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EFEITOS DE ALTERAÇÕES GENÉTICAS E AMBIENTAIS SOBRE A BIRREFRINGÊNCIA DA MATRIZ ORGÂNICA DO ESMALTE DENTÁRIO

Tese apresentada à 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: Prof. Dr. Sérgio Roberto Peres Line

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RESUMO

O esmalte envolve a porção coronária dos dentes e constitui a estrutura mais mineralizada do corpo vertebrado. Seu desenvolvimento tem início com secreção, processamento proteolítico e auto-agregação de uma complexa mistura de proteínas. O estabelecimento de uma matriz orgânica ordenada parece ser fundamental para a formação adequada da fase mineral do esmalte. Microscopia de luz polarizada mostra que a matriz orgânica do esmalte secretório (MOES) apresenta-se fortemente birrefringente em cortes não corados de 5 µm de espessura. Esta propriedade reflete alto grau de organização em nível molecular com possível relevância funcional. Alterações no brilho de birrefringência da MOES podem indicar desordens moleculares e estar associadas a alterações na formação da fase mineral. Atraso no processo de fixação da MOES pode levar a uma rápida perda de sua birrefringência, comprometendo o seu estudo por meio de microscopia de luz polarizada. No presente trabalho, analisaram-se os efeitos do nocauteamento dos genes *Amelx* e *Mmp20* (experimento 1), dos bisfosfonatos (experimento 2) e de inibidores de serina proteinases e metaloproteinases (experimento 3) sobre a birrefringência da MOES. O experimento 1 mostrou que camundongos fêmeas $Amelx^{+/-}$ apresentam redução significativa no brilho de birrefringência quando comparados aos animais $Amelx^{+/+}$ do mesmo gênero ($p=0,0029$). A MOES dos camundongos fêmeas $Amelx^{-/-}$ não exibiu birrefringência. Os camundongos $Mmp20^{-/-}$ mostraram uma expressiva diminuição nos valores de retardo ótico em comparação aos camundongos $Mmp20^{+/+}$ e $Mmp20^{+/-}$ ($p=0,0000$). Os animais $Mmp20^{+/+}$ e $Mmp20^{+/-}$ apresentaram birrefringência semelhante ($p=1,0000$). O experimento 2 mostrou que ratos tratados com alendronato de sódio não apresentam alterações morfológicas na MOES, mas exibem diminuição expressiva no brilho de birrefringência quando comparados a ratos controles ($p<0,01$). Interessantemente, os ratos tratados com etidronato dissódico apresentaram alterações morfológicas severas na MOES, mas mostraram brilho de birrefringência na matriz secretada semelhante ao dos ratos controles ($p>0,05$).

O experimento 3 mostrou que a fenantrolina (inibidora de metaloproteinases, como a Mmp20) e o fenilmetilsulfonil fluoreto (inibidor de serina proteinases, como a Klk-4) preservam a birrefringência da MOES *ex vivo*. Os resultados aqui apresentados sugerem que: 1) a birrefringência da MOES depende da organização supramolecular das amelogeninas e é influenciada pela atividade proteolítica da Mmp20; 2) o alendronato de sódio pode induzir alterações quantitativas na organização supramolecular da MOES; 3) o etidronato dissódico não altera a ordem molecular da matriz orgânica do esmalte secretada e pode induzir defeitos no esmalte maduro através de interferência direta sobre a atividade secretora dos ameloblastos; 4) a rápida perda de birrefringência da MOES em amostras não imediatamente fixadas é resultante da atividade de proteinases do esmalte.

Palavras-chave: Esmalte dentário, Birrefringência, Bisfosfonatos, Fenantrolina, Fenilmetilsulfonil fluoreto.

ABSTRACT

Enamel covers dental crown and is the most mineralized structure in the vertebrate body. Its development begins with the secretion, processing and self-assembly of a complex mixture of proteins. The establishment of an ordered organic matrix seems to be a crucial step for the proper formation of enamel mineral phase. Polarizing microscopy shows that the secretory-stage enamel organic matrix (SEOM) is strongly birefringent in non-stained 5 µm-thick sections. This property indicates high level of molecular organization, which may be physiologically important. Changes in SEOM birefringence brightness may reflect molecular disorders and may be associated with alterations in the forming enamel mineral phase. Delay in fixation of SEOM may lead to rapid loss of birefringence, impairing analysis of that tissue with polarized light microscopy. In the present work, we analyzed the effects of *Amelx* and *Mmp20* knocking out (experiment 1), bisphosphonates (experiment 2) and metallo and serine proteinases' inhibitors (experiment 3) on SEOM birefringence. Experiment 1 showed that $Amelx^{+/-}$ female mice exhibit a significant reduction in the birefringence brightness when compared to $Amelx^{+/+}$ female animals. ($p=0.0029$). The SEOM from $Amelx^{-/-}$ female mice did not show birefringence. $Mmp20^{-/-}$ mice presented an expressive reduction in the optical retardation values in comparison to $Mmp20^{+/+}$ and $Mmp20^{+/-}$ animals ($p=0.0000$). $Mmp20^{+/+}$ and $Mmp20^{+/-}$ mice presented similar birefringence ($p=1.0000$). Experiment 2 showed that rats treated with sodium alendronate do not present morphological alterations in the SEOM, but exhibit significant decrease in the birefringence brightness when compared to control rats ($p<0.01$). Interestingly, bisodic etidronate rats showed severe morphological alterations in the SEOM, but exhibited SEOM birefringence brightness similar to that observed in control rats ($p>0.05$). Experiment 3 evidenced that 1,10-phenanthroline (metalloproteinase inhibitor) and phenylmethylsulphonyl fluoride (serine proteinase inhibitor) preserve SEOM birefringence brightness *ex vivo*. The results presented here support the following conclusions: 1) SEOM birefringence results from amelogenin

supramolecular organization and is influenced by proteolytic activity of Mmp20; 2) sodium alendronate can induce quantitative changes in the supramolecular organization of the SEOM; 3) bisodic etidronate does not disturb molecular order of the secreted enamel organic matrix and may induce changes in mature enamel by affecting directly secretory activity of ameloblasts; 4) rapid loss of birefringence in no immediately fixed SEOM is caused by the activity of enamel proteinases.

Key Words: Dental enamel, Birefringence, Bisphosphonates, Phenanthroline, Phenylmethylsulphonyl Fluoride.

SUMÁRIO

INTRODUÇÃO

O esmalte envolve a porção coronária dos dentes, constitui a estrutura mais mineralizada do corpo vertebrado e contém os maiores cristais de hidroxiapatita biologicamente produzidos (Eisenmann, 1998; Paine & Snead, 1997). Em humanos, estes cristais estão organizados formando uma arquitetura prismática altamente complexa. Defeitos na formação do esmalte estão entre as alterações mais comuns da dentição humana e aumentam a predisposição à cárie (Ellwood & O'Mullane, 1996). Dessa forma, torna-se relevante o estudo da influência de alterações genéticas e ambientais sobre o desenvolvimento do referido tecido.

O esmalte dentário é produzido por células epiteliais denominadas ameloblastos. Sua formação ocorre extracelularmente, sendo dependente de secreção, processamento proteolítico e auto-agregação de uma complexa mistura de proteínas. O crescimento, a morfologia e a organização dos cristais de hidroxiapatita do esmalte parecem ser regulados por interações com a referida mistura de proteínas, conhecida como matriz orgânica do esmalte (Moradian-Oldak *et al.*, 2003; Fincham *et al.*, 2000; Moradian-Oldak *et al.*, 1994; Moradian-Oldak *et al.*, 1998; Gibson *et al.*, 2001; Paine et al., 2003; Paine *et al.*, 2000; Ravassipour *et al.*, 2000; Caterina *et al.*, 2002; Collier *et al.*, 1997; Lench & Winter, 1995).

