Marcos Roberto dos Santos Frozoni

Cirurgião Dentista
Especialista em Endodontia

EFEITO DO FLÚOR NA ORGANIZAÇÃO SUPRAMOLECULAR DA MATRIZ ORGÂNICA DO ESMALTE DENTÁRIO EM CAMUNDONGOS

Dissertação apresentada à Faculdade de Odontologia de Piracicaba, da Universidade Estadual de Campinas, para obtenção do título de Mestre em Biologia Buco-Dental na área de Histologia e Embriologia.

Orientador: Prof. Dr. Sérgio Roberto Peres Line

Piracicaba
2008

FICHA CATALOGRÁFICA ELABORADA PELA
BIBLIOTECA DA FACULDADE DE ODONTOLOGIA DE PIRACICABA
Bibliotecário: Marilene Girello – CRB-8ª. / 6159

F939e

Frozoni, Marcos Roberto dos Santos.
Efeito do flúor na organização supramolecular da matriz orgânica do esmalte dentário em camundongos. / Marcos Roberto dos Santos Frozoni. -- Piracicaba, SP : [s.n.], 2008.

Orientador: Sérgio Roberto Peres Line.
Dissertação (Mestrado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Birrefringência. 2. Microscopia. I. Line, Sérgio Roberto Peres. II. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. III. Título.

(mg/fop)

Título em Inglês: Fluoride effect on supramolecular organization of dental enamel organic extracellular matrix of mice
Palavras-chave em Inglês (Keywords): 1. Birefringence. 2. Microscopy
Área de Concentração: Histologia e Embriologia
Titulação: Mestre em Biologia Buco-Dental
Banca Examinadora: Sérgio Roberto Peres Line, Victor Elias Arana-Chaves, Livia Maria Andaló Tenuta
Data da Defesa: 29-02-2008
Programa de Pós-Graduação em Biologia Buco-Dental

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A Comissão Julgadora dos trabalhos de Defesa de Dissertação de MESTRADO, em sessão pública realizada em 29 de Fevereiro de 2008, considerou o candidato MARCOS ROBERTO DOS SANTOS FROZONI aprovado.

PROF. DR. SERGIO ROBERTO PERES LINE

PROF. DR. VICTOR ELIAS ARANA CHAVES

PROFa. DRa. LÍVIA MARIA ANDALÓ TENUTA

À minha mãe, Mônica Maryse dos Santos Frozoni, pelo amor, força e coragem de sozinha ter dado continuidade aos ensinamentos que me mostraram o caminho da verdade e integridade.

Ao meu pai, Newton Silvestre Frozoni, que enquanto esteve entre nós dedicou sua vida exclusivamente ao amor e educação de seus filhos.

À minha futura esposa, Daniele Bonfante, que por dez anos me dedicou amor e apoio, para que eu pudesse trilhar os caminhos mais árduos.

AGRADECIMENTOS

Ao Prof. Dr. Sérgio Roberto Peres Line, pela valiosa orientação científica, simpática amizade e exemplo de ponderação e dedicação à vida docente.

Ao Prof. Dr. Waldocyr Simões, pelo paternal afeto e concreta orientação durante os meus primeiros passos na docência e pesquisa científica.

Ao Prof. Dr. Alexandre Ribeiro do Espírito Santo, pela orientação e presença constante no desenvolvimento de todas as fases deste experimento.

Aos professores do Departamento de Morfologia da Faculdade de Odontologia de Piracicaba (FOP) – UNICAMP, pelo respeito e dedicação que sempre a mim dispensaram em todos os momentos de dúvidas e necessidades.

Aos colegas do Programa de Pós-Graduação em Biologia Buco-Dental da FOP-UNICAMP, pela inesquecível amizade e agradável convívio em todos os nossos momentos de alegrias e dificuldades.

Aos colegas de docência da Faculdade de Odontologia de Araras: Prof. Cid Alonso Manicardi, Prof. Homero Cassonato Jr., Prof. Roger Fabiano Esteves e Prof. Alex Corrarelo, por estes longos anos juntos, dedicados a fazer o melhor possível para levar o conhecimento e educação aos nossos alunos.

A Cidinha, Eliene e Ivani, técnicas do Laboratório de Histologia do Departamento de Morfologia da FOP-UNICAMP, pelo ótimo convívio, grande amizade e fundamental ajuda na realização dos procedimentos laboratoriais do experimento sem a qual não seria possível a realização do mesmo.

Aos meus futuros sogros Antonio Benedito Bonfante e Marilda Carandina Bonfante pelos bons momentos passados juntos, respeito e consideração com que sempre me trataram.

Aos meus irmãos Isabela, Marco Antônio e Celso Antônio por estarmos conseguindo juntos, apesar das dificuldades, darmos continuidade aos ensinamentos de nossos pais.

Aos amigos de São Sebastião da Grama, que desde a infância sempre me dedicaram carinhosa amizade, grande respeito e momentos inesquecíveis.

A todos aqueles que de forma direta ou indireta colaboraram por mais esta realização de um sonho.

E principalmente a DEUS, presença constante em todos os meus passos.

RESUMO

A biossíntese do esmalte dentário inicia-se pela secreção, processamento proteolítico e auto-agregação de uma complexa mistura de proteínas**,** sintetizadas pelos ameloblastos, conhecida como matriz orgânica do esmalte. A formação desta matriz ocorre em três estágios: secreção (inicial), transição e maturação e parece ser fundamental para o controle da orientação e morfologia dos cristais de hidroxiapatita, que constituem a fase mineral do esmalte em desenvolvimento. No estágio de secreção da amelogênese, a matriz orgânica do esmalte apresenta uma organização supramolecular birrefringente, dessa forma, a referida matriz pode ser observada e quantificada por meio de microscopia de luz polarizada. Alterações genéticas e ambientais podem induzir a distúrbios na organização molecular da matriz orgânica extracellular do esmalte (MOECE) dentário no estágio secretório, gerando modificações em sua birrefringência, tais distúrbios podem contribuir para alterações na estrutura do esmalte maduro. Altos níveis de ingestão de flúor causam mudanças na estrutura e concentração das proteínas da matriz orgânica do esmalte, induzindo a falhas na mineralização e formação desorganizada dos cristais do esmalte. Estas alterações caracterizam a fluorose de esmalte e incluem aumento da porosidade, redução do conteúdo mineral e diminuição da microdureza do esmalte maduro. O objetivo deste estudo foi analisar os efeitos do flúor sobre a birrefringência da MOECE no estágio secretório. Quinze camundongos da linhagem A/J foram divididos em 3 grupos e submetidos a um tratamento de 30 dias com dieta exclusiva de ração e água deionizada *ad libitum*. A água ingerida continha 0, 25, e 50 ppm de flúor (NaF) nos grupos A/J-Controle, A/J-Flúor 25 ppm e A/J-Flúor 50 ppm, respectivamente. Os mesmos procedimentos foram aplicados a quinze camundongos da linhagem NOD (Non Obese Diabetic), caracterizando os grupos NOD-Controle, NOD-Flúor 25 ppm e NOD-Flúor 50 ppm. Após o período acima mencionado, todos os animais foram perfundidos com uma mistura de paraformaldeído 2% com glutaraldeído

