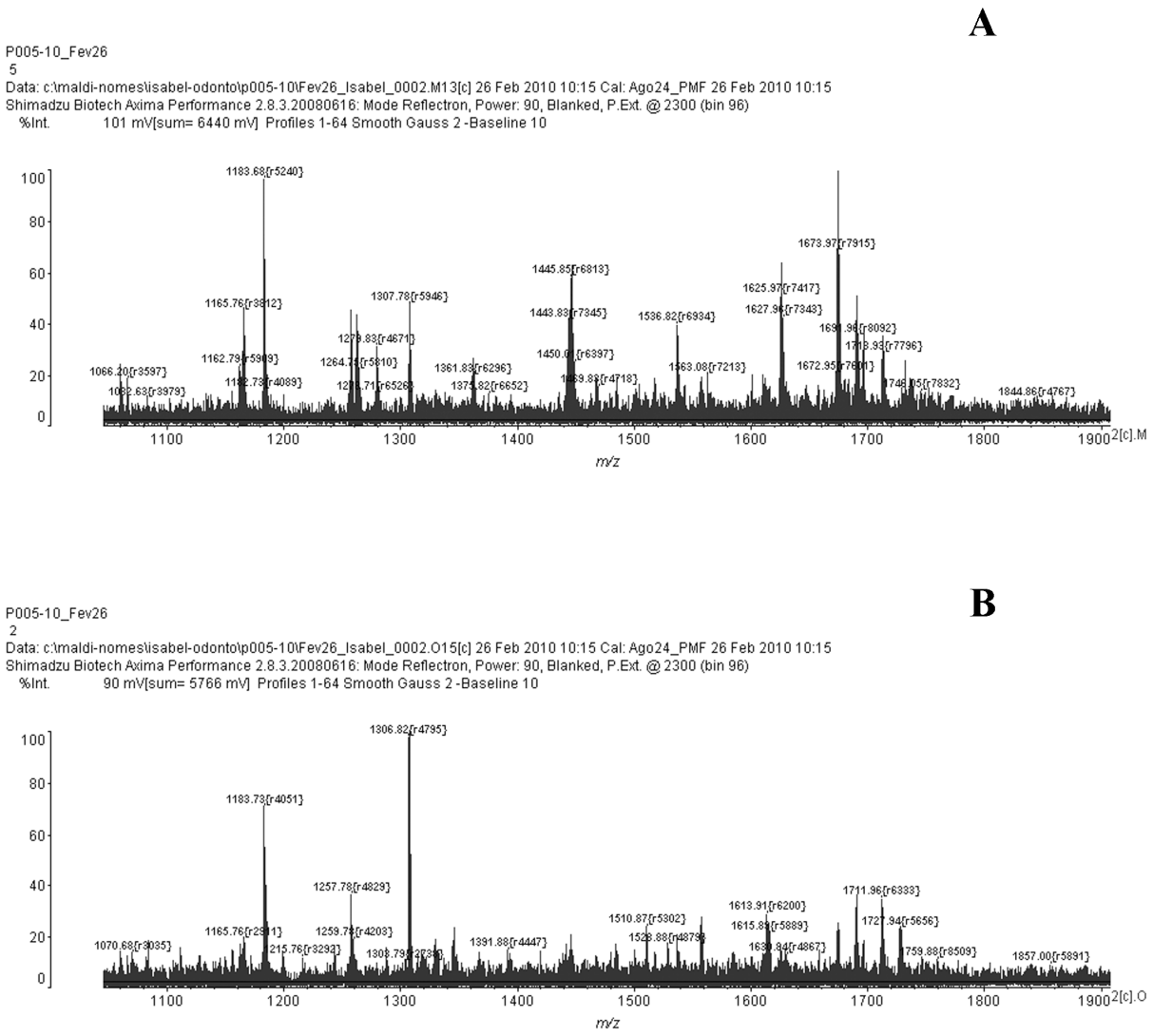

Figure 1. MS spectrum of a superficial enamel extract of mature human teeth. A) Non-erupted control teeth. Two Amelogenin –X isoform non tryptic peptides were identified (peaks *m/z* 1308.52 and 1674.92, peptide sequences K.WYQSIRPPYP and A.MPLPPHPGHPGYINF.S respectively). B) Non-erupted fluorotic teeth. Two Amelogenin –X isoform non tryptic peptides were identified (peaks *m/z* 1307.56 and 1690.92, peptide sequences K.WYQSIRPPYP and A.MPLPPHPGHPGYINF.S respectively).

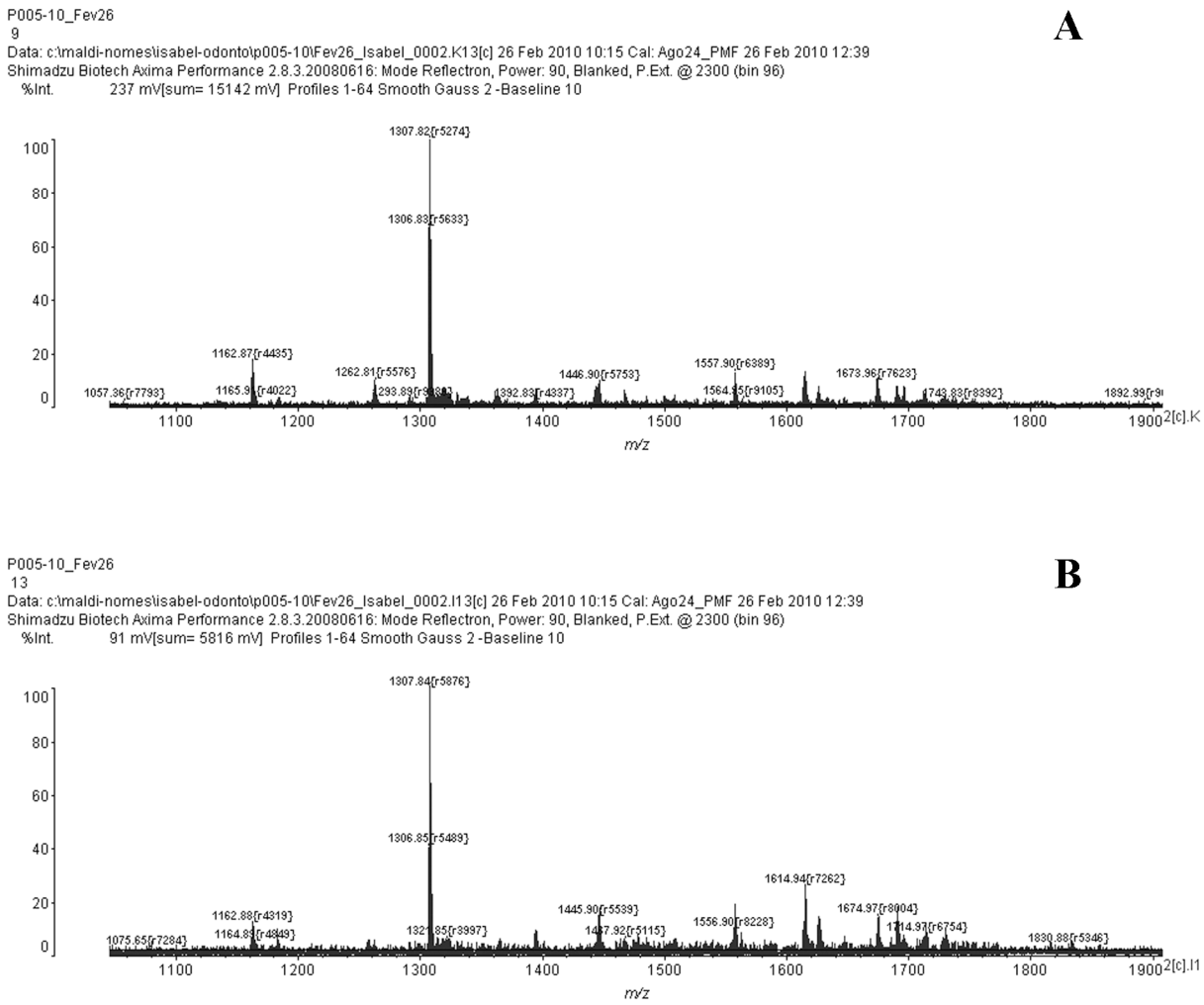

Figure 2. MS spectrum of a superficial enamel extract of mature human teeth. A) Erupted control teeth. One Amelogenin –X isoform non tryptic peptide was identified (peak *m/z* 1308.56, peptide sequences K.WYQSIRPPYP). B) Erupted fluorotic teeth. Two Amelogenin –X isoform non tryptic peptides were identified (peaks *m/z* 1308.53 and 1615.80, peptide sequences K.WYQSIRPPYP and A.MPLPPHPGHPGYINF.S respectively).

CAPÍTULO 4

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Manuscript ID:	JDR-10-0369
Title:	Fluorotic enamel composition reflects the maturation stage of enamel formation
Authors:	Porto, Isabel Sousa, FREDERICO Cury, Jaime Souza, Claudia Perez, Walter Kazarian, Sergei Bachmann, Luciano Gerlach, Raquel
Date Submitted:	03-Jul-2010

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Fluorotic enamel composition reflects the maturation stage of enamel formation

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Short title: Fluorosis reflects the maturation of enamel formation

Key words: fluoride, FTIR, BSE-SEM, microradiography, polarized light microscopy

Number of words in the abstract: 149.

Number of words in the abstract and the text: 2498.

Number of tables and figures: 4 figures.

Number of cited references: 20.

ABSTRACT

Since fluoride delays enamel maturation, we hypothesized that the composition of fluorotic lesions may resemble the enamel found during last wave of enamel mineralization. To test this hypothesis, spatially resolved quantitative data on mineral, organic, and water contents were measured in ground sections of erupted and nonerupted human normal (n=7) and fluorotic (n=7) teeth using polarized light microscopy, backscattered electrons scanning electron microscopy, microradiography, and Fourier Transform Infrared Spectroscopy (FTIR). Non-erupted and erupted fluorotic teeth presented hypomineralized areas in the outermost enamel (67.9% and 74.0% mineral content, respectively) /versus/ controls (97.5%) ($P > 0.05$). Water and organic matter contents were higher on the enamel surface, with fluorotic teeth exhibiting 13.4% (erupted) to 19.8% (non-erupted) of organic matter compared to the control (< 1%). The determination of these values for organic matter is novel. Data suggest that human fluorotic enamel lesions reflect the enamel composition of the late maturation stage of enamel formation.

INTRODUCTION

Excess fluoride causes enamel lesions known as enamel fluorosis. Fejerskov *et al* (1977) described the fluorotic defects in polarized light and showed that there is a subsuperficial hypomineralization, whereas the outermost enamel (about 30 μm) is normal (Eastoe and Fejerskov, 1984; Fejerskov et al., 1977). The fluorotic enamel has lower mineral content and higher protein content compared to the normal enamel (Den Besten, 1986; DenBesten and Heffernan, 1989; Shinoda, 1975; Shinoda and Ogura, 1978; Triller, 1979; Wright et al., 1996; Zhou et al., 1996). Post-eruptive changes alter both the organic and mineral content of the fluorotic enamel lesion, so the precise description of the biochemical components of fluorotic lesions that have never been exposed to the oral environment is still very relevant.

KLK-4 activity is essential for normal amelogenesis. Decreased levels of KLK-4 expression have been detected in enamel organ cells of rats exposed to 100 and 150 ppm fluoride (Sharma et al., 2010). Maturation stage ameloblasts probably have other important functions besides KLK-4 secretion, such as control of calcium influx (Smith, 1998a). A study found that enamel maturation does not take place when maturation stage ameloblasts are surgically removed, and the final mineral content of the enamel is only around 70% (Porto et al., 2009). The mechanism proposed for enamel fluorosis hypothesizes that fluoride toxicity would induce cell stress (Sharma et al., 2010), to which maturation stage ameloblasts are more sensitive than secretory stage ameloblasts, because of the low extracellular pH (Sharma et al., 2010). This mechanism nicely explains the long-known fact that fluoride predominantly affects maturation stage enamel (Sharma et al., 2010), but it also suggests the possibility that the histopathological appearance of the fluorotic lesions

would rather resemble enamel defect due to lack of completion of the final maturation stage. Such defects have been described in detail by Suga (1989), who carried out microradiography of ground sections. The final wave of mineralization has been reported to move from the inner enamel layer toward the surface of the enamel (Suga, 1989). Ground sections of rat incisor fluorotic lesions revealed that incisor fluorotic lesions are also superficial in the rat (Saiani et al., 2009), and not subsuperficial (Angmar-Mansson and Whitford, 1982). Such superficial lesions might indicate that enamel fluorosis indeed results from the direct toxicity of fluoride to the maturation stage ameloblasts, which are then not functional to regulate the final maturation of the enamel. The histopathological aspect and the determination of organic and mineral contents of the fluorotic enamel lesions with spatial resolution are thus very important for elucidation of the mechanism of fluorosis.

We hypothesized that the spatial distribution of biochemical contents in enamel fluorotic lesions resembles the one found in the last wave of mineralization during the maturation stage. To test this hypothesis, spatially resolved quantitative data on mineral, organic, and water contents were measured in ground sections of human fluorotic enamel from erupted and nonerupted teeth with normal and fluorotic enamel.

MATERIALS & METHODS

Tooth Extraction

This study was approved by the Ethics Committee for Human Research (protocol number 2003.1.1329.58.2). The fluorotic third molars (n=7 teeth) were extracted in the city of Venâncio Aires, State of Rio Grande do Sul, Brazil, in a district where severe fluorosis

had been described and which was due to the use of water containing approximately 8 ppm of fluoride (Marimon et al., 2007a). The control teeth (n=7) were extracted in the city of Ribeirão Preto, State of São Paulo, Brazil, at the Oral Surgery Clinics of the Dental School of Ribeirão Preto – University of Sao Paulo (Ribeirao Preto, SP, Brazil). Only patients for whom removal of the third molar was indicated for other reasons were recruited for this study. The patients were informed both verbally and in writing about the purposes of the research, and signed an informed consent document and a tooth donation term. Teeth were stored at -20 °C until processing.