A matriz orgânica do esmalte é composta principalmente pelas amelogeninas, proteínas estruturais que se reúnem para formar estruturas globulares conhecidas como nanosferas, que por sua vez se agrupam dando origem a uma estrutura supramolecular organizada (Eastoe, 1979; Ravindranath *et al.*, 2004; Fincham *et al.*, 1994; Fincham *et al.*, 1999). O estabelecimento desta estrutura parece ser fundamental para a formação adequada da fase mineral, uma

vez que o crescimento de cristalitos de esmalte *in vitro* ocorre paralelamente a polímeros de nanosferas de amelogeninas (Beniash *et al.*, 2005; Moradian-Oldak *et al.*, 2006). As principais proteinases da matriz do esmalte são a enamelisina (MMP-20) e a calicreína-4 (KLK-4) (Simmer & Hu, 2002). A MMP-20, presente nos estágios iniciais e intermediários da amelogênese, remove a porção carboxi-terminal da amelogenina gerando o fragmento *TRAP* (*Tyrosine-Rich Amelogenin Polypeptide*) (Ryu *et al.*, 1999), que permanece entre os cristais até o seu completo crescimento em altura evitando fusões prematuras (Moradian-Oldak *et al.*, 2003). Posteriormente, a KLK-4, presente no estágio de maturação, degrada as amelogeninas e outras proteínas do esmalte, facilitando a remoção das mesmas e permitindo um rápido crescimento dos cristais em largura, levando ao endurecimento do esmalte (Moradian-Oldak *et al.*, 1998).

O ponto de partida a realização do presente trabalho de tese foi a descoberta de que a organização supramolecular da matriz orgânica do esmalte pode ser avaliada por meio de microscopia de luz polarizada, uma vez que a referida estrutura apresenta-se fortemente birrefringente em cortes não corados de 5 µm de espessura (do Espírito Santo *et al.*, 2006). A birrefringência é uma propriedade anisotrópica que reflete alto grau de organização em nível molecular, com possível relevância funcional. Portanto, é plausível inferir que alterações no brilho de birrefringência da matriz orgânica do esmalte secretório possam refletir certo grau de desordem molecular, que por sua vez represente um ponto de partida para alterações na fase mineral do esmalte em formação.

Os bisfosfonatos são drogas que apresentam estrutura química análoga à do pirofosfato, ligam-se avidamente ao osso e inibem a reabsorção óssea osteoclástica (Fleisch, 1987). Esta propriedade os torna úteis para o tratamento de desordens esqueléticas como osteoporose, doença de Paget, osteogênese imperfeita e tumores ósseos metastáticos (Fleisch, 1998; Vasikaran, 2001). Os bisfosfonatos agem terapeuticamente causando efeitos tóxicos sobre os osteoclastos ou interferindo em mecanismos intracelulares específicos destas

células (Fleisch, 1997; van Beek *et al.*, 1999; van Beek *et al.*, 2002). Defeitos na mineralização do esmalte são possíveis efeitos adversos destas drogas (Simmelink, 1987; Wakamatsu, 1991; Weile *et al.*, 1990; Weile *et al.*, 1993; Yamada *et al.*, 2000; Fuangtharnthip et al., 2000). Apesar do mecanismo de ação dos bisfosfonatos ter sido exaustivamente estudado no tecido ósseo, seus efeitos sobre a formação do esmalte dentário permanecem pouco conhecidos.

A birrefringência da matriz orgânica do esmalte em fase de secreção pode ser completamente perdida como resultado de atraso ou falhas no processo de fixação das amostras (do Espírito Santo *et al.*, 2006). Incisivos de ratos devem ser imediatamente imersos em solução fixadora após extração e hemimandíbulas devem ser perfundidas para preservação adequada da matriz orgânica do esmalte a ser analisada em microscopia de luz polarizada (do Espírito Santo *et al.*, 2006).

Tomando-se como base as considerações dos parágrafos anteriores, três questionamentos foram elaborados:

1. A birrefringência da matriz orgânica do esmalte secretório é resultante da auto-agregação das amelogeninas e influenciada pela atividade proteolítica da MMP-20?

2. A exposição aos bisfosfonatos pode causar alterações na birrefringência da matriz orgânica do esmalte secretório e desta forma contribuir para defeitos no esmalte maduro?

3. A rápida perda da birrefringência da matriz orgânica do esmalte secretório de dentes não imediatamente fixados pode ser resultado da atividade proteolítica de enzimas da própria matriz?

Buscando respostas para estes três questionamentos, o presente trabalho teve como objetivos: 1) analisar a birrefringência da matriz orgânica do esmalte secretório e sua relação com a microdureza do esmalte maduro em camundongos *knockout* para os genes *Amelx* e *Mmp20*; 2) investigar a birrefringência da matriz orgânica do esmalte secretório e sua associação com a

microdureza e com a topografia superficial do esmalte maduro em ratos tratados com bisfosfonatos; 3) estudar o efeito de inibidores de serina proteinases e metaloproteinases *in situ* sobre a birrefringência da matriz orgânica do esmalte. Os desenhos experimentais do presente estudo foram aprovados pela Comissão de Ética na Experimentação Animal do Instituto de Biologia da Universidade Estadual de Campinas – IB-UNICAMP (protocolo n^{o} 1123-1) (ANEXO 1).

Esta tese está redigida de forma sucinta em três capítulos.

O primeiro capítulo, um artigo publicado na revista *Connective Tissue Research*, comprova que a birrefringência da matriz orgânica do esmalte secretório está relacionada com a organização supramolecular das amelogeninas e é influenciada pela atividade proteolítica da MMP-20.

O segundo capítulo, um artigo submetido para publicação na revista *Arquives of Oral Biology* (ANEXO 2), mostra que o alendronato de sódio, uma droga pertencente ao grupo dos bisfosfonatos, pode induzir alterações quantitativas na organização supramolecular da matriz orgânica do esmalte no estágio de secreção da amelogênese.

O terceiro capítulo, um artigo em vias de submissão para publicação na revista *European Journal of Oral Sciences*, confirma que a rápida perda da birrefringência em amostras não imediatamente fixadas de matriz orgânica do esmalte secretório é resultante da atividade proteolítica de enzimas da própria matriz. Além disso, o referido artigo mostra que a atividade proteolítica da enzima KLK-4 pode ser dependente de uma ação prévia da MMP-20 sobre as moléculas de amelogenina.

CAPÍTULO 1

Amelogenin- and enamelysin (Mmp-20)-deficient mice display altered birefringence in the secretory-stage enamel organic extracellular matrix.