0,5% em tampão fosfato 0,2 M e suas hemimaxilas foram extraídas e mantidas na mesma solução fixadora por 16h, as amostras foram então descalcificadas em mistura de ácido nítrico 5% com formaldeído 4 % por 6 h sob agitação. Após desidratação e inclusão em parafina, obtive-se cortes longitudinais de 5µm de espessura que foram desparafinizados, hidratados, montados em solução aquosa de glicerina 80% e analisados em microscopia de luz polarizada. Realizou-se a análise da matriz orgânica dos incisivos superiores de modo a se determinar o retardo óptico em nanômetros (nm) na área de maior birrefringência no estágio de secreção da amelogênese. Os valores de retardo ótico foram submetidos à análise estatística (Kruskal-Wallis) e os grupos A/J e NOD foram comparados separadamente. Observou-se um aumento, estatisticamente significante, dos valores de retardo ótico nos grupos A/J-Flúor 25 ppm e A/J-Flúor 50 ppm, quando comparados ao grupo A/J-Controle ($p<0,01$). O mesmo aconteceu com os grupos NOD-Flúor 25 ppm e NOD-Flúor 50 ppm que mostraram aumento, estatisticamente significante, dos valores de retardo ótico quando comparados ao grupo NOD-Controle ($p<0,01$). Os grupos A/J-Flúor apresentaram valores semelhantes ($p>0,05$) o que também ocorreu com os grupos NOD-Flúor. Os resultados do presente estudo mostram que o flúor induz a um aumento da birrefringência da MOECE no estágio de secreção, podendo estar associado ao mecanismo de desenvolvimento da fluorose de esmalte.

Palavras Chave: Amelogênese, Matriz orgânica extracelular do esmalte, Birrefringência, Flúor.

ABSTRACT

Dental enamel biosynthesis begins with secretion, proteolytic processing and self-assembly of a highly complex mixture of proteins, synthesised by ameloblast, which is known as the enamel organic matrix. This matrix formation occurs at three stages: secretion (initial), transition and maturation and seems to be essential for controlling orientation and morphology of the hydroxyapatite crystals that comprise mineral phase of developing enamel. In the secretory stage of amelogenesis, the enamel organic matrix presents a birefringent supramolecular organization. Therefore, it can be observed and quantified by polarizing microscopy. Genetic and environmental alterations may induce disturbances in the molecular organization of the secretory-stage enamel organic extracellular matrix (EOECM), producing birefringence changes, these disturbances may contribute to mature enamel alterations. High levels of ingested fluoride cause modifications in the structure and concentration of proteins of the enamel organic matrix inducing failures in the mineralization and disorganized enamel crystals formation. These alterations characterize enamel fluorosis, include increased porosity, mineral content reduction and diminished mature enamel micro hardness. The aim of the present study was to analyse the effects of fluoride on the birefringence of secretory stage EOECM. Fifteen A/J inbred mice strain were divided into 3 groups and submitted to a treatment during 30 days with exclusive diet of food and deionized water *ad libitum*. The ingested water contained 0, 25 and 50 ppm fluoride (NaF) in the groups A/J Control, A/J 25 ppm fluoride and A/J 50 ppm fluoride, respectively. The same procedures were applied to fifteen NOD (Non Obese Diabetic) mice, which formed the groups NOD Control, NOD 25 ppm fluoride and NOD 50 ppm fluoride. After the abovementioned period, all the animals were perfused with 2% paraformaldehyde, 0.5% glutaraldehyde in 0.2 M phosphate buffered solution. Its hemimaxillae were then extracted and maintained

in the same fixative solution for 16 h, the samples were decalcified under stirring in 5% nitric acid, 4% formaldehyde for 6 h. After dehydration and embedded in paraffin, longitudinal 5-µm-thick sections were obtained and deparaffined, hydrated and mounted with aqueous 80% glycerine as imbibing medium and analyzed with polarizing microscopy. Optical retardation (nm) of the area that showed the highest birefringence brightness in the EOECM of upper incisors was determined. Optical retardation values were submitted to statistical analysis (Kruskal-Wallis) and A/J and NOD groups were separately compared. An statistically significant increase in optical retardations values was observed in A/J 25 ppm Fluoride and A/J 50 ppm Fluoride, when compared to A/J Control group ($p<0.01$). The same happened with NOD 25 ppm Fluoride and NOD 50 ppm Fluoride groups which exhibited statistically significant increase in optical retardations values when compared to NOD Control group ($p<0.01$). A/J Fluoride groups presented similar optical retardation values ($p>0.05$) which occurred with NOD fluoride groups.

The results presented here show the fluoride induces an increase in the birefringence of secretory stage EOECM, which may be associated with enamel fluorosis development.

Key words: Enamel, Amelogenesis, Enamel Organic Matrix, Birefringence, Fluoride.

SUMÁRIO

INTRODUÇÃO

O esmalte é um tecido de origem ectodérmica, altamente mineralizado, que recobre os dentes dos vertebrados (Eisenmann, 1998) e a matriz extracelular que o origina é sintetizada e secretada por células especializadas do órgão dentário, os ameloblastos. O desenvolvimento do esmalte é caracterizado por interações complexas entre componentes desta matriz extracelular em fase orgânica, fase aquosa e fase mineral (Simmer & Fincham, 1995). Estas interações ocorrem em três estágios: o secretório, em que há deposição da matriz orgânica, concomitante com pequena quantidade de mineral; o de transição, onde se inicia a degradação protéica e aumenta muito a fase aquosa da matriz; e o de maturação, em que há extensa degradação da matriz orgânica e substituição por componente mineral (Robinson et al., 1979; Shore et al., 1993; Bronckers et al., 1995).