Macroscopic Analyses

Teeth were scored for fluorosis degree, using the Thylstrup & Fejerskov (TF) index (1978) (Thylstrup and Fejerskov, 1978), and root formation stage. Teeth were also photographed. Please see Supplementary Data.

Determination of Fluoride Ion

Dentine fluoride concentration was determined by means of a specific electrode coupled to an ion analyzer. Please refer to Supplementary Data.

Fourier Transform Infrared (FTIR) Spectroscopy

Six FTIR measurements were performed on the enamel of two non-erupted fluorotic teeth. Please see Supplementary Data.

Backscattered Electrons Scanning Electron Microscopy (BSE-SEM) and Determination of Mineral Volume

One hundred-micrometer thick midsagittal sections were analyzed by BSE-SEM using low vacuum. Measurements were performed at five points, located 100 µm from each other.

The point closest to the enamel surface was located at 15 μm off the enamel surface. See Supplementary Data for details on the determination of the mineral volume

Polarized Light Microscopy (PLM) and Determination of Volume of Water and Organic Matter:

Eighty-micrometer thick resin-embedded longitudinal crown sections were analyzed qualitatively with regard to sign of birefringence, using the red I filter under polarized light. Quantification of birefringence was performed with a Berek compensator and interpreted with the mathematical approach (Sousa et al., 2006). Analysis details are found in the Supplemental Data.

Microradiography (MRX):

Eighty-micrometer thick resin-embedded longitudinal crown sections were microradiographed in a Faxitron MX-20 equipment operating at 20 KeV. See Supplementary Data for details on the determination of the mineral volume.

Statistical Analysis:

All analyses were accomplished by ANOVA. A probability value of $P < 0.05$ was considered to be the minimum acceptable level of statistical significance.

RESULTS

All the erupted fluorotic teeth (n=3) had the root completely formed. Among the non-erupted fluorotic teeth (n=4), two had roots completely formed, one had 2/3 of the root formed, and one had 1/3 of the root formed. Control teeth (n=7) matched for root formation stage were used.

Analysis of the smooth face of the fluorotic teeth according to the TF index revealed that all non-erupted teeth displayed surface opacity and lesions compatible with TF degree 4 (Figure 1A). One of the erupted teeth had lesions compatible with TF degree 5, and its entire surface exhibited marked opacity and focal loss of the outer enamel (Figure 1C). The two other erupted teeth contained areas with irregular contours in which the outermost enamel had been lost, comprising more than half of the surface, being scored TF degree 7 (Figure 1E).

When crowns were cut longitudinally, the enamel of all the fluorotic teeth had an opaque "milky" appearance extending from the enamel surface towards 1/3 of the enamel width (Figures 1B, 1D, 1F). The inner enamel, starting midway from the surface toward the dentinoenamel junction – DEJ, displayed normal (translucent) appearance. The control teeth exhibited normal morphology and normal enamel appearance (Figures 1G, 1H).

Fluoride determination showed that the dentine of the non-erupted fluorotic and non-erupted control teeth presented 143 and 32 μg F/g dentine, respectively, while the erupted fluorotic and erupted control teeth exhibited 936 and 29 μg F/g dentine, respectively ($P < 0.05$) (Appendix Figure 1).

The mineral volume based on BSE-SEM images (Appendix Figure 2A) was decreased at 15 μm from the surface in the non-erupted and erupted fluorotic teeth (67.9% and 74.0%, respectively) *versus* controls (97.5%; $P < 0.0001$ and $P < 0.0005$, respectively) (Appendix Figure 2). The same was observed at 115 μm, where the mineral volume increased to 80.1% in the non-erupted fluorotic teeth ($P < 0.0001$ versus control), and to 81.1% in the erupted fluorotic teeth ($P < 0.01$ versus control). At 215 μm from the surface, the mineral content reached 84.7% and 82.4% in the non-erupted and erupted fluorotic

enamel, respectively. The mineral content of the control enamel was 92.4% at this point, and both the erupted and non-erupted fluorotic enamel still had decreased mineral content compared to the control ($P < 0.05$). No differences were found deeper than 315 µm between the fluorotic and control enamel. While a steady increase in the mineral volume of all fluorotic teeth was found from the surface toward the deeper enamel, the opposite trend was observed for the control teeth (Appendix Figure 2B).

The MRX results (Figure 2) revealed mineral contents that coincided very much with the results obtained by BSE-SEM image analysis. A steady rise in mineral percentage was observed in the non-erupted fluorotic teeth, starting with 66.3% at 15 µm and reaching 89.7% at 400 µm. The erupted fluorotic teeth had 79.4% mineral volume at 15 µm, and no appreciable elevation in mineral volume was detected toward the enamel, reaching 83.8% at 400 µm from the surface. Up to 200 µm, statistically significant differences between the fluorotic and control enamel were always noted ($P < 0.05$) (Fig. 2). No statistically significant differences found at 300 µm. A slight decrease in mineral volume was also observed in control teeth ongoing from the surface toward the inner enamel, although statistically significant differences were only found within the non-erupted control group when samples collected at ≤ 200 µm were compared to samples collected at deeper enamel layers (at 400 µm) ($P < 0.05$).

Under PLM, the fluorotic non-erupted and erupted teeth showed the lowest negative birefringence areas in the outer enamel (Figures 3A, 3B), which are areas characteristic of hypomineralized enamel. Both erupted and non-erupted control teeth, on the other hand, showed a uniform negative birefringence as expected for normal mineralized enamel (Figures 3C, 3D). The determination of the water and organic matter revealed a higher

content of organic matter on the fluorotic enamel surface in both erupted (19.8%) and non-erupted teeth (13.4%) compared to the control enamel (lower than 1%) ($P < 0.01$ for both comparisons to control). Although the differences did not reach statistical significance, there was a ~7% increase in the organic content in any of the analyzed points when non-erupted teeth were compared with erupted teeth. The organic content decreased toward the deeper enamel (Figure 3E). The organic content of the control teeth could not be determined at 15 µm because the Angmar formula does not allow for calculations of volumes below 1%. The water content was almost constant at all points analyzed for each of the groups of teeth. The fluorotic enamel contained augmented water volume (~ 7 to 9% in the erupted fluorotic teeth, and 10 to 12% in the non-erupted fluorotic teeth) compared with the control teeth (~5% at any point tested) ($P < 0.01$) (Figure 3F).

The FTIR analysis demonstrated that the C-H group content was higher on the surface of the fluorotic enamel (100 µm from the enamel surface) compared with the inner enamel (at 50 µm from the ADJ) ($P < 0.01$) (Fig. 4A). The opposite trend was observed for the carbonate group, which showed lower values on the enamel surface compared to the inner enamel (Fig. 4B).

DISCUSSION

Results from this study show that the composition of fluorotic lesions appears to be similar to the one described in the enamel found during last wave of enamel mineralization, confirming the working hypothesis. Data presented here lends support to the theory that enamel fluorosis may arise because maturation stage enamel ameloblasts are malfunctioning in a high fluoride environment (Sharma et al., 2010), so that the final

maturation stage is never adequately completed. Below we discuss the observations of this study in detail.

First, we found an increase in the organic content (13-20%) of the fluorotic enamel, in both erupted and non-erupted human third molars. This augmented organic content was determined by analysis of birefringence of the enamel together with the information of the mineral content obtained by BSE-SEM at the same points. Using an FTIR analysis equipment that enabled the spatial determination of the amount of C-H/Phosphate and Carbonate/Phosphate in non-erupted fluorotic molars, the same trend was observed, with larger C-H/Phosphate and lower Carbonate/Phosphate in the fluorotic lesions. Control enamel close the DEJ showed the opposite trend. Organic content determination in percentage in human non-erupted fluorotic teeth is a novel finding.

Second, data provides some additional data on the mineral content of fluorotic lesions in human teeth, showing that such lesions seem to start with ~70% of mineral content on the very surface of the enamel, followed by a steady rise toward the inner enamel, with no statistical difference from the control enamel at > 350 μm from the surface. At this point, it is not possible for us to determine whether the increased mineral content (~80%) found on the surface of the erupted fluorotic teeth is a result of post-eruptive maturation or if the teeth available for analysis presented less severe fluorosis (although this does not appear to be the case, since the erupted teeth displayed the more severe TF scores). This is in agreement with earlier studies on human fluorotic enamel, which showed that the hypomineralized lesions extend about halfway through the enamel in the more severe forms (Fejerskov et al., 1994).

The analysis by PLM microscopy revealed the outer layer of enamel with the lowest negative birefringence, indicating hypomineralization. This is not in agreement with the classical report of a relatively well-mineralized (i.e. more negatively birefringent) outer layer in human fluorotic enamel (Fejerskov et al., 1975). Recently we were also unable to find this normally mineralized outer layer in the enamel of the continuously growing rat incisor in fluorotic animals (Saiani et al., 2009). We have carefully observed all the fluorotic teeth (both erupted and non-erupted) by PLM. Neither this technique nor the other two techniques used to assess mineral content (microradiography and BSE-SEM analysis) indicated a rise in the mineral content of the superficial enamel. Precise determination of mineral of the superficial layer of fluorotic teeth is important. Sato et al (1996) have suggested that fluorotic teeth have premature mineralization of the outer enamel, restraining the proteins into the enamel and causing sub-superficial lesions that resemble a white spot caused by caries.

In conclusion, this study showed that the spatially-resolved distribution of biochemical components in human fluorotic enamel resembles the one found in the late maturation enamel.

ACKNOWLEDGEMENTS

This study was supported by The State of Sao Paulo Research Foundation (FAPESP) and the (Brazilian) National Research Council (CNPq). The authors thank Dr. Elcio Marcantonio Jr. (Dental School of Araraquara, UNESP) for the excellent resin embedded tooth preparations.

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FIGURES

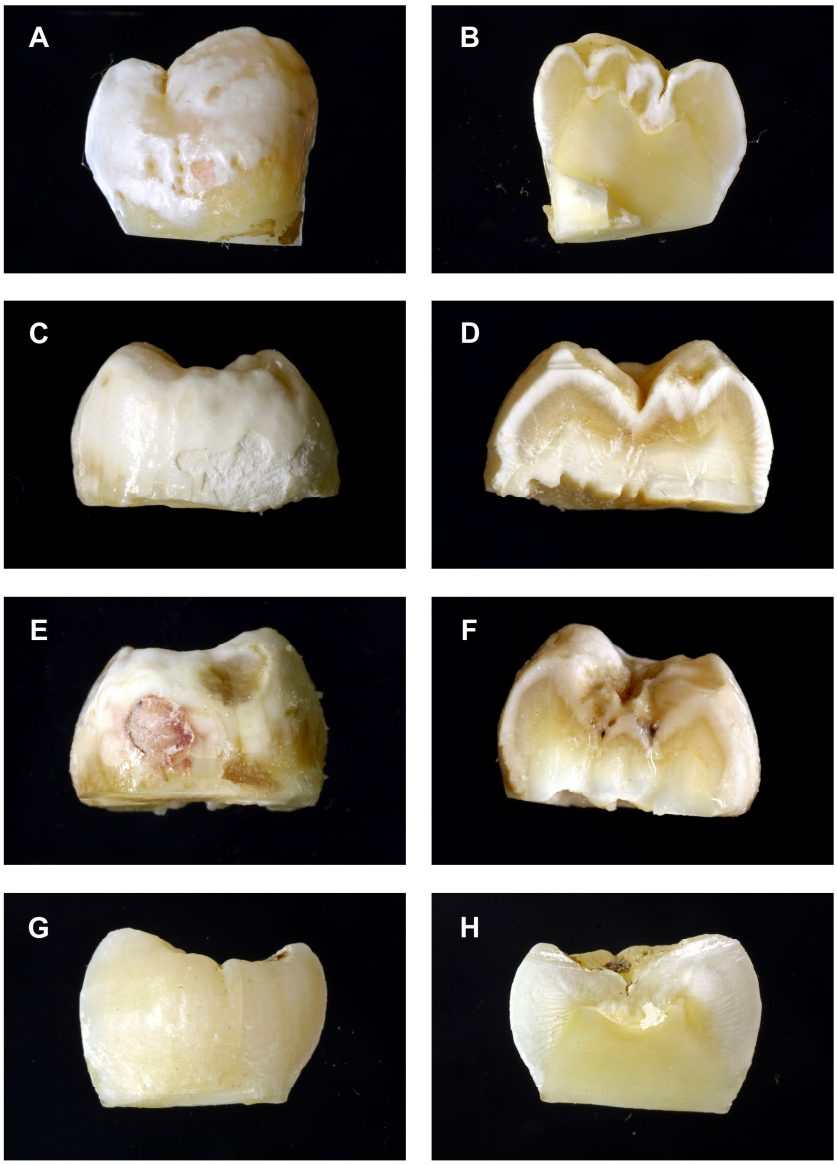

Figure 1. Macroscopic views of external and internal fluorotic and control human third molars. (A-B) Non-erupted fluorotic teeth corresponding to TF 4. (C-F) Erupted fluorotic teeth. Teeth show TF 7 (C-D) and TF 5 (E-F). Note that all the fluorotic teeth have an opaque surface. (G-H) Control tooth showing normal morphology and translucency.