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Amelogenin- and Enamelysin (Mmp-20)-Deficient Mice Display Altered Birefringence in the Secretory-Stage Enamel Organic Extracellular Matrix

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Dental enamel is the most mineralized tissue of vertebrate organisms. Enamel biosynthesis is initiated by the secretion, processing, and self-assembly of a complex mixture of proteins. The formation of an ordered enamel organic extracellular matrix (ECM) seems be a crucial step for the proper formation of mineral phase. Polarizing microscopy demonstrates that the ordered supramolecular structure of the secretory-stage enamel organic ECM is strongly birefringent. In the present work we analyzed the birefringence of secretory-stage enamel organic ECM in amelogenin (*Amelx*)- and enamelysin (*Mmp20*)-deficient mice. Female $Amelx^{+/-}$ animals showed significant reduction in optical retardation values when compared with the $Amelx^{+/+}$ subgroup ($p = 0.0029$). The secretory-stage enamel organic ECM of the $Amelx^{-/-}$ subgroup did not exhibit birefringence. The secretory-stage enamel organic ECM of $Mmp20^{-/-}$ mice showed a significant decrease in optical retardation as compared with $Mmp20^{+/+}$ and $Mmp20^{+/-}$ mice ($p = 0.0000$). $Mmp20^{+/-}$ and $Mmp20^{+/+}$ mice exhibited similar birefringence ($p = 1.0000$). The results presented here support growing evidence for the idea that the birefringence of secretory-stage enamel organic ECM is influenced by the ordered supramolecular organization of its components.

Keywords Amelogenin, Birefringence, Enamel Organic Extracellular Matrix, Enamelysin, Knockout

INTRODUCTION

Enamel biosynthesis is initiated by the secretion, processing, and self-assembly of a complex mixture of proteins. These are proteolytically cleaved and replaced by mineral ions that deposit to form fluoride containing carbonated apatite crystals. Enamel mineralization develops further with the thickening of these crystals, to give rise to the hardest mineralized tissue in the vertebrate body [1, 2]. In the case of rat incisor, these events take place in a time- and space-restricted fashion, as distinct zones of secreting, maturing, and mature enamel are noted depending on the developmental stage of the tooth [3].

The organization and growth of the hydroxyapatite crystals seems to be strictly regulated by interactions with the components of enamel organic extracellular matrix (ECM) [4–13].

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Enamel organic ECM is composed mainly of amelogenins [14]. Amelogenin molecules can assemble to form structures referred to as nanospheres that can further assemble to form an organized supramolecular structure [15–17]. The formation of an ordered enamel organic ECM seems to be a crucial step for the proper formation of the mineral phase as the growth of enamel crystallites in solution, in the presence of assembled amelogenin nanospheres, is organized parallel to their c-axial direction [18, 19]. It is plausible to assume that disturbances in the supramolecular organization of enamel organic ECM at the early phases of formation can lead to severe alterations in mature enamel.

Polarizing microscopy has been extensively used for the study of macromolecular ordering and aggregational states of organic molecules present in the cell and ECM. The ordered supramolecular structure of the secretory-stage enamel organic ECM can be analyzed by polarizing microscopy as it was shown to be strongly birefringent [20]. The analysis of enamel organic ECM birefringence allows performing *in situ* studies in tissue sections. Thus, we can obtain valuable quantitative and qualitative information on the effects of genetic mutations on the supramolecular structure of enamel organic ECM and its association with enamel defects. In the present work we analyzed the birefringence of secretory-stage enamel organic ECM in amelogenin (*Amelx*)- and enamelysin (*Mmp*-20)-knockout mice.

MATERIALS AND METHODS

Animals and Tissue Preparation

Thirty female mice of 8 weeks age were used in this study. Animals were divided into 6 subgroups ($Amelx^{+/+}$, $Amelx^{+/-}$, $Amelx^{-/-}$, $Mmp20^{+/+}$, $Mmp20^{+/-}$, and $Mmp20^{-/-}$). Generation of amelogenin and enamelysin knockout mice were previously described by Gibson et al. [7] and Caterina et al. [11], respectively. Animals were anesthetized with ketamine and perfused with 2% paraformaldehyde, and 0.5% glutaraldehyde in 0.2 M phosphate-buffered saline (PBS), pH 7.2. Hemimaxillae were then immersed in fixative solution for 72 hr. Incisor teeth of each hemimaxillae were sectioned transversely at the level of alveolar bone crest, using a hard tissue microtome. Mineralized samples were used for enamel cross-sectional microhardness testing. Decalcification was performed by immersion of hemimaxillae in 5% nitric acid, and 4% formaldehyde for 12 hr. After dehydration, decalcified samples were embedded in paraffin, and 5-μm thick longitudinal sections were obtained. The sections were treated with xylene for removal of the paraffin and hydrated.

Polarizing Light Microscopy of Secretory-Stage Enamel Organic Matrix

Unstained sections of the hemimaxillae from each animal were analyzed to determine optical retardation (nm) of the area that showed the highest birefringence brightness in secretory-stage enamel. Sections were immersed in 80% glycerin for 30 min and 40 measurements were performed in each tooth. A Leica DM LP microscope (Leica Microsystems) equipped with polarizing filters, a Brace-Köhler compensator (Wild Leitz, Wetzlar, Germany), and polychromatic light were used. *Amelx* and *Mmp20* groups were compared independently. Measurements were submitted to statistical analysis (t-test or ANOVA followed by Dunnett multiple comparison test). All the sections investigated with polarizing microscopy also were stained by hematoxylin and eosin (H&E) and analyzed with light microscopy.

Dispersion birefringence curves were obtained after determining optical retardations of the area that showed the highest birefringence brightness, as a function of each refractive index (n) of the following imbibing media: water; 30%, 40%, 60%, and 80% aqueous glycerin; 100% glycerin; mineral oil, Nujol® (Plough, Rio de Janeiro, Brazil); Entellan® resin (Merck, Darmstadt, Germany); immersion oil (Leica Microsystems, Wetzlar, Germany); Vetec® Synthetic Canada Balsam (Vetec, Duque de Caxias, Brazil); and Caedax® Synthetic Canada Balsam (Merck, Darmstadt, Germany). These fluids were used in the sequence they are quoted. Their correspondent refractive indices are reported later in Figure 3. Sections were immersed in the imbibing media for 30 min before measuring optical retardations, which were determined in nanometers (nm). A Leica DM LP microscope (Leica Microsystems) equipped with polarizing filters, Brace-Köhler compensator (Wild Leitz, Wetzlar, Germany), and polychromatic light was used.

Mature Enamel Microhardness Testing

Transversal sections of teeth fragments containing mature enamel (obtained before hemimaxillae decalcification) were embedded in acrylic resin and polished for microhardness testing (Model FMA-ARS, Future Tech Corp., Tokyo, Japan). The region selected for testing corresponded to the middle portion of the distance between dentine-enamel junction and enamel surface. Four indentations were made in this region, each separated 50 μm from another. The load used was 25 g and the time was 5 sec. Microhardness values for each animal represent the mean of 4 indentations. It was not possible to determine the microhardness of $Amelx^{-/-}$ and $Mmp20^{-/-}$ animals since enamel fractured during the analysis. *Amelx* and *Mmp20* groups were analyzed separately, comparing wild-type and heterozygous knockout subgroups (*t*-test).