A matriz orgânica do esmalte no estágio secretório é principalmente formada por um grupo heterogêneo de proteínas chamadas de amelogeninas que constituem 90% do total das proteínas neste estágio da amelogênese (Termine et al., 1980). Em solução, as amelogeninas formam agregados globulares chamados “nanosferas” e as implicações biológicas desta característica podem estar relacionadas com a habilidade das amelogeninas interagirem com os cristais, extremamente finos, de hidroxiapatita na formação do esmalte e protegê-los de fusões prematuras (Fincham et al., 1999).

Além das amelogeninas, a fase orgânica da matriz contém outras proteínas estruturais e várias proteinases (Overall & Limeback, 1988). As proteinases da matriz de esmalte já identificadas pertencem às famílias das serino-proteinases e de metaloproteinases (Carter et al., 1989; Denbesten & Heffernan, 1989; Moradian-Oldak et al., 1994; Coletta, 1996; Fukae et al., 1998). A serino-proteinase encontrada é conhecida como calicreína-4 (KLK-4, EMSP-1) (Simmer et al., 1998). A outra proteinase do esmalte mais bem caracterizada é uma

metaloproteinase conhecida como enamelisina e classificada como MMP-20 (Bartlett et al., 1996; Bartlett et al., 1998; Bartlett & Simmer, 1999).

A remoção das amelogeninas, pelos ameloblastos, durante a maturação parece ser um passo crítico para a mineralização do esmalte e dependente da ação de proteinases (Bartlett & Simmer, 1999), estas amelogeninas normalmente são hidrolisadas de modo lento no estágio secretório, principalmente pela MMP-20, e de modo bastante intenso nos estágios de transição/maturação precoce, essencialmente pela KLK-4 (Smith & Nanci, 1996; Smith, 1998).

A associação linear das nanosferas de amelogeninas forma um arranjo em forma de fita (Moradian–Oldak et al., 2006) e toda esta estrutura supramolecular altamente organizada, principalmente no estágio de secreção avançada, é altamente birrefringente, vista ao microscópio de polarização (Espírito Santo et al., 2006; Espírito Santo et al., 2007). É plausível considerar que defeitos no esmalte maduro, possivelmente, estejam associados a alterações na organização supramolecular birrefringente de sua matriz orgânica nos estágios iniciais da amelogênese e a inibição das proteinases presentes na matriz do esmalte é uma hipótese atrativa para explicar defeitos do esmalte e achados bioquímicos de acúmulo de proteínas na referida matriz (Smith, 1998; Ryu et al., 1999).

A fluorose dentária está diretamente relacionada a várias formas de exposição ao flúor durante a formação do esmalte e se caracteriza por uma camada externa de esmalte normalmente mineralizada e uma camada subjacente hipomineralizada (Weatherell et al., 1975; Aoba, 1997) sendo, esta característica, descrita por vários autores como conseqüência de um retardo na remoção de proteínas da matriz durante a formação de esmalte fluorótico (Larsen et al., 1985; Richards et al., 1986; Denbesten, 1986).

Uma vez que a fluorose de esmalte é caracterizada por uma alteração na matriz orgânica do esmalte em desenvolvimento, oriunda do aumento na secreção protéica, diminuição da quantidade de proteinases ou da atividade destas (Denbesten & Heffernan, 1989; Denbesten & Thariani, 1992), ela contribui para alterações na mineralização do esmalte dentário.

Outros autores declaram que a mais aceitável hipótese para explicar uma alteração na matriz orgânica do esmalte, é que o flúor incorporado ao cristal de hidroxiapatita aumenta a adesão das proteínas de matriz ao cristal, resultando em uma proteólise reduzida e uma retenção prolongada das proteínas na fase de secreção e maturação da amelogênese (Robinson et al., 2006; Aoba & Fejerskov, 2002).

Diante de trabalhos divergentes acerca do efeito do flúor sistêmico na constituição da matriz orgânica do esmalte ou na atividade de proteinases desta matriz, permanecendo não esclarecido o mecanismo patogênico da fluorose dentária, estudos que abrangem relatos de alterações na organização da matriz e na morfologia dos cristais de esmalte fluorótico, tendem a ganhar maior suporte.

Sabendo que a matriz orgânica do esmalte dentário é altamente birrefringente (Espírito Santo et al., 2006; Espírito Santo et al., 2007) pretendeu-se no presente estudo verificar o efeito do flúor sobre a birrefringência desta matriz orgânica no estágio secretório da amelogênese em camundongos A/J e NOD.

A metodologia aqui apresentada foi aprovada pela Comissão de Ética na Experimentação Animal do Instituto de Biologia da Universidade Estadual de Campinas – UNICAMP (protocolo nº 1011-2.) (Anexo 1).

Esta tese está redigida em formato alternativo, apresentando um artigo submetido para publicação na revista *European Journal of Oral Sciences*. Nele, pode-se notar que o flúor induz a um aumento da birrefringência da matriz orgânica do esmalte no estágio secretório da amelogênese.

CAPÍTULO 1

Fluoride effect on the birefringence of secretory-stage enamel organic extracellular matrix of mice.

Artigo submetido para publicação na revista ***European Journal of Oral Sciences*** (Anexo 2).

Fluoride effect on the birefringence of secretory-stage enamel organic extracellular matrix of mice

Marcos Roberto dos Santos Frozoni,
Alexandre Ribeiro do Espírito Santo,
Jaime Aparecido Cury,
Sérgio Roberto Peres Line.

Department of Morphology, Piracicaba Dental School, State University of Campinas, Piracicaba, São Paulo, Brazil

Running title:

Birefringence of the enamel organic matrix of fluoride-treated mice.

Correspondence to:
Sérgio Roberto Peres Line
Departamento de Morfologia, Faculdade de Odontologia de Piracicaba - UNICAMP, Av. Limeira, 901, Areião, CEP 13414-903, Caixa Postal 52, Piracicaba-SP, Brasil. Telefax: +55-19-2106-5333. E-mail address: serglin@fop.unicamp.br

Frozoni MRS, do Espírito Santo AR, Cury JA, Line SRP.

Fluoride effect on the birefringence of secretory-stage enamel organic extracellular matrix of mice.