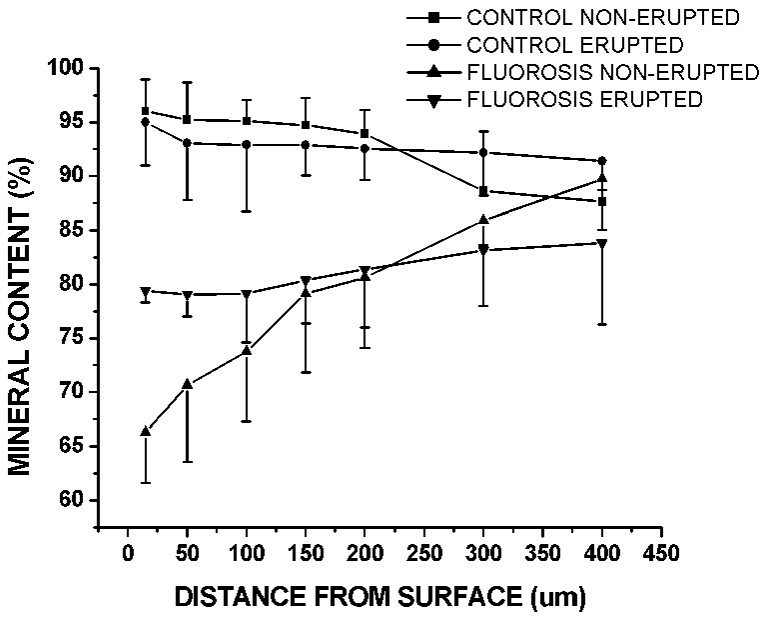

Figure 2. Determination of mineral content by microradiography. Lower mineral content is observed on the enamel surface of non-erupted and erupted fluorotic teeth, while the mineral content increases toward the inner enamel. These results are similar to the ones obtained by the mineral content analysis based on BSE-SEM (Appendix 2B).

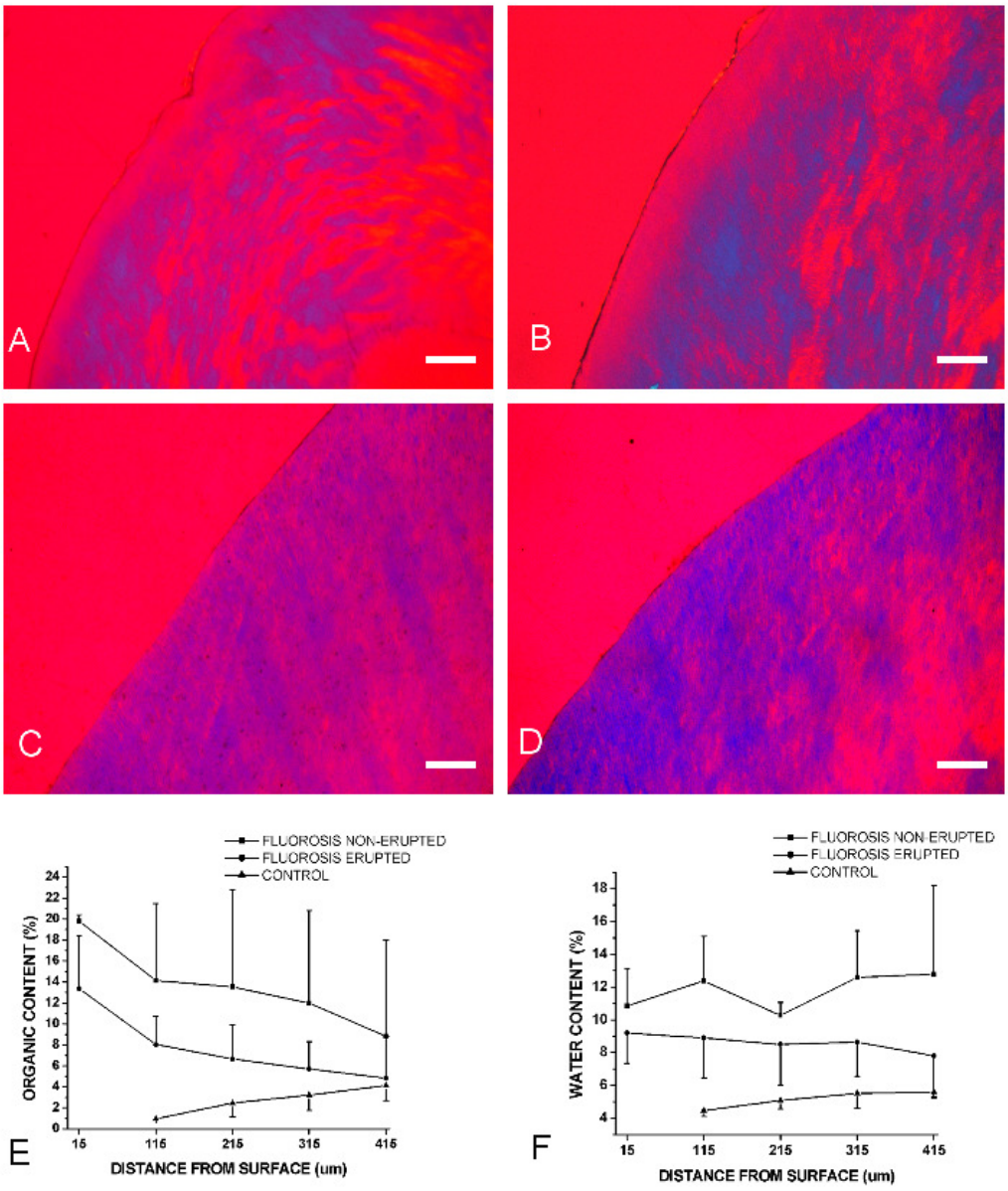

Figure 3. PLM of non-erupted fluorotic (A-B) and control teeth (C-D) in water. Fluorotic enamel show a pseudo-isotropic surface layer (red) overlying a negatively birefringent area (blue). Control enamel is entirely negatively birefringent. Plots of organic (E) and water (F) volumes. The organic volume is increased at the enamel surface and decreases towards the inner enamel. The water volume was relativity constant in all points measured in the enamel, being higher in fluorotic than in control teeth. Scale bar = 200 µm (A and C); = 100 µm (B and D).

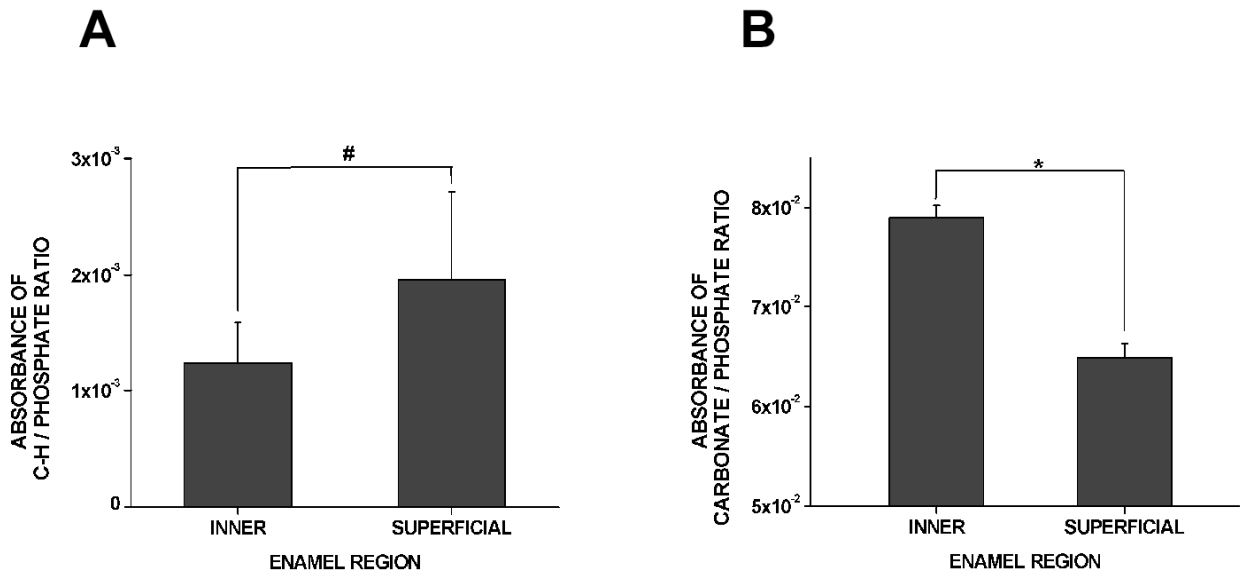

Figure 4. FTIR analysis of the superficial and inner enamel of fluorotic teeth (near the dentinoenamel junction). (A) C-H/Phosphate ratio is higher in the superficial enamel compared to the inner enamel. (B) Carbonate/Phosphate ratio is lower in the superficial enamel compared to the inner enamel. # $P < 0.01$ and * $P < 0.05$

SUPPLEMENTAL MATERIAL

MATERIALS & METHODS

Macroscopic Analyses

The teeth had the roots removed with a diamond disk in a sectioning machine and the crowns were photographed using a Macro 100EF Lens (Canon, Japan). Teeth were scored for fluorosis degree using the Thylstrup & Fejerskov index (1978) (Thylstrup and Fejerskov, 1978)and for root formation stage. Non-fluorotic erupted and non-erupted third molars were selected as controls for each of these teeth based on root formation stage.

Determination of Fluoride Ion

A 100 µm thick longitudinal section of each tooth crown was used for fluoride determination. Dentine was separated from the enamel, and a piece of dentine near the pulp was removed, weighed and transferred to a plastic test tube, to which 15.55 N nitric acid (HNO_3: Sigma Chemical Co. USA) was added in a proportion of 0.1 mL acid/1 mg of dentine for complete dissolution of the dentine. Thereafter the acid was neutralized with 15.55 N NaOH and the mixture was buffered with TISAB II (Total Ionic Strength Adjustment Buffer II). The fluoride concentration was determined by means of a specific electrode (Orion Research Inc., Model 96-09; Boston, United States) coupled to an ion analyzer (Orion Research Inc., Model EA 940, Boston, United States). Standard solutions (Orion #940907) in triplicates, at concentrations from 0.05 to 0.5 µgF/mL, were prepared in the same way as the samples. The results were expressed in µgF/g of dentine.

Fourier Transformation Infrared (FTIR) Spectroscopy

Midsagital 100 µm thick sections of non-erupted fluorotic teeth were used for those analyses, which had been polished up to 1200 mesh of granulometry. This experiment was

accomplished in micro ATR configuration coupled with an infrared microscope (Bruker Optics), equipped with a 20× objective and a Ge ATR crystal with 100 μm diameter contact area. This methodology has been extensively applied to biomedical materials and its advantages have been reported in the literature (Bartlett et al., 2005; Porto et al., 2010b; Saiani et al., 2009). The infrared spectra were obtained using a single element mercury cadmium telluride (MCT) detector. The spectra were acquired between 3800-700 cm^{-1}, with a resolution of 4 cm^{-1}.

The measurements were performed at three points on the superficial enamel and three points in inner enamel located close to the dentinoenamel junction (DEJ).

Back-Scattered Electron - Environmental Scanning Electronic Microscope (BSE-ESEM) and Determination of Mineral Volume

Midsagital 100 μm thick sections of all groups of teeth polished up to 1200 mesh of granulometry were analyzed by BSE-ESEM using low vacuum and an energy of 20 KeV. Measurements were done at five points, with distance of 100 μm from each other. The point closest to the enamel surface was located at 15 μm off the enamel surface. Image analysis using gray scale was done using of the image analysis program Image J. For measurement of mineral volume, the following empirical formulas were considered: $\mathbf{Ca}_{8.856\ 1}\mathbf{+Mg}_{0.088\ +}\ \mathbf{Na}_{0.292\ +}\ \mathbf{K}_{0.010\ +}\mathbf{P}_{5.592\ +}\mathbf{Cl}_{0.078\ +}\ \mathbf{O}_{25.561\ +}\ \mathbf{H}_{3.255\ +}\ \mathbf{C}_{0.457}$ for the mineral phase (Elliott et al., 1998); H_2O for water; and $C_{4.371}\ H_{7.044}\ N_{1.213}\ O_{1.344}\ S_{0.059}$ for the organic matter. Standards of aluminium oxide, berylium, carbon, calcium fluoride and chromium with known atomic weights were used to obtain a gray scale versus atomic number relationship. Using an image analysis freeware (ImageJ), gray levels were measured in standardized areas located at distances from the enamel surface as described above.

Conversion of atomic number to mineral volume was performed using the equation describing the variation of the water and organic contents as a function of the mineral content recently published (Sousa et al., 2009). Taking the densities of 1.0, 1.14 and 2.99 for water, organic matter and the mineral phase, respectively, and having the mean atomic number measured experimentally, the mineral volume could be determined.

Polarized Light Microscopy (PLM) and Determination of Volume of Water and Organic Matter:

Eighty-µm thick resin-embedded longitudinal crown sections were analyzed qualitatively with regard to sign of birefringence, using the red I filter under polarized light. Quantitative analysis were performed with the Berek compensator and the green interference filter (546 nm, and a bandwidth of 10 nm).

A piece of each tooth crown was previously fixed in 4% buffered paraformaldehyde for 48 h, dehydrated in crescent degrees of alcohol, embedded in glycol methacrylate resin (Technovit® 7200 VLC, Kulzer, Wehrheim, Germany) and cut using a high precision diamond disk (150 µm) in a sectioning machine (EXAKT, Germany). These sections were ground until the thickness of 80 µm. Thickness was measured to the nearest 1 µm using a light microscope equipped with calibrated reticle with the sampled positioned edge-on. Embedded sections were analyzed qualitatively with regard to sign of birefringence, using the red I filter (Axioskop 40, Carl Zeiss, Germany). The sections were also photographed in bright field.

For determination of volume of water and organic matter, the teeth analyzed in BSE-ESEM were used. The birefringence in water was quantified using a Berek compensator and green interference filter (546 nm, bandwidth of 10 nm) and interpreted

using the formula described by Sousa et al (2006), so that the content of water and organic matter was determined.