RESULTS

Amelx knockout mice exhibited extensive alterations in birefringence brightness in the secretory-stage enamel organic ECM (Figure 1). The $Amelx^{+/-}$ subgroup showed decreased secretory-stage enamel thickness, and a statistically significant reduction in optical retardation mean values when compared with the $Amelx^{+/+}$ subgroup (Figures 1A, 1A′; Figures 1B,

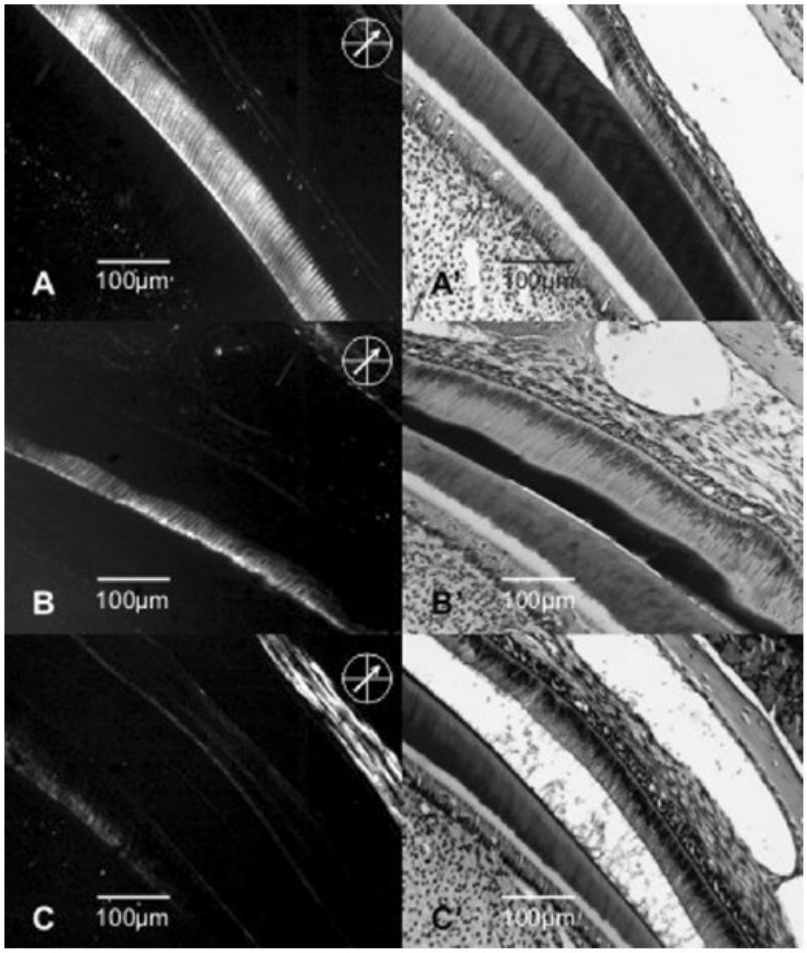

FIG. 1. Light and polarizing microcopies of the enamel organic ECM from mice included in the *Amelx* group. The analyzer and polarizer are denoted by crossed bars. The arrow at 45° with the polarizer and analyzer indicates position of maximum birefringence. (A) Birefringence of an unstained 5-μm section of the enamel organic ECM from an $Amelx^{+/+}$ mouse upper incisor. (A′) Bright field of section A, after staining with H&E. (B) Birefringence of unstained 5-μm section of the enamel organic ECM from an $Amelx^{+/-}$ mouse upper incisor. (B′) Bright field of section B, after staining with H&E. (C) Absence of birefringence in an unstained 5-μm section of the enamel organic ECM from an $Amelx^{-/-}$ mouse upper incisor. (C′) Bright field of section C, after staining with H&E. Note that heterozygous $Amelx^{+/-}$ mutant exhibits decreased thickness of the enamel organic ECM and weaker birefringence, whereas homozygous $Amelx^{-/-}$ mutant shows a small amount of disorganized biological material in the space where enamel is formed and lacks birefringence.

1B′; Figure 3, Table 1, *t*-test, $p < 0.01$). The secretory-stage enamel organic ECM of the $Amelx^{-/-}$ subgroup did not exhibit birefringence brightness, although a small amount of disorganized biological material could be observed in the space where enamel is formed (Figures 1C, 1C′; Figure 3, Table 1). This material probably corresponds to nonamelogenins that are included in 10% of secretory-stage enamel organic ECM.

The secretory-stage enamel of $Mmp20^{-/-}$ mice showed reduced thickness, and a statistically significant decrease in optical retardation mean values when compared with $Mmp20^{+/+}$ and $Mmp20^{+/-}$ mice (Figures 2A, 2A′; Figures 2B, 2B′; Figures 2C, 2C′; Figure 3, Table 2, ANOVA followed by Dunnett multiple comparison test, $p < 0.01$). The secretory-stage enamel organic ECM of $Mmp20^{+/-}$ and $Mmp20^{+/+}$ mice exhibited similar thickness, and birefringence (Figure 2A, 2A′; Figures 2B, 2B′; Figure 3, Table 2, ANOVA followed by Dunnett multiple comparison test, $p = 1.00$).

$Amelx^{+/+}$, $Mmp20^{+/+}$, and $Mmp20^{+/-}$ mice showed similar secretory enamel thickness and dispersion birefringence curves, which were clearly higher when compared with $Amelx^{+/-}$ mice data (Figures 1A, 1A′; Figures 2A, 2A′; Figures 2B, 2B′; Figures 1B, 1B′; Figure 3). $Mmp20^{-/-}$ animals presented diminished secretory enamel thickness (Figures 2C, 2C′), and lower dispersion birefringence curve even in comparison with

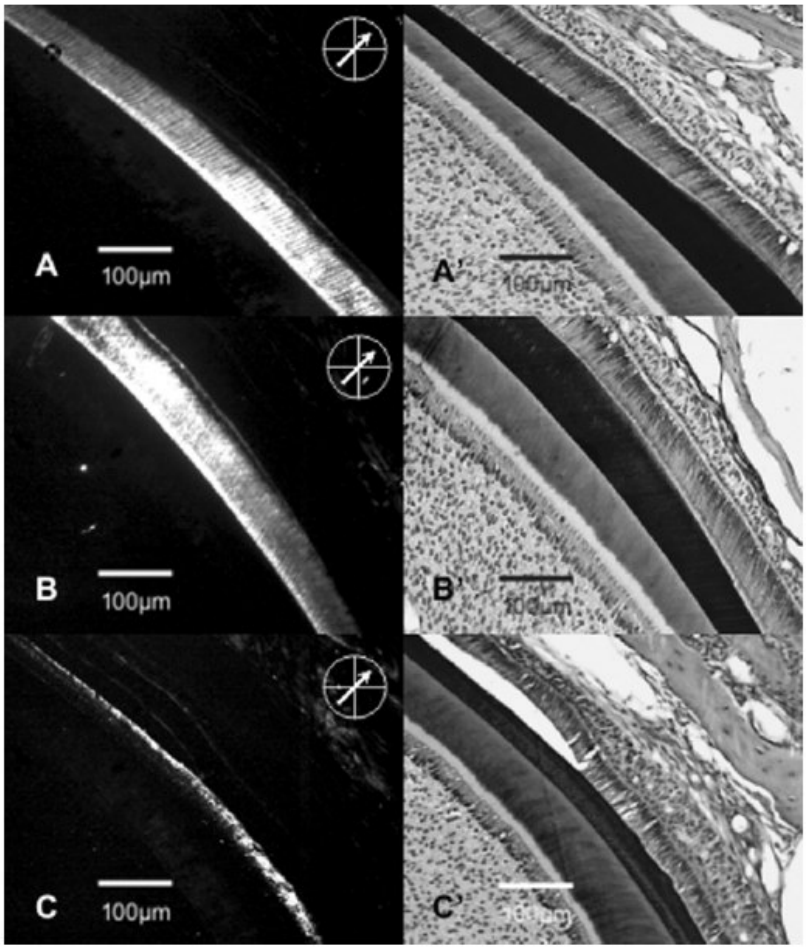

FIG. 2. Light and polarizing microcopies of the enamel organic extracellular matrix (ECM) from mice included in the *Mmp20* group. The analyzer and polarizer are denoted by crossed bars. The arrow at 45° with the polarizer and analyzer indicates position of maximum birefringence. (A) Birefringence of an unstained 5-μm section of the enamel organic ECM from an $Mmp20^{+/+}$ mouse upper incisor. (A′) Bright field of section A, after staining with H&E. (B) Birefringence of an unstained 5-μm section of the enamel organic ECM from an $Mmp20^{+/-}$ mouse upper incisor tooth. (B′) Bright field of section B, after staining with H&E. (C) Birefringence of an unstained 5-μm section of the enamel organic ECM from an $Mmp20^{-/-}$ mouse upper incisor tooth. (C′) Bright field of section C, after staining with H&E. Wild-type mouse and heterozygous $Mmp20^{+/-}$ mutant show similar thickness and birefringence of the enamel organic ECM, while homozygous $Mmp20^{-/-}$ knockout mouse exhibits thinner enamel organic ECM and weaker birefringence.