Eur J Oral Sci

Abstract

The formation of an ordered enamel organic extracellular matrix (EOECM) seems to be a crucial step for the proper formation of enamel mineral phase. The ordered supramolecular structure of the EOECM in the secretory-stage can be analyzed by polarizing microscopy, as it is strongly birefringent. High levels of fluoride (F) ingestion can interfere with the development of dental enamel, leading to increased porosity in mature enamel, which is known as enamel fluorosis. The aim of this study was to analyze the effects of F in the birefringence of the EOECM in the A/J and NOD (Non Obese Diabetic) inbred strains of mice given 0, 25 or 50 µg F/ml in drinking water. The results of the present study showed that experimental fluorosis leads to increased birefringence of the EOECM in the secretory stage of amelogenesis in both inbred mice strain.

Key words: Amelogenesis, Enamel organic extracellular matrix, Birefringence, Fluoride.

Correspondence to:

Sérgio Roberto Peres Line

Departamento de Morfologia, Faculdade de Odontologia de Piracicaba - UNICAMP, Av. Limeira, 901, Areião, CEP 13414-903, Caixa Postal 52, Piracicaba-SP, Brasil. Telefax: +55-19-2106-5333. E-mail address: serglin@fop.unicamp.br

Introduction

Fluoride is recognized worldwide an effective caries prophylactic agent. However, excessive intake of fluoride (F) during preeruptive tooth development induces physicochemical changes in the enamel which shows higher protein levels, reduction in mineral content, and consequent increased porosity and decrease in the hardness of the mature enamel, both in humans (1,2) and rats (3,4). Concentrations of fluoride in fresh water available in certain regions to human population oscillate from less than 0.1 ppm to > 100 ppm. The American Dental Association (ADA) recommends as optimal levels of F in drinking water 0.7 to 1.2 ppm (5), whereas the threshold for fluorosis risk in humans is in the range of 1.6 to 1.8 ppm from fresh water and others supplements as toothpaste (6). Fluoride ingestion is especially harmful to human dentition between 15-30 month-old, when dental enamel is been formed on unerupted permanent incisors (7,8). Several mechanisms have been proposed in order to explain the effects of F on enamel. It is known that F can affect ameloblast function (9) and interfere with the formation of enamel extracellular matrix components (10,11).

Polarizing microscopy has been extensively used for the study of macromolecular ordering and aggregational states of organic molecules present in cells and extracellular matrices. The ordered supramolecular structure manly formed by amelogenins' nanospheres in the enamel organic extracellular matrix (EOECM) can be analyzed by polarizing microscopy as it is strongly birefringent (12,13) .

The analysis of EOECM birefringence allows performing studies in tissue sections that may provide relevant quantitative and qualitative information on the effects of genetic and environmental factors on the supramolecular structure of EOECM and its association with enamel defects. In the present work, we analyzed the effects of high doses of fluoride on the birefringence of EOECM in mice.

Material and methods

EXPERIMENTAL DESIGN

This study is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA) and approved by the institutional Committee for Ethics in Animal Research (State University of Campinas – UNICAMP) with the protocol number 1011-2.

Fifteen (four-months-old) male and female mice of A/J inbreed strain with approximately 25 g were randomly divided into three groups and given either 0 (control group), 25 or 50 µg F/ml (as NaF) in deionized drinking water *ad libitum* during four weeks and the same was performed to fifteen (four-months-old) male and female mice of NOD (Non Obese Diabetic) inbreed strain

After treatment period, the animals were anesthetized with ketamine and intra cardiac perfused with 2% paraformaldehyde 0.5% glutaraldehyde in 0.2 M phosphate buffered saline solution (PBS), pH 7.2. Hemimaxillae were removed and then immersed in the same fixative solution for 16 h. The tibias of each animal were removed, wiped from soft tissue and immersed into the fixative solution for posterior bone F determination.

POLARIZING MICROSCOPY ANALYSES

The hemimaxillae were decalcified by immersion in 5% nitric acid and 4% formaldehyde for 6 h. After dehydration, the hemimaxillae were embedded in paraffin and 5 µm thick longitudinal serial sections of whole EOECM extension were obtained. The sections were treated with xylene for removal of the paraffin, and hydrated.

Unstained sections of the hemimaxillae from each animal were analyzed in order to determine optical retardation (nm) of the area that showed the highest birefringence brightness. Sections were immersed in 80% glycerine for 30 min and one measurement was performed in fifteen tissue sections of each animal. Optical

retardation measurements were performed using Leica DM LP polarizing microscope (Leica Microsystems) using a Brace-Köhler compensator (Wild Leitz, Wetzlar, Germany), and polychromatic light. The sections analysed with polarizing microscopy were also stained with haematoxylin and eosin and observed with bright field light microscopy.

BONE FLUORIDE CONCENTRATION

The entire tibias (whole) were removed from the fixative solution, rinsed in water and dried for 24 h at 90º C. They were powered and left at 90ºC for an additional 24 h, Duplicates of 5.0 mg (± 0.01) were weighed and transferred to plastic test tubes, to which 0.5 ml of 0.5 M hydrochloric acid (HCl) was added.

After 1 h, at room temperature and under agitation, 0.5 ml of TISAB (1.0 M acetate buffer, pH 5.0 with 1.0 M NaCl and 0.4 % cyclohexanediaminetetraacetic acid) was added. To this TISAB solution, NaOH was added at concentration of 0.5 M to neutralize the HCl. A specific electrode Orion 96-09, attached to an ion analyzer Orion EA-940, previously calibrated with various F standards (0.5-5.0 µg F/ml), and prepared under the same conditions as the samples, was used for the analysis. The results were expressed in µg F/g of bone and this determination was made according to BEZERRA DE MENEZES *et al* (14).

STATISTICAL ANALYSIS

Measurements were submitted to statistical analysis and the A/J and NOD groups independently compared. Since the optical retardations found here were non-parametrical, Kruskal-Wallis test was utilized to evaluate the EOECM data and the graphics were made using median as central tendency measure, with no expression of standard deviation. The significance limit was set at 5%, ($p<0.05$).

The data of F concentrations in bone were submitted to analysis of variance (ANOVA) followed by Bonferroni test. The significance limit was set at 5%, ($p<0.05$).