Microradiography:

Eighty-µm thick resin-embedded longitudinal crown sections (the same used for PLM) were mounted on high definition radiographic (High Resolution HD Plate, HTA Enterprises Microchrome Technology Products, CA, EUA) and submitted to an energy of 20 KeV and a time of 65min in a Faxitron MX-20 equipment (Faxitron X-ray, Lincolnshire, USA). An aluminum stepwedge of 10 steps (22 µm each) was also used to obtain a calibration curve between gray levels and aluminum thickness. The X-ray spectrum emitted has a peak at 8.5 KeV (Boone et al., 1997). Films were developed and photographed under a light microscope using a 20X eyepiece (Axioskop 40, Carl Zeiss, Germany) and a digital camera. For quantitative image analysis, uneven illumination was corrected in all digital images using the flat field correction procedure as described elsewhere (Murphy, 2001). A freeware (ImageJ) was used for all procedures of image processing and image analysis of gray levels at the following points from the enamel surface: 15 µm, 50 µm, 100 µm, 150 µm, 200 µm, 300 µm, and 400 µm. Mineral volume was measured using the Angmar formula (Angmar et al., 1963) with the following parameters: empirical formula for enamel apatite as described by Elliot (1997) (Elliot, 1997), with a mineral density of 2.99, linear attenuation coefficients of 212.356 and 112.948 for the enamel mineral and the aluminum, respectively.

RESULTS

Fluoride determination showed that the dentine of the non-erupted fluorotic and non-erupted control teeth presented 143 and 32 μg F/g dentine, respectively, while erupted fluorotic and erupted control teeth exhibited 936 and 29 μg F/g dentine, respectively ($P < 0.05$)(Appendix Fig. 1).

The mineral volume based on BSE-ESEM images (Appendix Fig. 2A) was decreased at 15 μm from the surface in fluorotic non-erupted teeth (67.9%) *versus* controls (97.5%) ($P < 0.0001$), and in fluorotic erupted teeth (74.0%) *versus* controls ($P < 0.0005$) (Appendix Fig. 2). The same was observed at 115 μm from the surface, where the mineral volume increased to 80.1% in the non-erupted fluorotic teeth ($P < 0.0001$ versus control), and to 81.1% in the erupted fluorotic teeth ($P < 0.01$ versus control). At 215 μm from the surface, the mineral content reached 84.7% and 82.4% in the non-erupted and erupted fluorotic enamel respectively. Control enamel showed 92.4% mineral content at this point, and both erupted and non-erupted fluorotic enamel still had a decreased mineral content when compared to control ($P < 0.05$). At 315 μm and at 415 μm from the surface, no statistical significant difference was found between fluorotic and control enamel ($P > 0.05$ for all comparisons). While there was a steady increase in mineral volume in all fluorotic teeth from the surface towards deeper enamel, the control teeth showed the opposite trend (Appendix Fig. 2B).

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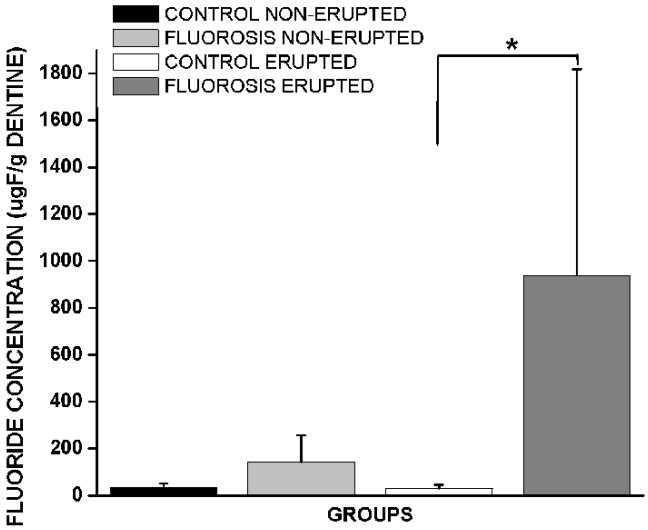
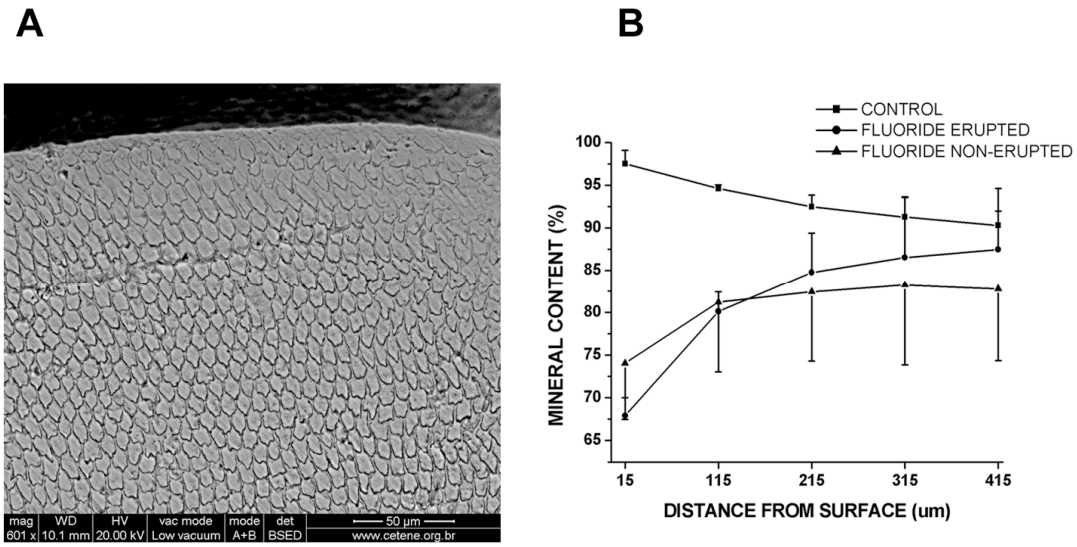
FIGURES



Appendix Figure 1. Concentration of fluoride in dentine (µgF/g dentine). Non-erupted fluorotic teeth had 5X times more fluoride concentration than control teeth than control teeth. The erupted fluorotic teeth have 30X more fluoride concentration than control teeth. In fluorotic group, erupted teeth had 4X more fluoride concentration than non-erupted teeth. *P<0.05.



Appendix Figure 2. (A) BSE-SEM analysis of non-erupted fluorotic teeth. (B) Determination of mineral volume. There is a hypomineralization in enamel surface of fluorotic teeth and fluorotic non-erupted teeth. The mineral volume increases across the inner enamel but does not reach the normal mineralization as control teeth.

DISCUSSÃO

Nos capítulos 1 e 2 mostramos técnicas de microataque ácido muito eficientes, conservadoras e de fácil manuseio para extrair proteínas de esmalte imaturo e maduro. Estas técnicas podem ser usadas para determinar o sexo na ciência forense já que o gene da amelogenina possui diferentes seqüências nos cromossomos X e Y (Steinlechner et al., 2002).

Devido ao seu alto grau de mineralização, o esmalte dental é o tecido que melhor preserva as proteínas por longos períodos, particularmente em ambientes secos, por isso é um bom material para se estudar isótopos no campo da antropologia, paleontologia e arqueologia (Dean, 2006; Smith & Hublin, 2008). Interessantemente, as taxas de isótopos de carbono e oxigênio, formados em diferentes épocas, preservam mudanças nas condições ambientais locais e na dieta de animais e hominídeos (Lee-Thorp and Sponheimer, 2006). A possibilidade de obter amostras de esmalte de diferentes camadas do esmalte também facilita a análise de microelementos em remanescentes arqueológicos. A relação entre as taxas de isótopos de estrôncio já foram determinadas em amostras de esmalte (Tykot, 2004).

Outra característica muito interessante do esmalte é que ele é produzido durante muitos anos; por isso, as marcas isotópicas encontradas no esmalte em diferentes épocas revelam informações dos componentes da dieta desde o nascimento até aproximadamente a idade de 17 anos (em humanos contemporâneos).

O uso de técnicas de microataque ácido pode ser utilizado para obter amostras de esmalte de pacientes com defeitos do esmalte como hipoplasia do esmalte, fluorose do

esmalte ou amelogênese imperfeita, já que não causa prejuízos à integridade deste tecido. No capítulo 3, verificamos que a técnica de imersão da coroa total de dente humano em ácido clorídrico preconizada por nós nos capítulos anteriores foi eficiente na análise das proteínas do esmalte de dentes humanos com fluorose. A grande novidade deste estudo é o uso de dentes inclusos, que ainda não foram expostos à cavidade oral, evitando a adsorção pós-eruptiva de proteínas exógenas. Neste estudo, não verificamos diferenças nos peptídeos resultantes da clivagem da amelogenina quando comparamos grupos controle e fluorótico, tanto nos dentes erupcionados quanto inclusos.

Porém, no nosso estudo do capítulo 4, encontramos maior conteúdo orgânico nos dentes com fluorose, tanto nos dentes erupcionados quanto inclusos. A determinação do conteúdo orgânico em porcentagem em dentes humanos não-erupcionados é um achado inédito. Verificamos também que há uma hipomineralização do esmalte mais externo, o que não está de acordo com a idéia clássica de que o esmalte fluorótico possui uma superfície hipermineralizada (Fejerskov et al., 1975). Em estudo anterior utilizando incisivos de ratos com fluorose, nós também não fomos capazes de detectar esta superfície hipermineralizada (Saiani et al., 2009). A determinação precisa do mineral na camada superficial do esmalte fluorótico é muito importante. Sato et al (1996) sugeriram que o esmalte fluorótico externo tem uma mineralização prematura, retendo as proteínas no esmalte, causando assim lesões sub-superficiais que se assemelham à lesão branca causadas por cárie.

CONCLUSÕES

Dos capítulos anteriormente apresentados, podemos concluir que:

Capítulo 1:

- Nossos resultados indicam que as técnicas de micro-ataque ácido superficial e dissolução do pó do esmalte são muito eficientes em gerar amostras adequadas para espectrometria de massa.

- O uso de ataque ácido em área restrita não fornece amostras com sinais adequados para a identificação de proteínas no caso de esmalte humano. Entretanto, esta técnica usada em esmalte imaturo pode ser útil para obter amostras de pequenas áreas de interesse. Sucessivos ataques ácidos em áreas restritas podem gerar massa de esmalte suficiente para uma análise em espectrômetro de massa adequada. Pode ser utilizada para obter amostras em áreas restritas de pacientes com defeitos de esmalte como, por exemplo, hipoplasia de esmalte, fluorose de esmalte ou amelogênese perfeita.

- Os procedimentos de microataque ácido são muito conservadores, removendo apenas a camada mais superficial do esmalte. Eles também são eficientes em gerar amostras de esmalte que são adequadas para a análise protéica, reduzindo drasticamente a necessidade de uma massa inicial grande de esmalte. Estas técnicas necessitam de menos tempo para obter as amostras. Por necessitar de menos manipulação, as chances de contaminação com proteínas exógenas (como a queratina, por exemplo) são muito menores.

Capítulo 2:

- A técnica de extrair proteínas da superfície do esmalte com procedimentos de micro-ataque ácido são adequadas em gerar amostras para análise em espectrômetro de

massa. Estas técnicas podem ser empregadas na ciência forense, na determinação do sexo, e na antropologia, paleontologia e arqueologia por trazer informações sobre tempo e condições ambientais por meio da análise de isótopos.

Capítulo 3:

- A técnica de microataque ácido descrita nos capítulos anteriores se mostrou eficaz na análise das proteínas do esmalte com fluorose;

- Um aumento no conteúdo orgânico nos dentes com fluorose não está relacionado com um problema na proteólise da amelogenina. Neste estudo, a clivagem da amelogenina não é afetada pelo flúor em dentes humanos fluoróticos erupcionados e inclusos. Possivelmente, a clivagem da amelogenina não é afetada, porém os peptídeos originados desta quebra protéica podem não conseguir deixar a matriz do esmalte, permanecendo assim, retidos no esmalte maduro.

Capítulo 4:

- Há um aumento do conteúdo orgânico (13-20%) no esmalte fluorótico, tanto nos terceiros molares erupcionados quanto inclusos. Esta determinação do conteúdo orgânico em porcentagem em dentes humanos fluoróticos inclusos é um achado inédito;

- As lesões fluoróticas apresentam ~70% de conteúdo mineral na superfície do esmalte. Este valor aumenta em direção ao esmalte mais interno (em direção à junção amelodentinária);

- A análise em microscopia de luz polarizada revelou que o esmalte mais externo apresenta um birefringência negativa menor, indicando hipomineralização desta camada;

- Os dados sugerem que a lesões fluoróticas em esmalte humano refletem a composição do esmalte no período tardio de maturação da amelogênese.

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ANEXO 1

Spectrochimica Acta Part A 77 (2010) 59–63

Contents lists available at ScienceDirect

Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

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Organic and inorganic content of fluorotic rat incisors measured by FTIR spectroscopy

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ARTICLE INFO

Article history:
Received 3 November 2009
Received in revised form 15 April 2010
Accepted 27 April 2010

Keywords:
Enamel
Fluorose
FTIR
Organic matter

ABSTRACT

Details on how fluoride interferes in enamel mineralization are still controversial. Therefore, this study aimed at analyzing the organic contents of fluorosis-affected teeth using Fourier Transformation Infrared spectroscopy. To this end, 10 male *Wistar* rats were divided into two groups: one received 45 ppm fluoride in distilled water for 60 days; the other received distilled water only. Then, the lower incisors were removed and prepared for analysis by two FTIR techniques namely, transmission and micro-ATR. For the first technique, the enamel was powdered, whereas in the second case one fluorotic incisor was cut longitudinally for micro-ATR. Using transmission and powdered samples, FTIR showed a higher C–H content in the fluorotic enamel compared with control enamel ($p < 0.05$, $n = 4$ in the flurotic, and $n = 5$ in the control group). Results from the micro-ATR-FTIR spectroscopic analysis on one longitudinally cut incisor carried out at six points reveal a higher C–H bond content at the surface of the enamel, with values decreasing toward the dentine–enamel junction, and reaching the lowest values at the subsuperficial enamel. These results agree with the morphological data, which indicate that in the rat incisor the fluorotic lesion is superficial, rather than subsuperficial, as in the case of human enamel. The results also suggest that the increased C–H bond content may extend toward the more basal enamel (intraosseous), indicating that fluorotic enamel may intrinsically contain more protein. Finally, particularly when coupled to ATR, FTIR is a suitable tool to study the rat incisor enamel, which is a largely used model of normal and abnormal amelogenesis. Further studies along this line may definitely answer some questions regarding protein content in fluorotic enamel as well as their origin.