$Amelx^{+/-}$ mice (Figure 3). The thickness of secretory enamel from $Amelx^{-/-}$ mice was not clearly assessed, since it comprised a small amount of disorganized biological material (Figures 1C, 1C′). However, as they exhibited no birefringence, their dispersion birefringence curve comprised a segment in the x axis (Figure 3).

Enamel microhardness results are in agreement with light and polarizing light microscopic analysis. $Amelx^{+/-}$ mice showed a statistically significant reduction in the microhardness values when compared with wild-type mice (Figure 4, *t*-test, $p < 0.01$). The $Mmp20^{+/-}$ subgroup did not exhibit altered microhardness values compared with $Mmp20^{+/+}$ subgroup (Figure 5, *t*-test, $p > 0.05$).

DISCUSSION

We have previously shown that the organic ECM of secretory enamel is highly birefringent [20]. The birefringence of enamel organic ECM starts at early secretory phase and disappears during the beginning of maturation stage. Birefringence is an optical phenomenon caused by double refraction of polarized light wavefronts in molecularly ordered structures. It is caused by

TABLE 1
Optical retardations (nm) of unstained 5-μm-sections from the enamel organic matrix of amelogenin (*Amelx*) group

Subgroups	*n*	Optical retardation (nm) (mean ± SD)
$Amelx^{+/+}$	5	14.4* ± 0.18
$Amelx^{+/-}$	5	11.9* ± 0.8
$Amelx^{-/-}$	5	0 ± 0

Measurements were made with a Brace-Köhler compensator and polychromatic light. Each value is the mean of 40 measurements.

*p = 0.0029 (t-test); SD = standard deviation, n = number of animals analyzed.

TABLE 2
Optical retardations (nm) of unstained 5-μm-sections from the enamel organic matrix of enamelysin (*Mmp20*) group

Subgroups	*n*	Optical retardation (nm) (mean ± SD)
$Mmp20^{+/+}$	5	14.7^{a} ± 0.93
$Mmp20^{+/-}$	5	14.9^{a} ± 0.95
$Mmp20^{-/-}$	5	5.6^{b} ± 0.39

Measurements were made with a Brace-Köhler compensator and polychromatic light. Each value is the mean of 40 measurements.

Different letters express statistical difference; p = 0.0000 (ANOVA followed by Dunnett multiple comparison test); SD = standard deviation. n = number of animals analyzed.

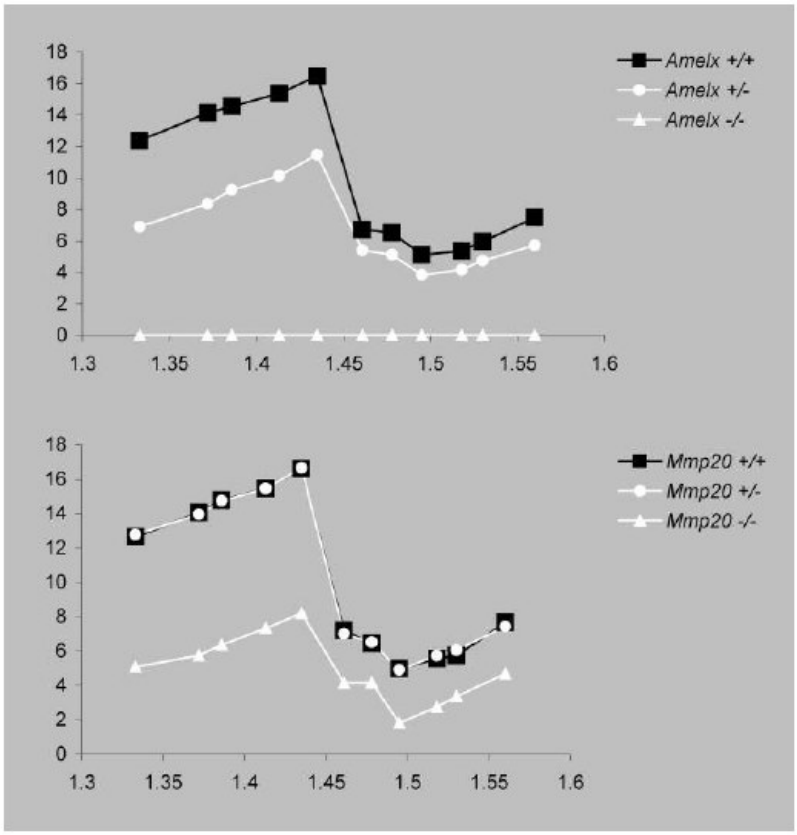

FIG. 3. Dispersion birefringence curves of *Amelx* and *Mmp20* knockout mice. Optical retardations (nm) of unstained 5-μm thick sections of the enamel organic ECM from mouse upper incisors, as a function of the refractive indices (n) of the following imbibing media: water (n = 1.333), 30% aqueous glycerin (n = 1.372), 40% aqueous glycerin (n = 1.386), 60% aqueous glycerin (n = 1.413), 80% aqueous glycerin (n = 1.435), 100% glycerin (n = 1.461), Nujol® mineral oil (n = 1.478), Entellan® resin (n = 1.495), Leica oil (n = 1.518), Vetec® Synthetic Canada Balsam (n = 1.53) and Caedax® Synthetic Canada Balsam (n = 1.56). Measurements of optical retardations were made with Brace-Köhler compensator with polychromatic light. Each point in the curve is the mean of 10 measurements. Note that the highest and the smallest (intrinsic birefringence) retardations for the series of refractive indices correspond respectively to n = 1.435 (80% aqueous glycerin) and n = 1.495 (Entellan® resin). $Amelx^{+/-}$ mouse shows weaker form and intrinsic birefringence and $Amelx^{-/-}$ mouse does not exhibit birefringence. $Mmp20^{-/-}$ mouse exhibits weaker form and intrinsic birefringence.