Results

EOECM BIREFRINGENCE

The birefringence of EOECM could be clearly observed in the decalcified tissue sections. The maximum birefringence occurs when prisms are aligned at approximately 45^0 with the polarizer and analyzer. Birefringence appears initially as a thin layer near dentin-enamel junction in the early secretory phase of amelogenesis, expanding progressively towards enamel surface. (Fig. 1, thin arrow). The strongest birefringence was observed in the late secretory stage when the ameloblast begins decrease its height and reduces the secretion of EOECM. In this region, enamel has already reached full thickness (Fig. 1, thick arrow). The optical retardations (nm) were recorded in the midst of late secretory stage, where the highest birefringence brightness was observed.

The late secretory-stage EOECM of A/J inbreed mice strain given 25 and 50 µg F/ml of fluoride in drinking water showed statically significant increase in optical retardation when compared with the A/J control group ($p= 0.000$, Fig. 2 and Fig. 4). The same occurred with NOD inbreed mice strain ($p= 0.000$, Figs. 3, and 5). Neither statically significant changes were noted in the optical retardation values of A/J fluorotic group (25 and 50 µg F/ml, $p = 0.365$), (Fig. 2C,D,E,F, and Fig. 4) nor in NOD fluorotic group (25 and 50 µg F/ml, $p = 0.0567$), (Fig. 3C,D,E,F; and Fig. 5).

The birefringence of EOECM was higher in NOD than A/J mice (Figs. 3 and 4). However, the median increase in birefringence brightness of fluorotic mice was higher in A/J (2.16 fold in 25 ppm and 2.53 fold in 50 ppm) than in NOD mice (1.4 fold in 25 and 50 ppm).

BONE FLUORIDE CONCENTRATION

Higher F concentrations were found in the bone of the groups treated with fluoride which differed statistically from the control group ($p<0.05$), but did not

between them ($p>0.05$). These findings were observed for both inbred mice strains (Figs. 6 and 7).

Discussion

The present study indicates that F ingestion in high levels increases significantly the birefringence of the EOECM in late secretory stage of amelogenesis. This increase in the birefringence was consistently found in animals of both inbred strains A/J and NOD, subjected to 25 and 50 µg F/ml in drinking water. During secretory phase, the enamel organic matrix is composed mainly by amelogenins, which account for more than 90% of the protein content in this tissue (2,15). These proteins can form globular structures known as nanospheres (16). Amelogenins' nanospheres have a tendency to assemble into collinear arrays forming an ordered supramolecular matrix (13,17), which accounts for the birefringence of the enamel organic matrix. In tissue sections with standardized thickness and imbibing medium, the strength of birefringence brightness will depend on the degree of structural organization of its components, i.e. more organized are the molecules higher is the birefringence brightness. It will also depend of the amount birefringent components in the light path as well. Therefore, the increase in birefringence observed in fluorotic EOECM can be due to an increase in the structural organization or in the amount of ordered organic components. Previous studies showed that F ingestion causes retention of amelogenin in late secretory and early maturation stages due a delay in its removal (2,10) The most accepted hypothesis is that F incorporation into hydroxyapatite crystals increases the bind of enamel proteins to the mineral phase resulting in reduced proteolysis and prolonged retention of proteins in enamel matrix (18). The results presented here are compatible with this hypothesis and add to the present knowledge showing that besides the increased amount of the organic material, ordered supramolecular structure of the EOECM is maintained in fluorotic mice.

The increase in the birefringence caused by F ingestion was higher in A/J than in NOD mice. A previous study showed that A/J strain is highly susceptible to

dental fluorosis, presenting a rapid onset and severe development of dental fluorosis when compared with other 11 inbred mouse strains (19). These results may suggest that the fluoride induced birefringence increase in the secretory-stage of EOECM could be associated to the susceptibility to dental fluorosis in mice strains.

No significant differences were observed in the birefringence between the fluorotic groups (25 and 50 µg F/ml) in both inbred strains. This finding can be explained by the metabolism of fluoride. When the amounts of fluoride overcome the tissue uptake capacity, a plateau is reached, and there would be no further increase in incorporation (14,20). In fact, there were no significant differences in bone fluoride concentrations between 25 and 50 µg F/ml groups.

Our findings showed that NOD inbred strain has EOECM at the late secretory stage of amelogenesis more birefringent than A/J inbred strain in all fluorotic groups (25 and 50 ppm) and even in the control group, although A/J inbred strain mice be considered a dental fluorosis susceptible mice strain (19), diabetes mellitus seems to increase the retentions of fluoride on tissues (21) and persons with diabetes mellitus seems to be more susceptible to dental fluorosis development (22, 23), however we have to take account that differences between related strains might be an indication of the actions of a small number of genes in determining susceptibly (19).

In both inbred mice strains the control group animals presented fair amounts of fluoride in bone, which was probably due to fluoride in diet (24) and by the fluoride incorporated in bone before the animals were given distilled water. Similar findings were also observed in other studies (14,19).

In previous studies we have considered the possibility that the ordered structure of EOECM is influenced by the presence of organized crystals that are immediately formed at early secretion stage of amelogenesis (12,13). Immediate fixation and demineralization can preserve this order in the remaining organic matrix. However, the crystals from fluorotic tissue are significantly rougher than controls at all stages of development and present morphological abnormalities

(25,26). This situation should contribute to disorganize the supramolecular assembly of amelogenin nanospheres and probably decrease the birefringence of EOECM at late secretory stage of amelogenesis. Since this was not observed here, it is likely that the ordered supramolecular arrangement of the EOECM occurs due to an intrinsically organized assembly of its organic components into collinear arrays, as it has been proposed by other authors (17,27).

Acknowledgements

This study was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP (04/10994-4) and Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq. MRS Frozoni was supported by CNPq.

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Figures and legends

Figure 1. Birefringence of the EOECM. Thin arrow indicates the beginning birefringence brightness in the early secretory stage of amelogenesis. Thick arrow indicates the area of the highest birefringence brightness (where optical retardation measurements have been conducted) in the late secretory stage of enamel formation.