1. Introduction

Fluoride alters the maturation process of the enamel and reacts with its structure to form fluorapatite ($Ca_5(PO_4)_3F$), which is more stable and more resistant to acid corrosion than hydroxyapatite [1,2]. This is the principle of the topical application or systemic use of fluoride, which prevents and diminishes the risk of dental caries. However, excess fluoride during enamel formation, particularly during the maturation phase of amelogenesis, causes both esthetic and functional defects in teeth [3].

Polarized light has revealed a subsuperficial hypomineralization in human enamel fluorotic defects, whereas the outermost enamel (about 100 μm) is normal [4]. In a study about the enamel mineralization of human and monkey teeth, it has been shown that hypomineralization is not always accompanied by hypoplasia, and could thus be associated with a disturbance in the degradation of the enamel matrix rather than with matrix secretion [5].

The mechanism responsible for fluorotic enamel is not known. The hypomineralized region of the fluorotic enamel displays modifications that are similar to caries with respect to crystal dissolution, and it has been reported that the mineralized surface of the fluorosed enamel is formed or modified by remineralization [6].

Details on how fluoride interferes in enamel mineralization are still controversial. According to a recent review [1], among the main hypotheses suggested to explain dental fluorosis are: (i) alteration in the conformation and association of proteins from the enamel

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doi:10.1016/j.saa.2010.04.024

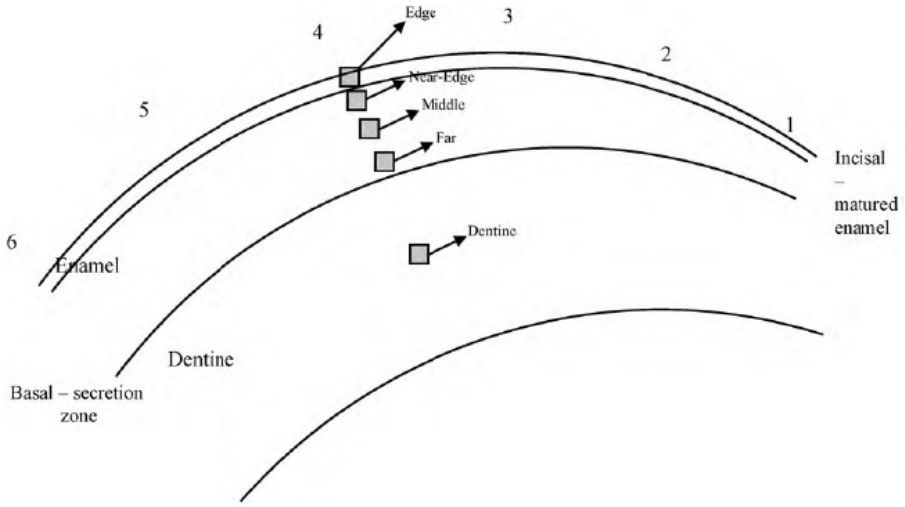

Fig. 1. Representation of sites selected for spatial analysis of one fluorotic tooth cut longitudinally by Fourier Transformation Infrared (FTIR) spectroscopy coupled with an attenuated total reflection (ATR) accessory, also named micro-ATR. Six points along the labial incisor surface indicated in the figure (numbered 1–6) were analyzed. At each point, five areas at different depths were analyzed, with four sites within the enamel, and one site within the dentine tissue.

matrix, (ii) binding of fluoride to proteins and proteases from the enamel matrix, (iii) alteration in protein–crystal interactions, (iv) processing alterations or altered proteolytic activity, and (v) alteration in the proteolytic activities sensitive to calcium levels.

To determine the precise mechanism of dental fluorosis, a more detailed description of the biochemical aspects of teeth affected by fluorosis is needed. Therefore, this study aimed to analyze the organic contents of control and fluorosis-affected teeth using Fourier Transform Infrared (FTIR) spectroscopy. As described before, the current hypothesis is that dental fluorosis is based on a complex biochemical process taking place in the organic matrix of the enamel tissue. The present work intends to start a biochemical characterization by applying a well established analytical technique to obtain important information on fluorotic rat incisor teeth, since such information is lacking and is very important for the fluorosis research area.

2. Materials and methods

2.1. Rat management

This study was approved by the Animals Ethics Committee of the University of São Paulo, protocol number 01.1364.53.4. Ten male *Wistar* rats were used and divided into two groups: the experimental group received 45 ppm fluoride (sodium fluoride) in distilled water for 60 days; the control group received distilled water only. Water and food were administered *ad libitum*. After 60 days, the rats were killed and their lower incisors were removed.

2.2. Sample preparation for FTIR spectroscopy

The samples were analyzed by two different FTIR spectroscopy techniques. The first consisted in the macroscopic transmission analysis of the enamel powder obtained from four sound teeth and five fluorotic teeth; the second analysis was conducted *in situ* in a longitudinal section of the tooth with a microscopic instrumentation using an attenuated total reflection (ATR) accessory, also named micro-ATR. This second measurement enables the spatial characterization of the fluorotic enamel.

An FTIR spectrometer (Nicolet 380, USA) was employed for transmission measurements. The enamel from the control and fluorotic groups was obtained using a dental diamond bur. Approximately 1 mg enamel powder from each sample was mixed with 100 mg potassium bromide (KBr) powder. After mixing, the powder was pressed into a ½-inch diameter pellet and compacted with 4 ton. The infrared spectra of all the samples were acquired with a 2 cm^{-1} resolution, in the 4000–400 cm^{-1} spectral range. For this analysis, five samples from the control group and four samples from the experimental group were selected.

After transmission analysis, an evaluation of one fluorotic tooth with higher spatial resolution was conducted. The sample was cut longitudinally, thereby exposing the enamel and the dentine. Before analysis, the sample had been polished up to 12,000 mesh of granulometry. This experiment was accomplished in the micro-ATR configuration coupled with an infrared microscope (Bruker Optics) equipped with a 20× objective and a Ge ATR crystal with 100 μm diameter contact area. This methodology has been extensively applied to biomedical materials and its advantages have been reported in the literature [7,8]. The infrared spectra were obtained using a single point mercury cadmium telluride (MCT) detector. The spectra were acquired between 3800 and 700 cm^{-1}, with a resolution of 4 cm^{-1}.

The measurement was performed at six points spaced approximately 4 mm between each measurement, along the labial surface of the rat lower incisor, in the direction going from the mature enamel to the secretion zone. In each position, a measurement was performed on the surface of the tooth by positioning the crystal tip at the fluorotic region of the enamel. After this measurement, different positions were selected at different enamel depths up to a measurement in the dentine tissue. The visual description of this procedure can be seen in Fig. 1.

After acquisition of the spectra, chemical composition analysis was carried out from the relative evaluation of the band areas. For this work, the area of the water bands (3700–2400 cm^{-1}), amide I bands (1800–1580 cm^{-1}), ν(C–H) bands positioned at 3000–2820 cm^{-1}, carbonate bands at 890–820 cm^{-1}, and phosphate bands at 750–490 cm^{-1} were analyzed by means of the Microcal Origin 6.0 software. The areas of the bands were measured relative to the phosphate band. This relative evaluation

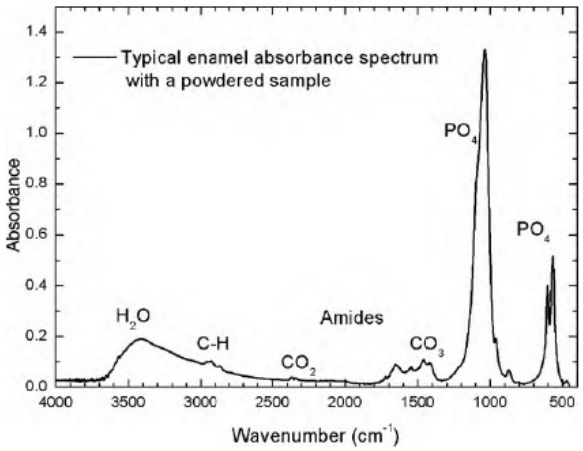

Fig. 2. Typical enamel absorption spectrum acquired in the transmission mode of FTIR spectroscopy. It is possible to observe bands assigned to water, C–H, CO_3, amides, and PO_4.

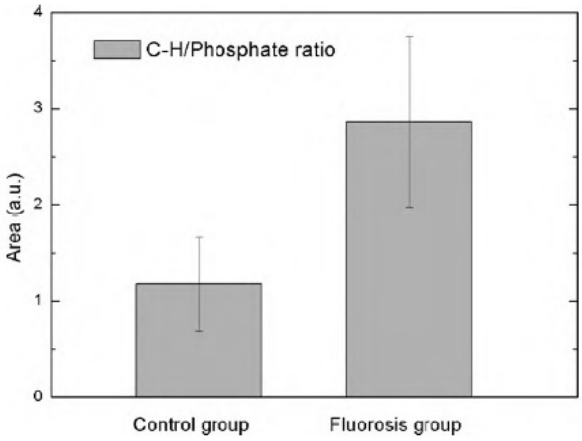

Fig. 3. C–H content in the control and fluorosis groups. The C–H content is higher in the fluorosis group compared with the control group ($p < 0.05$).

should remove signal changes originated from errors of the powder selection or changes due to the bad crystal contact in the ATR experiment. After selection of the bands, the background signal was subtracted and the area was integrated for area determination. Statistical difference between groups of transmission analysis was evaluated by ANOVA, and considered significant when $p < 0.05$.

3. Results

The transmission mode revealed a typical absorbance spectrum, shown in Fig. 2. This spectrum allows visualization of all the major chemical groups found in the enamel. In this work, the water content (3700–2400 cm^{-1}), C–H bonds (3000–2820 cm^{-1}), amide I (1800–1580 cm^{-1}), carbonate (890–820 cm^{-1}), and phosphate (750–490 cm^{-1}) contents were evaluated.

The ratio values with the respective standard deviations obtained for water, C–H, amide I, and carbonate are shown in Table 1; no significant differences were observed ($p < 0.05$) for the water, amide I, and carbonate values. A statistical significant difference between the control and experimental group was detected only for the C–H content. The ratio between C–H and phosphate indicates the composition of the organic matrix relative to the inorganic matrix. The difference between the C–H content of the control and fluorotic groups can also be observed in Fig. 3.

After the transmission measurement, we attempted to characterize the difference in the C–H content spatially. To this end, the absorption spectra were acquired by means of the ATR technique in one fluorotic tooth only. The typical spectrum described in Fig. 4 was obtained in the micro-ATR mode and is similar to the one acquired in the transmission mode (Fig. 1). In the micro-ATR study, only the C–H content relative to the content of phosphate was determined, since only changes in the C–H content had been observed in the previous transmission analysis.

The C–H/phosphate ratio can be visualized in Fig. 5 and Table 2 and represents the distribution of the organic matter along the labial surface of the incisor and into the enamel and dentine. Since only one tooth was investigated, reaching statistical significant differences at the usual 5% level is rather difficult. Therefore, the findings will be summed up in a descriptive way. Even though only one tooth was studied, results appear to be very important in the light of the recent morphological description of the fluorotic lesions found in the rat incisors, as discussed later.

In Fig. 5 and Table 2 a tendency toward a decrease in the C–H content is observed at the subsuperficial enamel, compared to both the superficial enamel and the deeper enamel layers and dentin. Region 1 is the most mature enamel (already inside the mouth of the animal, with a C–H content of 9.37 at the superficial enamel), while region 6 comprises the least mature enamel. Accordingly, this enamel exhibits higher organic content as judged by the C–H content (29.5 at the superficial enamel) Suggesting that this region may be close to the transition stage where the enamel starts to acquire mineral to a higher percentage (>40%). This observation points to increased C–H values in the dentine at any analyzed point. In the enamel, the higher C–H values are found in region 6, and a much higher value is found in the superficial layer (29.5) compared to the subsuperficial (13.8) and deeper enamel layers (7.3 and 7.8). Though descriptive, these data are in full agreement with the enamel maturation process described in recent years, where the inner enamel layers mineralize first, followed by mineralization of the outer enamel layers [5].

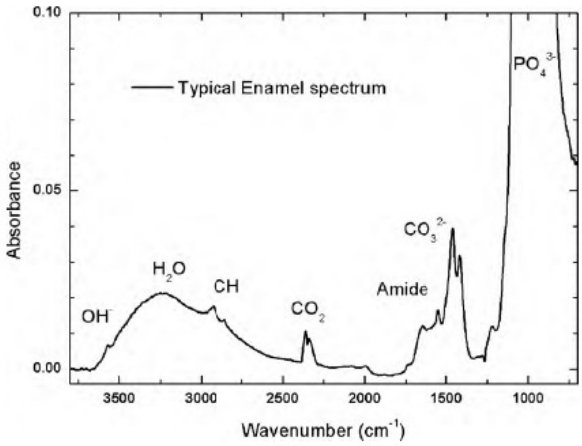

Fig. 4. Typical micro-ATR spectrum of the enamel tissue acquired in the enamel tooth cut longitudinally. The observed bands are the same described before in the absorption spectrum (Fig. 2) acquired in the transmission mode.

Table 1
Water, C–H, amide I, and carbonate content of control and fluorotic teeth. Only the CH/PO_4 ratio is higher in the fluorosis ($n=4$) compared with the control group ($n=5$).

	Water	SD	CH	SD	Amide I	SD	Carbonate	SD
Control group	7	3	1.2*	0.5	0.2	0.1	0.27	0.01
Fluorosis group	10	4	2.9*	0.9	0.4	0.2	0.24	0.03

* Statistical difference with ANOVA analysis ($p<0.05$).

Table 2
C–H/PO_4 ratio at different anatomical locations of a fluorotic tooth as depicted the Fig. 1. The mean values correspond to the five points (1–5) without the measurement at the tooth root.