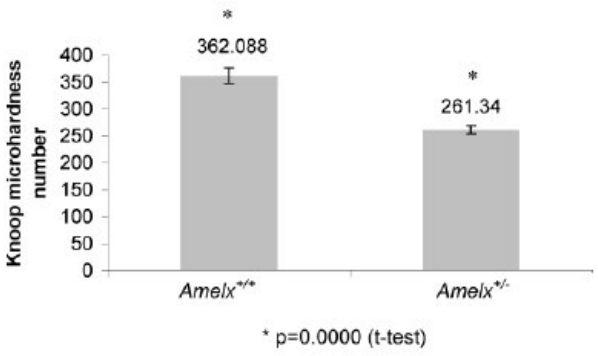

FIG. 4. Enamel microhardness values of $Amelx^{+/+}$ and $Amelx^{+/-}$ subgroups. $Amelx^{+/-}$ subgroup shows a statistically significant reduction in microhardness. This result is in agreement with the weaker birefringence observed in this group (Figures 1 and 3).

the existence of orientation-dependent differences in refractive index in anisotropic substances. In organic biological tissues, birefringence occurs as a consequence of ordered polymerization of macromolecules. In this sense the birefringence of secretory enamel organic ECM is likely to be imparted by the polymerization of amelogenin nanospheres [19]. Our *in situ* analysis of amelogenin knockout mice support this hypothesis since heterozygous $Amelx^{+/-}$ knockout mice had a significantly weaker form and intrinsic (minimal retardation) birefringence whereas the secretory enamel formed in *Amelx* null mice lacked birefringence.

In both, wild type and $Amelx^{+/-}$ mutants, the maximum birefringence was attained when enamel rods were oriented at 45° with respect to the polarizer and analyzer filters. Our research shows that despite of the decreased birefringence there were no significant statistical changes regarding the macromolecular orientation of the matrix components. This suggests that the observed differences in optical retardations are caused by changes in the aggregational status of the molecular components from the enamel organic ECM.

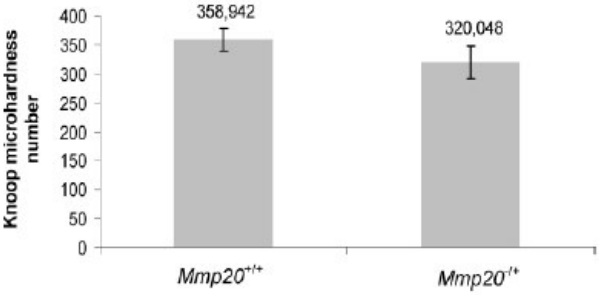

FIG. 5. Enamel microhardness values of $Mmp20^{+/+}$ and $Mmp20^{+/-}$ subgroups. Statistical difference was not observed (t-test; $p = 0.1264$). This finding is in agreement with polarizing microscopy analysis, that shows these two groups exhibit similar birefringence of the enamel organic ECM (Figures 2 and 3).

The birefringence brightness is first observed in the early secretory stage near the dentin-enamel junction [20]. The fact that birefringence is not initially observed near secreting ameloblasts indicates that the assembly of secretory enamel organic ECM into high-order structures does not occur immediately after secretion. This pattern suggests that this organic matrix must be processed to form an ordered supramolecular structure that causes strong birefringence. The Mmp-20 proteolytic cleavage of amelogenins during early secretory-stage may influence the increasing of enamel organic ECM birefringence. Mmp-20 is present in the mineralizing front, and it is thought to participate in the early cleavage events that generate smaller nanospheres that allow the crystals to grow in length but not in thickness, leading to the thickening of the enamel layer [21]. In fact, the *in situ* analysis of $Mmp20^{-/-}$ mice shows that secretory enamel organic ECM from these mice is thin and exhibits reduced form and intrinsic birefringence.

It is interesting, however, that the organic ECM still presents a weak birefringence. This is possibly caused by full-length uncleaved amelogenin molecules, which also are able to assemble to form chains of nanospheres [16, 22]. These results indicate that the proteolytic cleavage of amelogenin by Mmp-20 may be associated with the formation of a strongly birefringent secretory-stage enamel organic ECM. It also is possible that the organization of organic ECM is influenced by the presence of organized crystals that are secreted immediately after secretion. Immediate fixation and demineralization can preserve this order in the remaining organic matrix.

The birefringence and thickness of the enamel organic ECM of heterozygous $Mmp20^{+/-}$ mice did not differ from wild-type animals. In addition, microhardness of mature enamel of $Mmp20^{+/-}$ mutants was not affected. These findings are in accordance with Caterina et al. [11] showing that the ultrastructural aspects of mature enamel and enamel matrix from heterozygous mice did not differ from wild-type animals. Heterozygous knocking out of *Amelx* gene, which encodes a structural protein, provided diminished birefringence and thickness of the enamel organic ECM and affected microhardness of mature enamel. In fact, most autosomal recessive anomalies are caused by mutations in genes that encode enzymes. In these cases, a single functional allele seems to be enough for providing normal function in heterozygous individuals [23].

Microhardness is related to the level of mineralization and the amount of organic matrix left. The evidence that the microhardness data are in agreement with the light and polarizing microcopies is support for the idea that the differences in birefringence might be the result of differences in mineralization, which in turn affects the ultrastructure of the remaining matrix.

Most studies on supermolecular structure of the enamel organic ECM were performed using *in vitro* systems with purified amelogenin molecules. Besides amelogenin, little is known about the precise function of other organic components expressed during the early phases of enamel development.

In this scenario, the birefringence analysis of the secretory-stage enamel organic ECM of mutant mice models bearing amelogenesis imperfecta may give important insights into the biological function of these molecules or reflect alterations in the mineral phase of developing enamel.

ACKNOWLEDGMENTS

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CAPÍTULO 2

Birefringence of the secretory-stage enamel organic extracellular matrix from rats submitted to successive injections of bisphosphonates.

Artigo submetido à publicação na revista ***Archives of Oral Biology*** (Anexo 2).

Birefringence of the secretory-stage enamel organic extracellular matrix from rats submitted to successive injections of bisphosphonates

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Running title:

Birefringence of the enamel organic ECM from bisphosphonates-treated rats

Keywords: Amelogenesis, Enamel, Enamel organic extracellular matrix, Birefringence, Bisphosphonates.

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ABSTRACT

Objective: The aim of the present study was to assess birefringence of the secretory-stage enamel organic extracellular matrix (ECM) from rats treated with successive injections of bisphosphonates.

Design: Unstained 5-µm-thick longitudinal sections were obtained from properly fixed, decalcified, dehydrated and paraffin-embedded upper incisors of rats that had been daily submitted to subcutaneous injections of bisodic etidronate (8 mg/Kg/day), sodium alendronate (30 µg/Kg/day) or sodium chloride as control (8 mg/Kg/day) for 42 days. Sections were immersed in 80% glycerin for 30 min and optical retardation of the area that showed the highest birefringence brightness in secretory-stage enamel organic ECM was determined in nanometers. Data were compared using ANOVA followed by Dunnett multiple comparison test.

Results: Etidronate-treated rats exhibited extensive morphological changes in the secretory-stage enamel organic ECM and presented optical retardation values similar to those showed by Control rats ($p>0.05$). Alendronate-treated rats did not show morphological alterations in the secretory-stage enamel organic ECM, however, presented statistically significant reduction in optical retardation of birefringence brightness when compared with Control and Etidronate rats ($p<0.01$).

Conclusions: Bisodic etidronate may induce changes in mature enamel by affecting directly ameloblasts' secretion of structural proteins and not by disturbing molecular order of the secreted enamel organic matrix. Sodium alendronate may induce quantitative changes in the supramolecular organization in the secretory-stage enamel organic ECM.

Keywords: Amelogenesis, Enamel, Enamel organic extracellular matrix, Birefringence, Bisphosphonates

1. Introduction

Enamel is the hardest mineralized tissue in the vertebrate body and contains the largest known biologically formed hydroxyapatite crystals,[1,2] which are assembled forming a highly complex prismatic architecture in humans.[3] Since enamel covers dental crown, defects in its complex structure are associated with a higher susceptibility to caries development.[4] Therefore, it is important to investigate the effects of genetic and environmental alterations on the formation of this tissue.