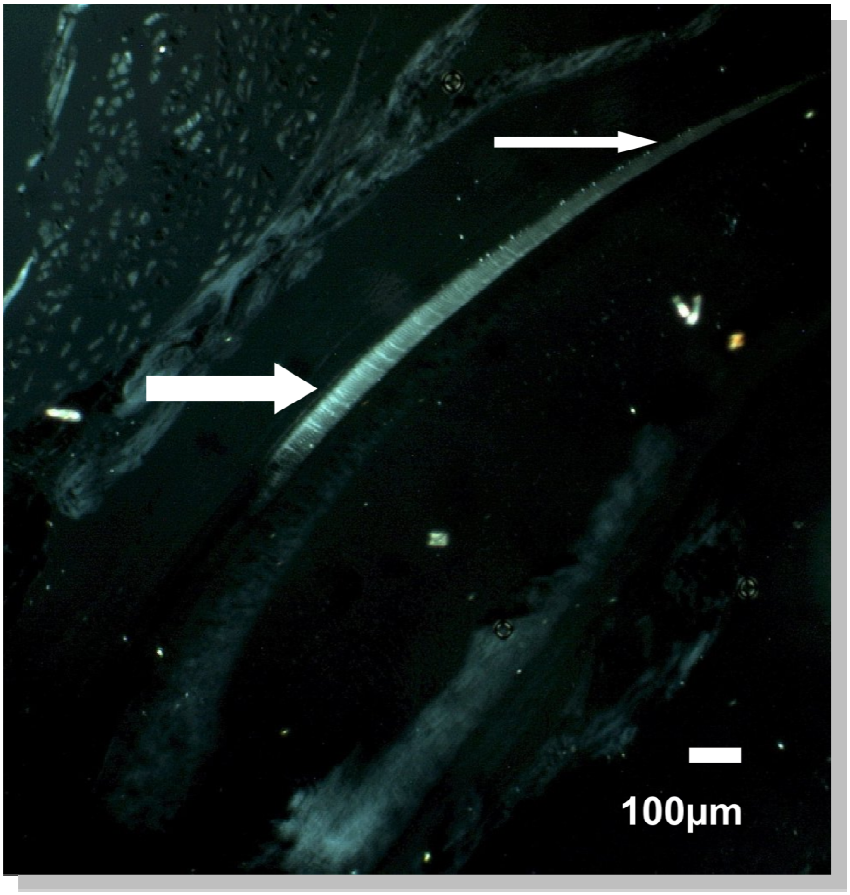


Figure 2. Birefringence micrographs of the secretory-stage EOECM from A/J mice upper incisor of unstained 5-µm section imbibed in 80% aqueous glycerine: **A.** A/J control group (0-ppm fluoride), **B.** Bright field of section A, after staining with H&E, **C.** A/J 25-ppm group, **D.** Bright field of section C, after staining with H&E, **E.** A/J 50-ppm group, **F.** Bright field of section E, after staining with H&E, Analyzer and polarizer are signalized by crossed bars and the arrow at 45^0 with the polarizer and analyzer indicates the position of maximum birefringence (Upper left corner). Note that birefringence brightness of 25-ppm and 50-ppm is increased when comparing to the A/J control group (0-ppm fluoride) indicating more organized material extracellular of EOECM (Kruskal-Wallis test $p<0.05$). Bars 100µm (Lower right corner).

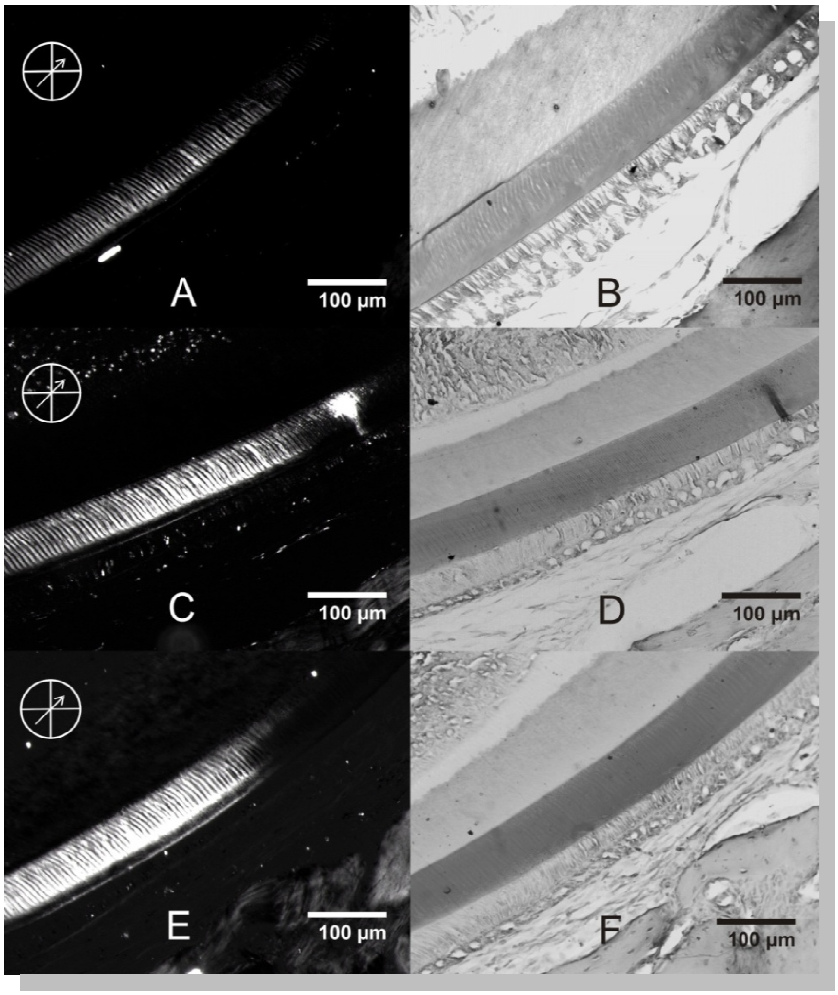


Figure 3. Birefringence micrographs of the secretory-stage EOECM from NOD mice upper incisor of unstained 5-µm section imbibed in 80% aqueous glycerine: **A.** NOD control group (0-ppm fluoride), **B.** Bright field of section A, after staining with H&E, **C.** NOD 25-ppm group, **D.** Bright field of section C, after staining with H&E, **E.** NOD 50-ppm group, **F.** Bright field of section E, after staining with H&E, Analyzer and polarizer are signalized by crossed bars and the arrow at 45^0 with the polarizer and analyzer indicates the position of maximum birefringence (Upper left corner). Note that birefringence brightness of 25-ppm and 50-ppm is increased when comparing to the NOD control group (0-ppm fluoride) indicating more organized material extracellular of EOECM (Kruskal-Wallis test $p<0.05$). Bars 100µm (Lower right corner).