Analyzed positions	Tooth tip	Intermediate values				Mean	Tooth root
	1	2	3	4	5	1–5	6
		Ratio values (a.u., $\times 10^{-3}$)					
Superficial	9.37	3.58	7.29	9.64	6.79	7.2 ± 2.4	29.55
Subsuperficial	4.99	1.07	3.64	2.30	1.48	2.7 ± 1.6	13.86
Middle	4.78	5.01	4.20	5.13	4.96	4.8 ± 0.4	7.34
Inner	7.05	6.59	4.03	5.70	5.45	5.8 ± 1.2	7.79
Dentine	12.35	8.44	7.24	11.50	8.35	9.6 ± 2.2	12.89

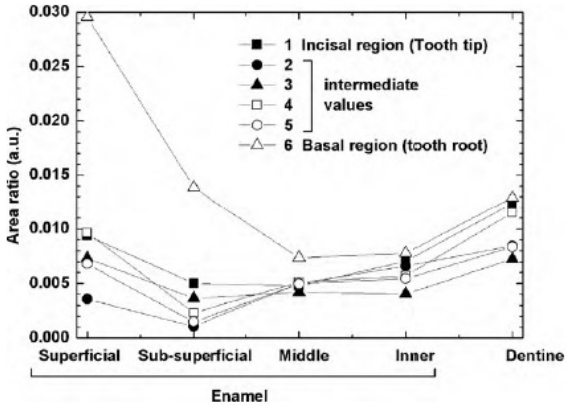

Fig. 5. Ratio of the C–H/phosphate at different sites in a longitudinally sectioned tooth. The longitudinal section of the tooth was analyzed in six different positions, from the incisal region of the tooth up to the basal region. For each position (along incisal–basal line, 1–6), measurements were made at five different depths: superficial, subsuperficial, middle, and inner enamel (near the enamel–dentin junction) and finally within the dentine tissue.

The subsuperficial layer exhibits the lowest C–H values at any given point from 1 to 5, with a mean value of 2.7 (±1.6), while the superficial layers lead to a mean value of 7.2 (±2.4) for points 1–5; the other two deeper regions of the enamel give mean value of 4.8 (±0.4) and 5.8 (±1.2), respectively. The data also indicate areas with higher and lower C–H values, suggesting that some of the low C–H values may derive from normal areas. This may indeed happen, since the fluorotic rat enamel contains normal areas mixed with areas of altered enamel [9]. In the case of the animals of this study, the exposure to this level of fluoride would result in successive bands of normal (pigmented) and abnormal (white) enamel [9]. Since this measurements were taken without knowing whether the point was in the normal or abnormal enamel, the increased variability in superficial enamel is expected.

4. Discussion

A higher C–H content was observed by means of the transmission mode using powder samples and by micro-ATR analysis using a cut tooth and mapping the changes longitudinally. The latter technique revealed that the C–H content is higher at the surface and decreases toward the dentine–enamel junction. These results are in agreement with those in the literature describing that fluorotic enamel has increased protein content [15,16,17].

In a study on the morphological characterization of the rat incisor fluorotic enamel, only the outer enamel (corresponding to the superficial enamel in this study) presented less birefringent areas (abnormal) under polarizing light microscopy, which is consistent with less mineralized areas [9]. Therefore, the increased C–H content found in the superficial enamel in the present study fully agrees with results relative to abnormal enamel lying on the very surface of the rat incisor enamel.

The increased C–H/PO_4 ratios observed in the superficial and subsuperficial enamel in region 6 (Table 2) also appear to be consistent with less mineralized enamel in the basal region of the tooth, as described before [10].

Other studies have shown that the fluorotic enamel is less mineralized and more porous than the normal one [11,12,13]. However, whether the increased organic content of the fluorotic enamel originates from exogenous organic matter incorporated into the enamel through these porosities or the retention of organic matter is intrinsic to the enamel and results from changes in amelogenesis caused by fluoride are still controversial. In this study, the FTIR-results suggest that there may be an increased organic content at any part of the intraosseous superficial enamel, since this superficial enamel exhibits higher absolute values of C–H bond content throughout regions 1–5. Nevertheless, definitive data based on a larger sample size and the precise location of the analysis point are needed, whether in the normal or abnormal enamel, since both are found in successive bands of the fluorotic rat incisor enamel.

It has been previously demonstrated that no differences in the protein pattern of the enamel can be found between the fluorotic and control enamel [14]. Other studies have shown that fluoride inhibits the mechanisms involved in protein removal during enamel maturation [13,15,16]. However, no differences in the activity of enamel proteinases in vitro were observed using a wide range of fluoride concentrations [14].

Studies on moderately fluorotic enamel of extracted teeth showed no differences between the control and fluorotic enamel [17]. Wright et al. [18] suggested that fluorotic human enamel contains increased amounts of protein (0.27%) compared with normal mature enamel (0.1%); however, the small number of investigated teeth and the high variability do not support a definite conclusion. Therefore, a controversy exists regarding the true existence of increased organic matter in the fluorotic enamel defects. The fact that the mature enamel contains 95% of mineral by weight and

less than 1% organic matter [19] turns enamel into a very difficult matrix for organic matter studies.

Recently, an increase in the protein content of fluoride-treated zebrafish teeth has been shown by FTIR spectroscopy [7]. Along this line, the present study is the first using a widely employed amelogenesis model namely, the continuously growing rat incisor, which also reveals a rise in the amount of C–H bonds in the teeth from fluoride-treated rats. Microscopic analysis suggests that this increase occurs on the enamel surface. These findings support the notion that fluorotic defects in rat incisors are superficial defects [9], whereas in humans fluorotic defects are subsuperficial [3,4]. The precise morphological and chemical characterization of the defects with spatial resolution is necessary for understanding the mechanism that leads to the formation of these defects. This study shows at least three important new aspects: (1) the superficial defects found by polarizing light may contain larger amounts of C–H bonds, (2) the increased C–H bond content may extend toward the more basal enamel (intraosseous), suggesting that the fluorotic enamel may intrinsically contain more protein, and (3) FTIR, particularly when coupled to ATR, is a suitable tool to study the rat incisor enamel, which is a largely used model of normal and abnormal amelogenesis.

The present result does not differentiate the C–H content from organic and inorganic components. Considering that the inorganic matrix is composed of hydroxyapatite, the major C–H content observed in this work has certainly originated from the organic matrix of the enamel tissue.

There may also exist a difference in the amide I band, but this difference did not reach statistical significance at the 5% level and, therefore, further studies are needed to adequately investigate this possibility.

The applied methodology is adequate for identification of the organic composition of the fluorotic enamel. Results are more consistent because conventional infrared spectroscopy has been used together with microscopy techniques. In general terms, the macroscopic results obtained in the transmission mode agree with those from the ATR microscopic analysis. Because the fluorotic lesions in the rat incisor enamel are not continuous on the outer (superficial) enamel, the precise location of the ATR tip is needed for comparison of true fluorotic with normal areas, which are also present in fluorotic incisors.

Thus, more detailed studies are necessary to obtain spatial information on organic matter. This will allow for determination of the origin of the organic material. Micro-ATR, on the other hand, provides the necessary information about how the ratio of organic/inorganic components changes along as well as across the tooth.

In conclusion, a higher C–H content was observed in the fluorotic enamel compared with control enamel by FTIR using transmission and powdered samples. The results from the micro-ATR-FTIR analysis on one longitudinally cut incisor made at 6 reveals a higher C–H bond content at the surface of the enamel, with values decreasing toward the dentine–enamel junction, and the lowest values being detected at the subsuperficial enamel. These results agree with the morphological data, which indicate that in the rat incisor the fluorotic lesion is superficial rather than subsuperficial, as in the case of the human enamel. The results also suggest that the increased C–H bond content may extend toward the more basal enamel (intraosseous), indicating that the fluorotic enamel may intrinsically contain more protein. Finally, FTIR, particularly when coupled with ATR, is a suitable tool for the study of the rat incisor enamel, which is a largely used model of normal and abnormal amelogenesis. Further studies along this line may definitely answer some questions regarding protein content and in the flurotic enamel, as well as their origin.

Acknowledgements

This study was supported by The State of Sao Paulo Research Foundation (FAPESP) and the (Brazilian) National Research Council (CNPq).

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ANEXO 2

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Morphological characterization of rat incisor fluorotic lesions

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ARTICLE INFO

Article history:
Accepted 30 August 2009

Keywords:
Fluorosis
Polarized light microscopy
SEM
BSE-SEM
Birefringence

ABSTRACT

The morphological characterization of fluorotic rat incisor enamel was carried out. Experimental adult animals received drinking water with 45 mg F/L of fluoride, and the control group received distilled water. Fluoride concentrations found in the control and fluorosis groups were 0.04 and 0.09 μg/mL (plasma), 0.26 and 0.66 μg/mg (whole tibia), and 0.24 and 2.3 μg/mg (tibia surface), with $P \leq 0.001$ for all comparisons between the groups. A succession of white and pigmented bands was observed in the fluorotic rat incisors. Under polarizing light microscopy, cross-sections of superficial areas corresponding to the white bands (from the surface to ~20 μm) showed high positive birefringence. These fluorotic lesions also exhibited the lowest resistance to superficial acid etching. No morphological differences in inner enamel were seen under scanning electron microscopy. In fluorotic enamel, only the surface layer related to the white areas presented lower birefringence compared with the enamel of control teeth and the surface layer of the pigmented areas (normal ones) of fluorotic teeth. In conclusion, the white bands of fluorotic rat enamel represent hypomineralized superficial areas and are not subsurface lesions. The detailed description of these lesions is important to understand dental fluorosis.

1. Introduction

Enamel fluorosis is a consequence of impaired maturation of the dental enamel by systemic fluoride, causing a poorly mineralized tissue with normal thickness.[1] Although several studies have described retention of proteins in fluorotic enamel,[2–5] the majority of these reports have employed rat enamel in the early maturation stage.

A recent review on dental fluorosis[6] includes the following hypotheses for dental fluorosis: (i) alteration in the conformation or aggregation of enamel matrix proteins, (ii) binding of fluoride ion to enamel matrix proteins and proteases, (iii) alteration in protein–crystal interaction, (iv) different processing or lifetime of proteases *in situ* and (v) alteration of proteolytic activities sensitive to calcium levels. In addition, fluoride-induced early mineralization of the enamel surface

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0003–9969/$ – see front matter
doi:10.1016/j.archoralbio.2009.08.009

layer, which might impede complete removal of organic matter from the inner layers, has been suggested as a contributing factor[7] to fluorosis.

Both human and rat fluorotic enamel lesions have been histologically described as a subsurface hypomineralized area underneath a relatively well-mineralized surface layer; i.e., subsurface lesions.[8,9] Such surface layer appears radiopaque in a microradiography, and negatively birefringent in water under examination by polarizing microscopy. The description of these lesions as being radiolucent inside and covered by normal enamel[9] is consistent with the hypothesis that fluorotic enamel lesions are caused by impaired removal of organic matter from inner enamel layers resulting from the early mineralization of the surface layer of the maturing enamel.

The rat incisor is widely employed for studies on amelogenesis, but few literature reports describe the histopathology of fluorotic enamel lesions in the rat incisor. Regarding the subsurface nature of the fluorotic lesion in the rat incisor enamel, published microradiographs[9,10] do not give unequivocal evidence of the presence of a relatively well-mineralized surface layer. While the presence of this layer would suggest that a physical barrier exists in the outer enamel that might impede the removal of organic matter from the inner enamel, the lack of such mineralized layer would indicate that this physical barrier does not exist. Therefore, the in-depth characterization of the mature fluorotic enamel may aid understanding of the mechanism underlying the development of fluorotic enamel defects, particularly in the rat incisor amelogenesis model.

Therefore, we have carried out the detailed morphological characterization of fluorotic rat incisor enamel by bright field, polarized light microscopy and scanning electron microscopy (secondary and backscattered electron modes).

2. Materials and methods

This study was approved by the University's Ethical Committee for Animal Research (CEEUA – USP, protocol number 01.1364.53.4). Forty one-month-old male *Wistar* rats were used and divided into two groups: the experimental group, which received 45 mg fluoride (100 mg sodium fluoride)/L distilled water for 60 days; and the control group, which received distilled water. Water and food were administered *ad libitum*. After 60 days, the rats were killed, and their upper incisors were removed for morphological analysis. The labial surface of both control and fluorotic enamel of incisors was photographed under low magnification prior to other analyses.

2.1. Determination of fluoride ion

2.1.1. Plasma

The blood of five control and five fluorotic rats was extracted from the jugular vein and transferred to a tube containing ethylenediamine tetraacetic acid (EDTA: Sigma Chemical Co., USA), to avoid blood coagulation. The blood was centrifuged for 10 min at 2000 × *g*, and 1 mL plasma was placed on Petri dishes (Falcon 1007), to which 2 mL deionized water was added. A 0.075N sodium hydroxide (NaOH, A.R.: Sigma Chemical Co., USA) trap solution (50 μL in five drops) was placed on the Petri dish lid, and after addition of 1 mL 3N sulphuric acid (H_2SO_4: Sigma Chemical Co., USA) saturated with hexamethyldisiloxane (HMDS), each dish was immediately sealed. During overnight diffusion, fluoride ion released by acid hydrolysis was collected in the NaOH trap. The trap was then recovered and buffered to pH 5.2 with 25 μL acetic acid 0.20N (CHCOOH: Sigma Chemical Co., USA), and the recovered solution was adjusted to a final volume of 100 μL with deionized water. The fluoride concentration was determined by means of a specific electrode (Orion Research Inc., Model 96-09, Boston, USA) coupled with an ion analyzer (Orion Research Inc., Model EA 940, Boston, USA). Standard solutions (Orion #940907) in triplicates, at concentrations ranging from 0.031 to 0.5 μg F/mL, were also acid-diffused. Blanks were subjected to the same procedure.