Enamel is produced by specialized cells called ameloblasts and its formation occurs extracellularly, comprising a typical process of biomineralization that begins with the secretion, processing and self-assembly of a complex mixture of proteins.[2,5] These are proteolytically cleaved and replaced by mineral ions, which deposit to form the abovementioned crystals. As these crystals are completely grown in length, width and thickness, enamel formation is finished. In the case of rat incisor, enamel production takes place in a time- and space-restricted fashion, as distinct zones of secreting, maturing and mature enamel are noted depending on the developmental stage of the tooth.[6]

The establishment of an ordered enamel organic extracellular matrix (ECM) seems to regulate organization, growth and morphology of enamel crystals.[7-16] Polarizing light microscopy (PLM) analysis demonstrates that the ordered supramolecular structure of the secretory-stage enamel organic ECM is strongly birefringent.[17] Possibly, birefringence occurs as a consequence of the organized polymers of nanospheres generated after cleavage of full-length amelogenins by enamelysin.[18] Analysis of the enamel organic ECM birefringence allows performing *in situ* studies in tissue sections. It makes possible to obtain valuable quantitative and qualitative information on the effects of genetic and environmental disturbances on the supramolecular structure of the enamel organic ECM and its association with enamel defects.

Bisphosphonates are pyrophosphate analogs that avidly bind to bone and inhibit osteoclastic bone resorption,[19] making them useful for treatment of skeletal disorders such as osteoporosis, Paget's disease, osteogenesis imperfecta and metastatic bone diseases.[20-22] Bisphosphonates therapeutically act by causing toxic effects on osteoclasts or interfering with specific intracellular pathways in those cells.[23-25] Defective enamel mineralization is one of the possible adverse effects of these drugs.[26-31] Although bisphosphonates' mechanism of action is exhaustively studied in bone, their effects on the enamel formation are still poorly understood.

To test the hypothesis that bisphosphonates may disturb molecular order of the secretory-stage enamel organic ECM, in the present PLM study, we investigated the birefringence of this tissue on 5-µm-thick longitudinal sections of upper incisors from adult rats that had been treated with clinically used bisphosphonates, bisodic etidronate[32] or sodium alendronate.[33,34]

2. Materials and Methods

Animals, treatments and tissue preparation

Ethical approval for the present study was granted by Ethical Committee for Animal Research of the State University of Campinas - UNICAMP, Brazil.

Twenty-nine male Wistar rats weighing ≈ 200 g were divided into 3 groups: Control (n=9), Etidronate (n=11) and Alendronate (n=10). Rats in the Etidronate Group were subcutaneously injected with bisodic etidronate (Farmacotécnica - Instituto de Manipulações Farmacêuticas Ltda, Brasília-DF, Brazil) at a dose of 8 mg/Kg/day[31] for 42 consecutive days. Animals in the Alendronate Group were subjected to daily subcutaneous injections of 30 µg/Kg/day[33] of sodium alendronate (Farmacotécnica - Instituto de Manipulações Farmacêuticas Ltda,

Brasília-DF, Brazil) for forty-two days. Control rats were subcutaneously injected with sodium chloride at a dose of 8 mg/Kg/day for forty-two consecutive days. All the solutions were made using water as solvent. Animals in the three groups were provided with food and water *ad libitum*. Twenty-four hours after the last injection, animals were anesthetized with ketamine and perfused with 2% paraformaldehyde, 0.5% glutaraldehyde in 0.2 M phosphate-buffered saline (PBS), pH 7.2. Hemimaxillae were then extracted and immersed in fixative solution for 24 h. Incisor tooth of each hemimaxilla was sectioned transversally at 2 mm above the alveolar bone crest, using a hard tissue microtome. Anterior mineralized fragments from right upper incisors were used for enamel cross-sectional microhardness testing. Anterior mineralized fragments from left upper incisors were used for analysis of enamel surface topography by scanning electron microscopy (SEM). Hemimaxillae containing posterior fragments of the incisors were subjected to decalcification by immersion in 5% nitric acid, 4% formaldehyde under continuous shaking for 24 h. After dehydration and clarification, decalcified samples were embedded in paraffin, and 5-µm-thick longitudinal sections were obtained. The sections were treated with xylene for removal of the paraffin, and hydrated.

Polarizing light microscopy of secretory-stage enamel organic matrix

Unstained longitudinal 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 in secretory-stage enamel of upper incisors. 15 sections from each tooth were immersed in 80% glycerin for 30 min and 10 measurements were performed by an observer blind to the studied groups. A mean value was obtained for each animal. A Leica DM LP microscope (Leica Microsystems) equipped with polarizing filters, a Brace-Köhler compensator (Wild Leitz, Wetzlar, Germany), and polychromatic light, was used. Measurements were submitted to statistical analysis (ANOVA followed by Dunnett multiple comparison

test). All the sections investigated with polarizing microscopy were also stained by HE and analyzed with bright field light microscopy.

Dispersion birefringence curves were obtained after determining optical retardations of the area that showed the highest birefringence brightness, as a function of each refractive index (n) of the following imbibing media: water, 80% aqueous glycerin, 100% glycerin, Entellan® resin (Merck, Darmstadt, Germany) and Caedax® Synthetic Canada Balsam (Merck, Darmstadt, Germany). These fluids were used in the sequence they are quoted. Their correspondent refractive indices are reported in Fig. 2. Sections were immersed in the imbibing media for 30 min before measuring optical retardations, which were determined in nanometres (nm). A Leica DM LP microscope (Leica Microsystems) equipped with polarizing filters, Brace-Köhler compensator (Wild Leitz, Wetzlar, Germany) and polychromatic light was used.

Mature enamel microhardness testing

Mineralized fragments from upper right incisors containing mature enamel (obtained before hemimaxillae decalcification) were embedded in acrylic resin and had their distal extremity polished for enamel cross-sectional microhardness testing (Model FMA-ARS, Future Tech Corp., Tokyo, Japan). Five indentations, each separated 50 µm from another, were made at 15, 30 and 50 µm from dentine-enamel junction (DEJ), comprising a total of 15 indentations per tooth. The load used was 25 g and the time was 5 s. Knoop microhardness value for each abovementioned zone from each animal represents the mean of 5 indentations. Microhardness testing was conducted by an observer blind to the studied groups. Control, Etidronate and Alendronate groups were compared, submitting microhardness values to statistical analysis (ANOVA followed by Dunnett multiple comparison test).

Scanning electron microscopy of mature enamel surface

Mature enamel of mineralized fragments from upper left incisors had its surface topography analyzed with SEM. The fragments were air-dried, fastened to stubs, sputter-coated and examined using a JEOL JSM-5600LV scanning electron microscope (JEOL LTD., Tokyo, Japan). Control, Etidronate and Alendronate groups were compared by descriptive analysis.

3. Results

Alendronate-treated group did not exhibit morphological alterations in the secretory-stage enamel organic ECM (Fig. 1E, F). However, that group showed a statistically significant reduction in optical retardation of birefringence brightness of about 63% at n=1.435 (imbibing medium's refractive index of maximum birefringence) when compared with Control and Etidronate groups (Fig. 1; Fig. 2; Table 1, ANOVA followed by Dunnett multiple comparison test, $p<0.01$). The secretory-stage enamel organic ECM of the Etidronate-treated group exhibited extensive morphological modifications (Fig. 1C, D) and presented optical retardation values similar to those showed by Control group (Fig. 1A, B, C, D; Fig. 2; Table 1, ANOVA followed by Dunnett multiple comparison test, $p>0.05$). Control and Etidronate rats exhibited similar dispersion birefringence curves, which were clearly higher when compared to Alendronate rats' data (Fig. 2).