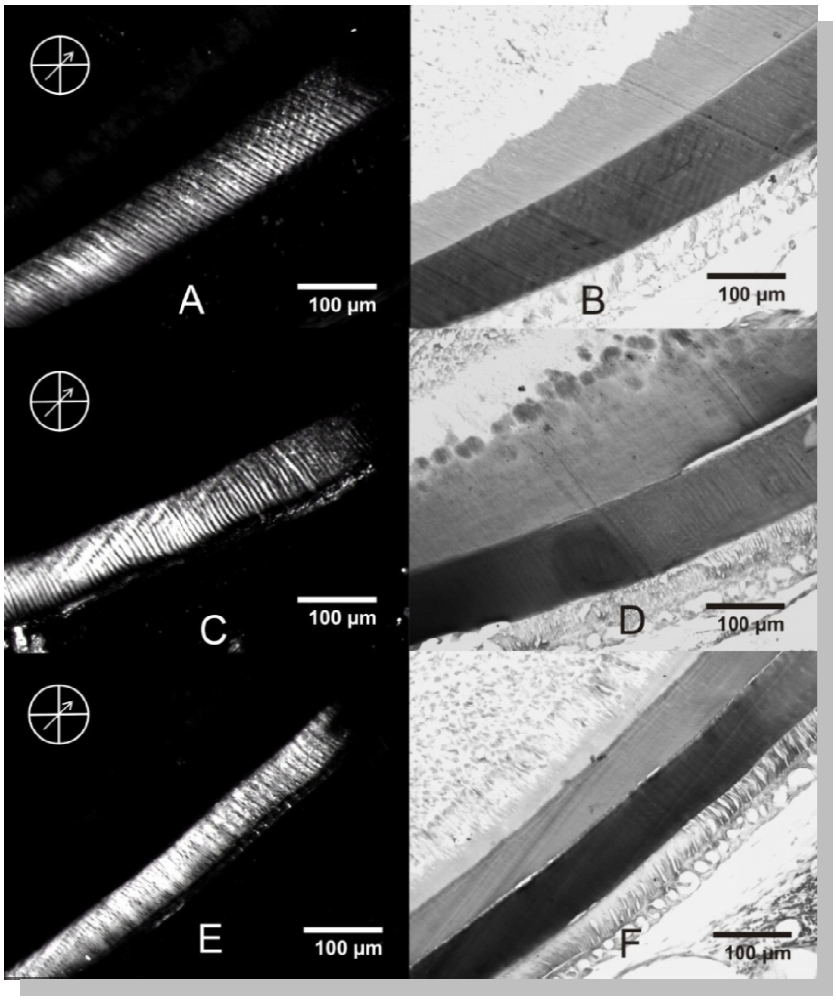


Figure 4. Optical retardations (nm) of unstained 5 µm-sections imbibed in 80% aqueous glycerine from the secretory-stage EOECM of A/J inbred mice strain group. Different letters express statistical difference. Note that fluoride treatment increased optical retardation median values of birefringence brightness.

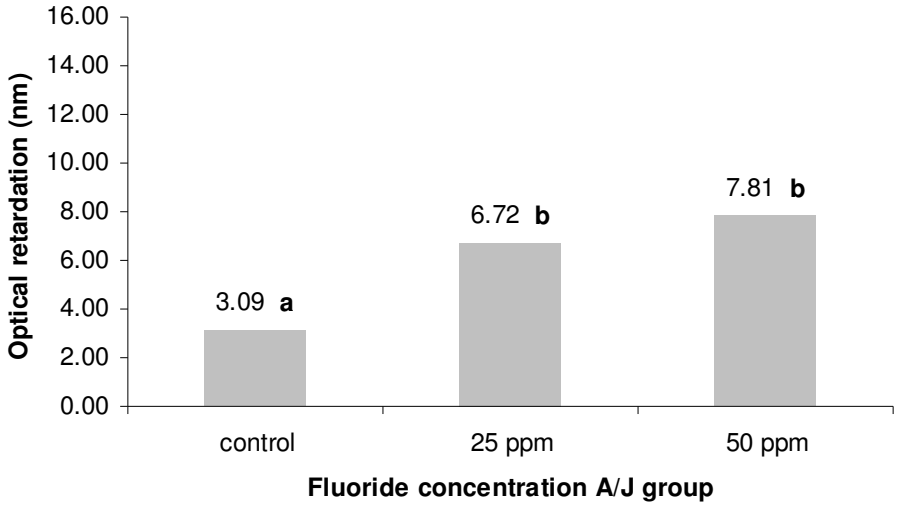


Figure 5. Optical retardations (nm) of unstained 5 µm-sections imbibed in 80% aqueous glycerine from the secretory-stage EOECM of NOD inbred mice strain group. Different letters express statistical difference. Note that fluoride treatment increased optical retardation median values of birefringence brightness.

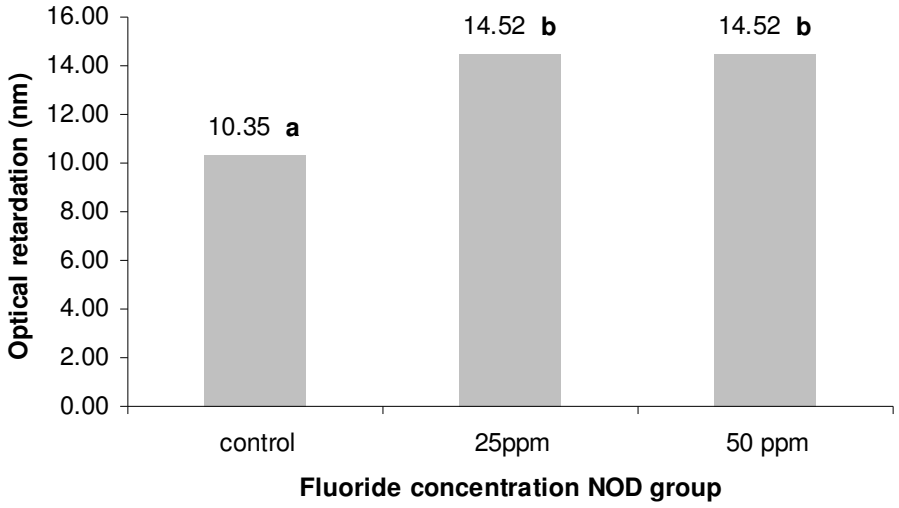


Figure 6. Means of fluoride concentration (µg F /g of dry weight) in tibias of A/J inbred strain mice according to treatments. Different letters express statistical difference between treatment groups ($p<0.05$) and bars the SD.