2.1.2. Bone

The femurs of five control and five fluorotic rats were dried for 24 h at 90 °C. Then, the femurs were transversally sectioned, leading to two slices measuring approximately 5 mm from the mid-diaphysial region of each bone.[11] One slice was dissolved in acid, to obtain the whole bone fluoride concentration; the other one was used to evaluate fluoride concentration on the bone surface. For whole bone determinations, the femur slices were left at 90 °C for 24 h.[11] The slices were weighed and transferred to plastic test tubes, to which 15.55N nitric acid (HNO_3: Sigma Chemical Co., USA) was added in a 0.1 mL acid/mg bone ratio. After 24 h at room temperature, the acid was neutralized with 15.55N NaOH, and the mixture was buffered with TISAB II. The extracted fluoride was determined as described above, on the basis of a fluoride standard curve built with fluoride standard solutions (0.05–0.5 μg F/mL) prepared in the same way as the samples. The results are expressed in μg F/mg bone.

To determine fluoride concentration on the bone surface, a circular hole (2.0 mm diameter) was punched in an adhesive tape, which was applied to the surface of the bone slice.[11] A window of 3.14 mm^2 area was exposed, and 5 μL 1.6N hydrochloric acid (HCl: Sigma Chemical Co., USA) in 70% glycerol was applied for 1 min, under agitation. The extract was transferred to a tube containing 190 μL deionized water. This etching step was repeated, and the second 5 μL etching solution was directly added to the tube. In the end, 5 μL of a 70% glycerol solution was applied to the window for 30 s and added to the same tube. The extracts were neutralized with 0.105 mL 0.228N NaOH, and buffered with TISAB II. The extracted fluoride was determined as described above. The amount of dissolved bone was inferred from the amount of P present in the acid extracts, assuming that its concentration in the bone is 13.5%. The P contents of the samples were determined colorimetrically.[12] Fluoride concentrations in the bone are expressed as μg F/mg bone.

2.2. Preparation of hard tissue sections of incisors

Analyses of enamel in hand-ground longitudinal sections (100 μm thick) of five rat incisors in a pilot study showed that the thickness of the enamel varied from 100 μm to more than 250 μm in animals of the same age group. The variability in the

thickness suggested that the method employed for preparation of the sections was not satisfactory. To determine the best method to prepare adequate midsagittal sections of enamel we prepared transversal sections at three different points of the incisor length and measured the enamel width, which was smallest at the most central aspect of the enamel.

To ensure that midsagittal sections of incisors would be obtained, the more apical 1/3 of the tooth was removed. Then, the remaining incisal 2/3 were hand-ground, so that a longitudinal section reaching the central aspect of the tooth was achieved, on the basis of the anatomical aspect of the pulp cavity. At this point, the other side of the tooth was exposed to sand paper, so that this side would be ground until a midsagittal 100 μm section was obtained. Thereafter, the section was polished and cleaned for quantitative polarized light microscopy and scanning electron microscopy analyses.

2.3. *Polarized light microscopy (PLM)*

For qualitative PLM, five control and five fluorotic incisors were previously fixed in 4% paraformaldehyde for 48 h, dehydrated in increasing degrees of alcohol, embedded in glycolmethacrylate resin (Technovit® 7200, Heraeus Kulzer GmbH, Wehrheim, Germany),[13] and cut by means of a high precision diamond disk (100 μm) in a sectioning machine (EXAKT, Germany). These sections were ground until a thickness of 80 μm was achieved. The thickness was measured with a digital paquimeter (accuracy of ±10 μm). Embedded sections were qualitatively analysed for birefringence sign using a Red I Plate. Qualitative analyses were performed after immersion of the sections in distilled water for 24 h, followed by immersion in Thoulet's solution with a refractive index of 1.62 for 48 h. Enamel birefringence is the result of the intrinsic (due to the mineral content, with refractive index of 1.62) and the form (due to the non-mineral content—water and organic matter, with refractive indexes of 1.33 and 1.56, respectively) birefringences.[14] When the tissue presents increased amounts of larger pores than normal human enamel, it is believed that an aqueous solution with the same refractive index of the mineral content can replace water. The Thoulet's solution, which is a solution of potassium iodide and mercurial iodide in water, and has a refractive index of 1.62. Using this solution the form birefringence is almost completely eliminated, so that the observed birefringence is directly proportional to the mineral content, as experimentally shown with Thoulet's 1.62 in carious enamel.[15]

For the quantitative analysis of enamel birefringence, longitudinal ground sections of ten control and ten fluorotic incisors were employed. To this end, the teeth were firstly fixed in 4% paraformaldehyde, and ground sections were then manually prepared without previous embedding in resin. The thickness was measured edge-on to the nearest 2 μm in a light microscope equipped with a reticule. The thickness of the sections ranged from 80 to 110 μm. Only sections immersed in Thoulet's 1.62 were analysed. The refractive index of the immersion medium was determined by an Abbe refractometer. Quantitative birefringence analyses were performed in a polarizing microscope equipped with a Sénarmont compensator and interference green filter (546 nm). The following histological layers were analysed in the area covering a distance of ~100 μm from the enamel surface to the enamel–dentine junction (EDJ): the outer enamel, the superficial 30 μm enamel layer; the middle enamel, the 20 μm layer just beneath the outer layer; and the inner layer, which comprised the innermost 20 μm enamel layer, close to the EDJ.

2.4. *Statistical analysis*

Analyses of fluoride concentration in the plasma and bone were accomplished by unpaired *t*-test. Comparisons between the fluoride concentration in the surface and whole bone were done by paired *t*-test. Analyses of differences in enamel birefringence were carried out using one-way analysis of variance (ANOVA). A probability value of $P < 0.05$ was considered to be the minimum acceptable level of statistical significance.

2.5. *Scanning electron microscopy—secondary electrons (SEM) and backscattered electrons (BSE-SEM)*

Twenty control and twenty fluorotic incisors (from ten animals of each group) were removed and stored at −20 °C until preparation for SEM, when the teeth were randomly divided into four groups. Group I: teeth were individually ultrasonicated for 10 min and then fixed in 4% buffered paraformaldehyde, being then used for surface analysis; Group II: the enamel was treated with 37% phosphoric acid for 30 s and teeth were ultrasonicated for 10 min, being then fixed as above for surface analysis; Group III: teeth were

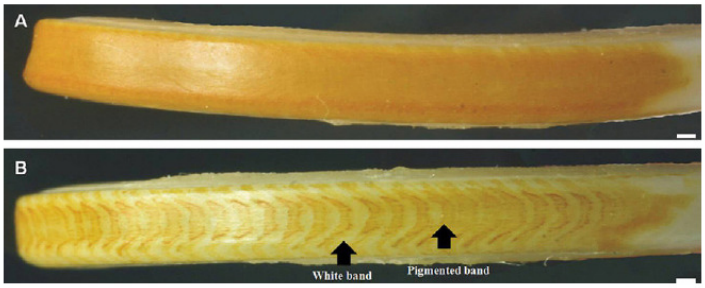

Fig. 1 – Stereomicroscope images of control (A) and fluorotic (B) rat incisors, the incisal edge on the left (4×). While normal incisor enamel displays an even orange colouration, fluorotic rat incisor enamel is characterized by a succession of white and orange bands, which appear heterogeneous in colour and do not show precise limits. Bars = 300 μm.

Table 1 – Fluoride concentration in the plasma, whole tibia and tibia surface (average ± S.E.).

	Control ($n = 5$)	Fluorosis ($n = 5$)	P value
Plasma (μg/mL)	0.04 ± 0.01	0.09 ± 0.01	<0.0001
Whole tibia (μg/mg)	0.26 ± 0.03	0.66 ± 0.07*	0.001
Tibia surface (μg/mg)	0.24 ± 0.26	2.30 ± 0 71*	<0.0001

* $P < 0.001$ fluoride concentration of whole tibia *versus* tibia surface in the fluorosis group.

ground until the midsagittal plane was reached, followed by individual ultrasonication for 10 min and fixation as above, being then used for internal enamel analysis; Group IV: teeth were ground until the midsagittal plane was reached, being the internal surface of the enamel then treated with 37% phosphoric acid for 30 s, and teeth ultrasonicated for 10 min, being then fixed as above for internal enamel analysis. The teeth were dehydrated with increasing concentrations of acetone, mounted in stubs, covered with carbon, and analysed in a scanning electron microscope (Jeol JSM – 5600LV, Tokyo, Japan) using both secondary electrons and backscattered electrons detectors with an energy of 20 keV.

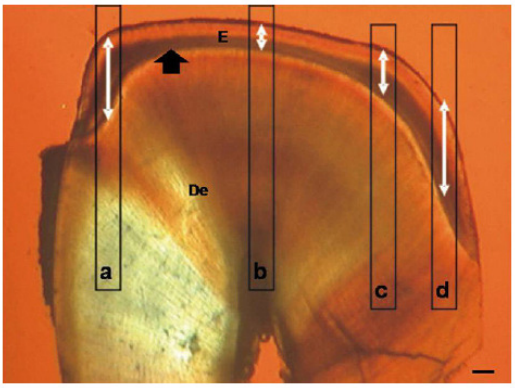

Fig. 2 – Cross-section of an upper rat incisor under polarized light microscope (10×). Rectangles 'a'–'d' represent possible sagittal sections obtained while the incisor was being ground or sectioned. Arrows inside the rectangles represent the different widths the enamel would show on longitudinal sections (a–d) observed under a microscope. Bar = 100 μm. E: enamel; De: dentine; black arrow: dentine–enamel junction.

3. Results

The upper incisor photographs of control and experimental animals exposed to 45 mg F/L in the drinking water (Fig. 1)

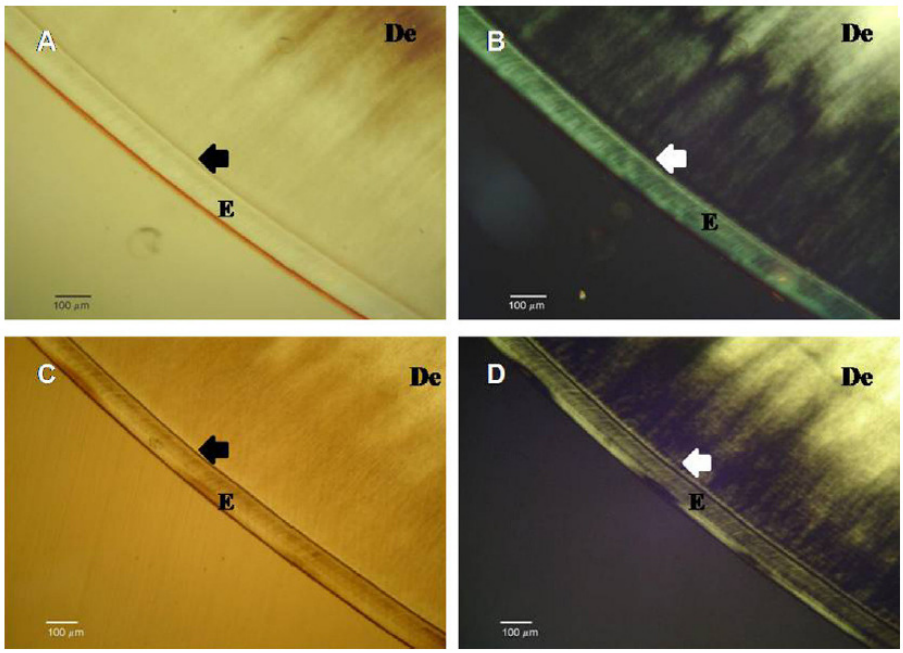

Fig. 3 – Longitudinal sections of control (A and B) and fluorotic (C and D) upper rat incisors (10×). Pictures A–C were taken under bright light; pictures B–D under polarized light in Thoulet's 1.62 medium. Fluorotic enamel displays bands in the case of the outer enamel, which are dark under white light and less birefringent under polarized light, consistent with less mineralized areas. E: enamel; De: dentine; arrows: dentine–enamel junction.

showed the classical fluorosis pattern of rat incisor enamel in the animals exposed to fluoride, with lack of iron pigmentation in some areas, forming a succession of white and pigmented bands (Fig. 1B).

The determination of fluoride ion in the plasma and in the bone of control and fluoride-exposed animals revealed a fluoride concentration of 0.04 *versus* 0.09 μg/mL in the plasma of control and fluorosis animals, respectively ($P < 0.0001$); 0.26 *versus* 0.66 μg/mg in the whole tibia ($P \leq 0.001$); and 0.24 *versus* 2.30 μg/mg in the tibia surface ($P < 0.0001$). In the fluorosis group, there was also ~3.5 times more fluoride on the bone surface compared with the whole bone (0.66 *versus* 2.30, respectively) ($P < 0.001$) (Table 1).

Variation in the enamel thickness in the mesiodistal direction is shown in Fig. 2. White double headed arrows inside rectangles represent the enamel thickness that would be shown in sections located in positions 'a'–'d' (black rectangles mimic 100 μm thick longitudinal sections). Once this was established, only sections in position 'b' were prepared (yielding a 100 μm thick enamel layer ongoing from the surface to the EDJ). This was also important for preparation of the midsagittal plane samples for SEM.

Embedded midsagittal sections of control and fluorotic enamel under bright field and PLM are shown in Fig. 3. Histological features observed via polarizing microscopy of water-immersed sections revealed that the three histological layers of both control and fluorotic teeth had *positive birefringence*. In fluorotic teeth, the white areas presented a more positive birefringent surface layer if compared with the surface layers of the pigmented areas of the same sections and those of normal enamel. The more birefringent areas were located in the very superficial aspect of enamel to a depth of ~30 μm. When immersed in Thoulet's solution, the three layers (outer, middle, and inner enamel) of control and fluorotic teeth displayed *negative birefringence*. However, the surface layer of fluorotic teeth related to the white areas presented a less negative birefringent value than the other layers of the same sections and the control teeth (Fig. 3). Apart from such differences, no other qualitative differences between fluorotic and control sections were observed.