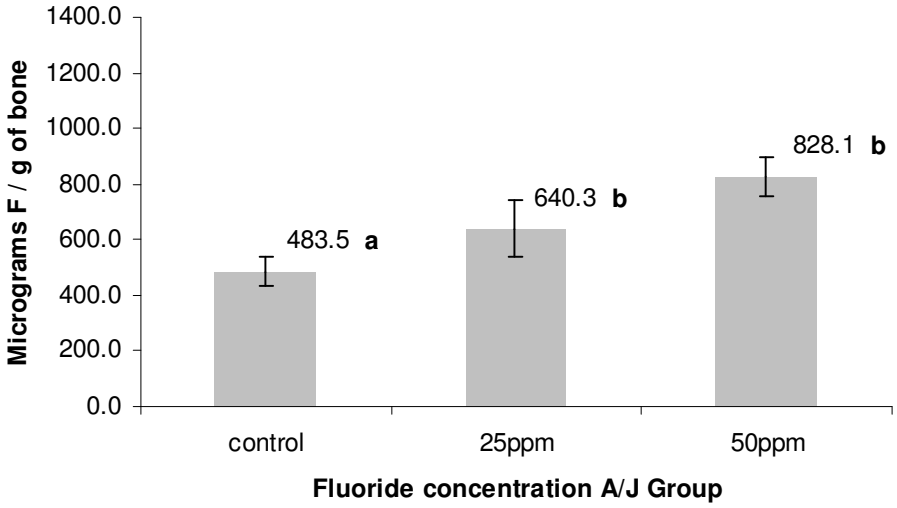
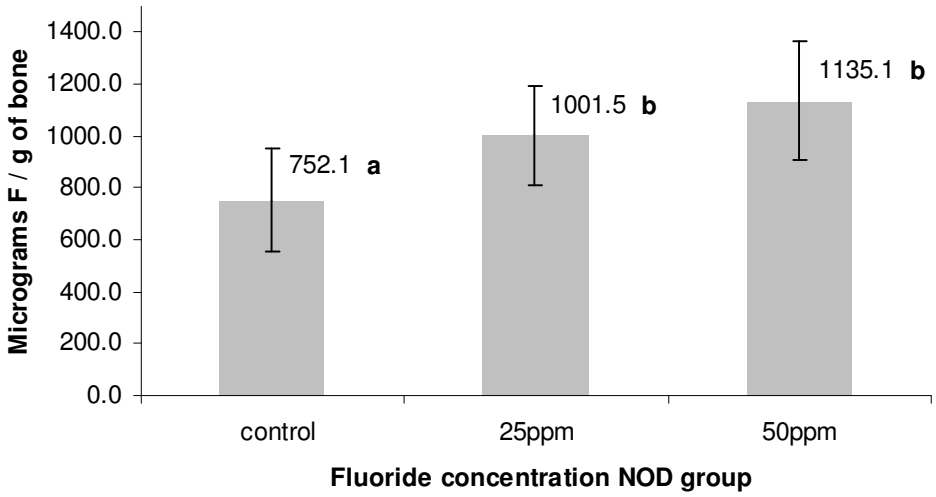


Figure 7. Means of fluoride concentration (µg F /g of dry weight) in tibias of NOD inbred strain mice according to treatments. Different letters express statistical difference between treatment groups ($p<0.05$) and bars the SD.



CONCLUSÃO

Os achados deste estudo dão suporte as seguintes conclusões:

1- A fluorose experimental não inibiu a birrefringência da matriz orgânica extracelular do esmalte dentário no estágio secretório da amelogênese em camundongos da raça A/J e NOD.

2- A fluorose experimental está associada ao aumento da birrefringência na matriz orgânica extracelular do esmalte dentário no estágio secretório da amelogênese em camundongos da raça A/J e NOD.

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* De acordo com as normas da UNICAMP/FOP, de acordo com o *International Committee of Medical Journal Editors* - Grupo de Vancouver. Abreviatura dos periódicos em conformidade com o Medline.

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ANEXOS

ANEXO 1

UNICAMP

Universidade Estadual de Campinas
Instituto de Biologia

CEEA-IB-UNICAMP

Comissão de Ética na Experimentação Animal
CEEA-IB-UNICAMP

CERTIFICADO

Certificamos que o Protocolo nº **1011-2**, sobre **"EFEITO DO FLÚOR NA ORGANIZAÇÃO DA MATRIZ EXTRACELULAR DO ESMALTE DENTAL EM CAMUNDONGOS"** sob a responsabilidade de **Prof. Dr. Sérgio Roberto Peres Line / Marcos Roberto dos Santos Frozoni**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética na Experimentação Animal (CEEA)-IB-UNICAMP em 30 de outubro de 2006.

CERTIFICATE

We certify that the protocol nº **1011-2**, entitled **"FLUORIDE EFFECT ON DENTAL ENAMEL EXTRACELLULAR MATRIZ ON MICE"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - UNICAMP) on October 30, 2006.

Campinas, 30 de outubro de 2006.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

Fátima Alonso
Secretária Executiva

CEEA/IB – Unicamp
Caixa Postal 6109
13083-970 Campinas, SP – Brasil

Telefone: (19) 3788-6359
Telefax: (19) 3788-6356
E-mail: ceea@cemib.unicamp.br
http://www.ib.unicamp.br/institucional/ceea/index.htm

ANEXO II

European Journal of Oral Sciences

European Journal of Oral Sciences

Fluoride effect on the birefringence of secretory-stage enamel organic extracellular matrix of mice

Journal:	*European Journal of Oral Sciences*
Manuscript ID:	EOS-3318-MAN-08
Manuscript Type:	Original Manuscript
Date Submitted by the Author:	12-Feb-2008
Complete List of Authors:	Frozoni, Marcos; Piracicaba Dental School/UNICAMP, Morphology do Espirito Santo, Alexandre; Piracicaba Dental School/UNICAMP, Morphology Cury, Jaime; Piracicaba Dental School/UNICAMP, Biochemistry Line, Sergio R. P.; Piracicaba Dental School/UNICAMP, Morphology
Research Area:	Animal experiment, Structural biology
Keywords (Please write 3 to 5 keywords according to Index Medicus):	Amelogenesis, Enamel organic extracellular matrix, Birefringence, Fluoride

European Journal of Oral Sciences