Quantitative birefringence data in Thoulet's 1.62 for the control teeth showed that the outer layer presented the lowest values, followed by the inner layer, while the middle layer presented the highest values (Fig. 4). Surface layer related to the white areas of fluorotic teeth presented lower birefringence values compared with the other layers of fluorotic and control teeth. Birefringence of the surface layer of the white areas of fluorotic teeth was different from that of the surface layer of control teeth ($P < 0.001$) and also from that of the surface layer of the pigmented areas of the fluorotic teeth ($P < 0.05$). There were no statistically significant differences between teeth from the two groups with respect to the other layers.

SEM analysis showed that the labial surface of control and fluorotic enamel is homogeneous when specimens are prepared without acid etching (Fig. 5). Acid etching revealed bands with the same pattern observed in Fig. 1. Careful analysis evidenced that white bands seen with the naked eye, observed as darker areas in bright field microscopy and less birefringent areas by PLM, were less resistant to acid and appeared as depressions under SEM (Fig. 5B and C). These areas appear more porous than those of the control teeth, since they displayed a rougher surface after acid etching. BSE-SEM analysis indicated that white bands of the labial surface, seen in Fig. 1B, are darker than the pigmented bands, thus reflecting a higher mean atomic number in the latter. In groups treated with 37% phosphoric acid, these differences disappeared in the midsagittal sections (Fig. 6).

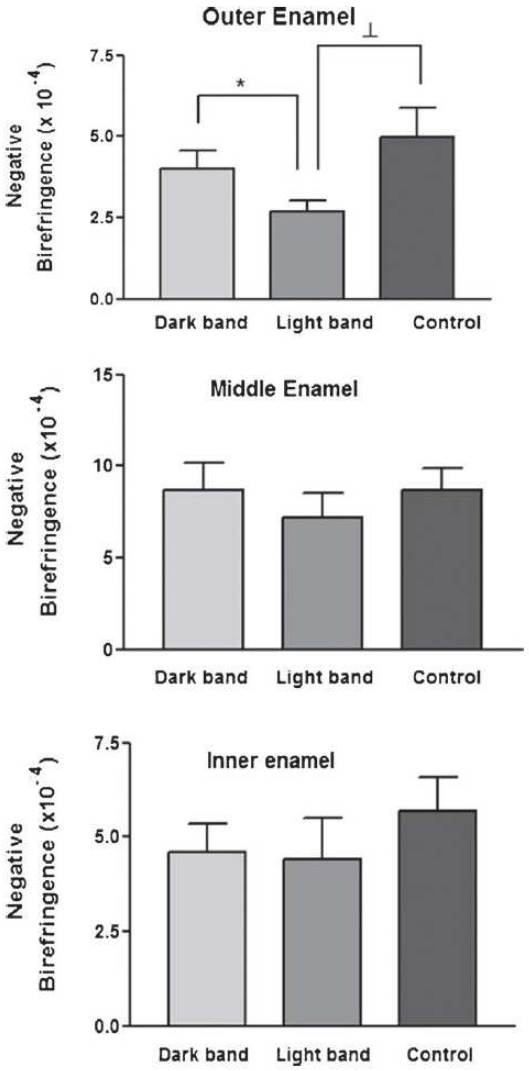

Fig. 4 – Birefringence values of sites in rat enamel related to the pigmented and white bands of fluorotic teeth and from control teeth, located in the outer, middle and inner enamel histological layers. Only in the outer layer were differences found, represented by a lower negative birefringence in the white bands relative to the pigmented bands/control areas. *$P < 0.05$ dark band *versus* light band; ⊥$P < 0.001$ light band *versus* control.

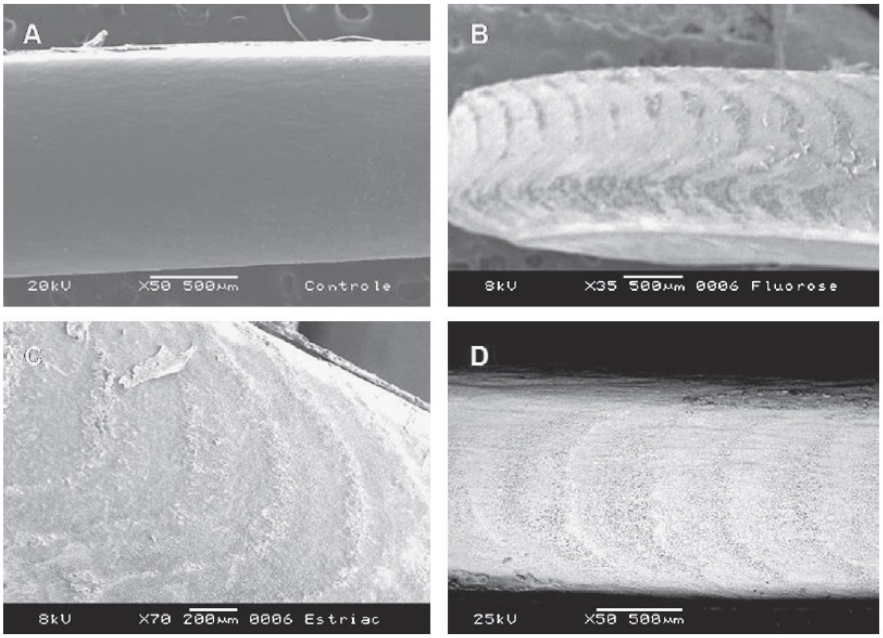

Fig. 5 – Panel of SEM (A–C) and BSE-SEM (D) photographs showing the surface of control (A) and fluorotic incisor enamel (B–D). (A–C) Phosphoric acid (37%) etching. (D) Without treatment. White bands are more susceptible to acid etching and show a rougher appearance when observed by SEM (C), while the pigmented bands are more resistant to acid and exhibit a brighter appearance by BSE-SEM, indicating that these pigmented bands have a more mineralized structure in comparison to the less bright bands, with increased organic content.

4. Discussion

Excess fluoride results in a pattern of repeated pigmented and white bands in the labial surface of rat incisors (Fig. 1B).

In an attempt to characterize the degree of fluorosis in the animals, fluoride was determined in the plasma, as well as in whole and surface bone. While plasma fluoride levels only doubled in the fluorosis group compared with the control (due to exposure to fluoride from other sources), fluoride concentration was 2.5 times higher in the whole bone and almost 10 times higher on the surface of the tibia. Therefore, surface bone may be a good marker of the amount of fluoride accumulated by the animals in studies in which this parameter is critical. In our case, the values obtained in this study will enable future reliable comparisons.

Acid etching followed by SEM analysis showed that pigmented bands are more resistant to acid attack, as already described.[16] This agrees with the interpretation that pigmented bands resemble the control enamel, which has the same colour and is also more resistant to acid. Our BSE-SEM findings showed that the white bands present a lower quantity of backscattered electrons; i.e., they are darker, which reflects a lower mean atomic number. This is probably due to a decrease in iron content and decreased mineralization. In a previous study, we demonstrated that enamel mineralized without post-secretory ameloblasts shows a whitish colour, due to absence of iron on the enamel surface, and a decrease in the enamel mineralization.[17]

For light microscopy and SEM analysis of inner enamel, the thinnest enamel (position "b" in Fig. 2) was chosen to obtain good reproducibility of ground sections perpendicular to the enamel surface. Our findings show that the rat incisor fluorotic lesions are superficial. This description is important for understanding of possible mechanism responsible for dental fluorosis, but it is in contrast with what has been previously described for the rat incisor fluorotic enamel.[9] Our longitudinal sections of fluorotic enamel under bright field and PLM (Fig. 3C and D) resemble published micrographs of fluorotic rat incisor enamel[9,10] which, in spite of authors' descriptions, do not give clear evidence of subsurface lesions. The careful grinding of midsagittal enamel sections was essential to avoid artifacts that could lead to an erroneous description of subsurface rat enamel fluorotic lesions resembling those that are well known in human fluorotic teeth, and do have a well-mineralized surface layer. Evidence of abnormal mineralization in the white bands of the rat incisor fluorotic enamel is important, because some hypotheses concerning fluorosis suggest that inner enamel proteins might be entrapped within the enamel by a more mineralized outer enamel layer in fluorotic teeth,[7] which is not the case with the rat incisor.

Our sections also revealed the fluorotic lesions as darker areas under light microscopy in bright field. These sections also displayed a higher positive birefringence in water and a lower negative birefringence under PLM in Thoulet's solution 1.62. The behaviour of the rat enamel birefringence mimics the

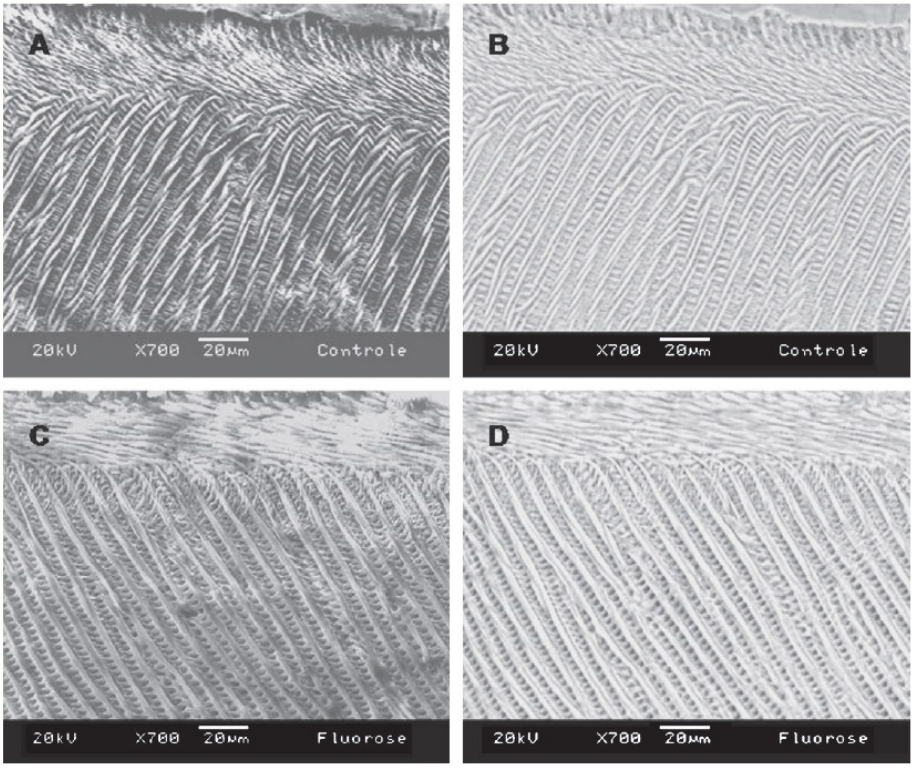

Fig. 6 – Panel of SEM (A and C) and BSE-SEM (B and D) photographs showing the internal aspect of a midsagittal section of the control (A and B) and the fluorotic enamel (C and D) after 37% phosphoric acid etching for 30 s (700×). No differences in the internal enamel could be observed between the groups.

behaviour of the birefringence of the body of the lesion of carious human enamel, which also presents positive sign in water and negative sign in Thoulet's 1.62.[15] It is also the same birefringence pattern that we found in a previous study, in which the absence of post-secretory ameloblasts caused a defect in enamel mineralization.[17]

Therefore, we decided to perform quantitative analysis of the birefringence of rat enamel in Thoulet's 1.62, which reasonably relates to the mineral content obtained by microradiography.[15] The outer layer of the white areas in fluorotic enamel showed a lower birefringence in Thoulet's 1.62, consistent with a lower mineral content.

The positive birefringence in water of both control and fluorotic rat enamel differs from that of mature sound human enamel, which presents negative birefringence in water.[18] This difference may be due to a lower mineral content in rat enamel compared with human enamel, leading to a higher contribution from the positive form of birefringence to the observed birefringence. This in turn results in a higher positive birefringence in water and a lower negative birefringence in Thoulet's 1.62, which is consistent with the interpretation that the outer layer of the white areas in fluorotic teeth can be regarded as hypomineralized.

These defects are visible in all specimens of satisfactorily prepared fluorotic rat incisors, and they clearly show that the defect is located in the outside enamel only. Observing fluorotic defects by PLM may be a very useful tool for researchers trying to better characterize these defects.

Another interesting finding of the present work is that data from control teeth suggest that the different histological layers present different mineral contents, with the middle enamel layer having the highest mineral content, followed by the inner layer and the outer enamel layer. This is in agreement with previous experimental data indicating that the outermost and the innermost 20 µm of rat enamel present the lowest mineral content, as shown by electron microprobe analysis.[19] The outer and inner layers analysed in our study were within the abovementioned locations. It is noteworthy that PLM is more sensitive to mineral loss than microradiography.[20]

As described in a previous study,[19] administration of different substances to rats induces a decrease in the mineral content of the outer enamel, suggesting that they may disturb normal enamel maturation and mineralization of the outer enamel, which is the last layer to mineralize.

In conclusion, the fluorotic lesions in the enamel of rat incisors displayed a pattern of repeated white and pigmented bands in the labial surface. We found evidences that under the fluorosis inducing conditions used here, the white bands of fluorotic enamel represent hypomineralized superficial areas

of the enamel. No subsurface lesions were identified when the midsagittal section of each tooth was analysed.

Acknowledgements

The authors thank the excellent technical assistance of Mrs. Ana Cláudia Gregolin Costa Miranda, Department of Diagnosis and Surgery, Dental School of Araraquara, UNESP; and Waldomiro Vieira Filho, Department of Biochemistry, Dental School of Piracicaba, FOP/UNICAMP, during preparation of the specimens and fluoride determinations, respectively. The authors state that there is no possible conflict of interest such as patent, ownership, stock ownership, consultancies or speaker's fee.

Funding: This study was supported by The State of Sao Paulo Research Foundation (FAPESP) and the (Brazilian) National Research Council (CNPq).

Competing interests: We state that none of the authors have any conflict of interests.

Ethical approval: University's Ethical Committee for Animal Research (CEEUA – USP, protocol number 01.1364.53.4).

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