Similar to polarizing light microscopy analysis, Alendronate group showed a statistically significant reduction of about 10-15% in Knoop microhardness values at 15 and 30 µm of distance from DEJ, compared with Control group (Fig. 3; ANOVA followed by Dunnett multiple comparison test, $p<0.01$). Although bisodic etidronate did not expressively diminish birefringence brightness of the secretory-stage enamel organic ECM, it induced a statistically significant decrease of about

10% in mature enamel microhardness at 15 and 30 µm of distance from DEJ (Fig. 3; ANOVA followed by Dunnett multiple comparison test, $p<0.01$). Control, Etidronate and Alendronate groups presented similar Knoop microhardness values at 50 µm of distance from DEJ (Fig. 3; ANOVA followed by Dunnett multiple comparison test, $p>0.05$).

SEM findings are in accordance with bright field and polarizing light microcopies. Enamel surface of upper incisors from Control and Alendronate rats did not show any relevant deffect, whereas Etidronate group exhibitted extensively altered enamel layer, with the presence of large enamel free-zones which seem to be resulted from failure of enamel protein secretion.

4. Discussion

Birefringence is the anisotropy caused by the difference between the two refractive indices of a substance. When passing through birefringent substances, polarized light propagates as two wave fronts. The relative displacement of these two wave fronts is called optical retardation, which is given as a length in nm and is directly proportional to birefringence brightness intensity. In organic biological tissues, birefringence occurs as a consequence of ordered polymerization of macromolecules. The birefringence of the enamel organic ECM starts at early secretory phase and disappears during the beginning of maturation stage.[17] It seems to be imparted by polymerization of amelogenin nanospheres[17,35] and is increased after cleavage of full-length amelogenins by enamelysin (MMP-20).[18] Therefore, changes in the birefringence of the secretory-stage enamel organic ECM may reflect qualitative and/or quantitative changes in the supramolecular organization of its components, which in turns may influence growth and morphology of mineral phase, leading to significant defects in mature enamel. This

is a possible mechanism by which environmental factors may cause enamel defects.

Bisphosphonates are therapeutic drugs used in many bone pathologies and may be administered to pregnant women or children during deciduous and permanent teeth development.[22,33,34] These drugs are analogues of pyrophosphate (P-O-P) in which the geminal oxygen has been substituted by carbon, originating the P-C-P bond, which is resistant to enzymatic hydrolysis.[36] The basic P-C-P structure allows a great number of variations, either by changing the two side chains R1 and R2 on the carbon atom, or by esterifying or altering the phosphate groups.[19] In the present study, we used two bisphosphonates: bisodic etidronate and sodium alendronate, which have different chemical structures that are associated with different antiresorptive potencies.[37] Bisodic etidronate presents two Na atoms substituting one H atom from each phosphate group. Its R1 and R2 are respectively OH and CH_3. Sodium alendronate exhibits a Na atom substituting one H atom from a phosphate group. Its R1 and R2 are respectively OH and $(CH_2)_3NH_2$. Although these two drugs avidly bind to hydroxyapatite crystals, the presence of a nitrogen atom in R2 of sodium alendronate makes this drug much more potent than bisodic etidronate in regarding to antiresorptive action in bone.[24,25,37,38] These drugs are both internalized by osteoclasts during bone resorption and perturb metabolism of those cells, eventually inducing apoptosis.[37] Etidronate can be metabolically incorporated into nonhydrolyzable analogs of ATP that may inhibit ATP-dependent intracellular enzymes. Alendronate, which is more potent, is not metabolized this way but can inhibit prenylation of GTP-binding proteins that are responsible for cytoskeletal integrity and intracellular signaling of osteoclasts.

Although there is a clear advance in the knowledge of bisphosphonates' mechanisms of action in bone cells and ECM, the effects of these drugs on the enamel formation are still poorly understood. This is the first PLM study that accesses the effects of bisphosphonates on the secretory-stage enamel organic

ECM. As will be discussed below, the results presented here corroborate with previous studies and allow new insights into the mechanisms by which bisphosphonates may interfere with enamel formation.

The enamel organic ECM deposited by secretory ameloblasts from Etidronate rats exhibited form and intrinsic (minimal optical retardation) birefringences similar to those observed in Control rats. Its maximum birefringence brightness was attained when enamel rods were oriented at 45^{o} with respect to the polarizer and analyzer filters. These findings show that bisodic etidronate does not induce significant changes in the orientation and aggregational status of the molecular components of the secretory-stage enamel organic ECM. However, morphological alterations in the secretory enamel matrix in the Etidronate group could be clearly noted in the polarized light microscope analysis. These alterations were characterized by enamel-free zones that interrupt the continuity of enamel layer, like those observed in previous studies.[27,30,31] Moreover, the enamel organic matrix from that group showed non-birefringent longitudinal lines that indicate abrupt changes in the secretory activity of ameloblasts. These results indicate that bisodic etidronate acts directly on ameloblast metabolism, and does not seem to affect the assembly of the enamel organic ECM components.

Different from Etidronate group, no morphological alterations were observed in the secretory-stage enamel organic ECM of alendronate-treated rats. However, those rats presented significantly weaker form and intrinsic birefringences when compared to Control animals. Alendronate rats also presented maximum birefringence brightness when enamel rods were oriented at 45^{o} with respect to the polarizer and analyzer filters. Immunocytochemical analysis showed a higher synthesis/secretion of amelogenins by ameloblasts from alendronated-treated rats when comparing to control animals.[22] However, the presence of large patches of granular material formed by amelogenin molecules, as reported by Massa, Bradaschia-Correa and Arana-Chavez (2006)[22], indicates that alendronate also induced changes in the aggregational properties of the enamel organic ECM. In

addition, alendronate inhibits enamelysin (MMP-20) activity.[39] As a final consequence, there may be a prevalence of full-length amelogenins over products of amelogenin cleavage by MMP-20 in the extracellular space where enamel is formed. This disturbance could be associated with the observed decrease in birefringence brightness of the secretory-stage enamel organic ECM from rats treated with alendronate. In fact, there is evidence that proteolytic cleavage of amelogenin by MMP-20 is related to the formation of a stronger birefringent secretory-stage enamel organic ECM.[18]

Microhardness is related to the level of mineralization and the amount of organic matrix left. A 10-15% reduction in microhardness was induced by etidronate and alendronate in more internal layers of mature enamel. In the case of Alendronate group, the decrease in enamel microhardness values may be explained by morphologic alterations of hydroxyapatite crystals as a result of changes in the molecular order of the secretory-stage enamel organic ECM. The reduction in mature enamel microhardness values of Etidronate rats can be related to the presence of the large incremental lines. These lines are likely to be similar to hypocalcified pathologic Retzius lines formed as a response to physiological stress, such as neonatal line.[40] Decrease in birefringence brightness of the enamel organic ECM has been previously associated with reduction of mature enamel microhardness.[18]

SEM analysis showed that while bisodic etidronate induces severe alterations in the final morphology of mature enamel, sodium alendronate does not seem to significantly alter that tissue. These results are in agreement with the light microscopy study applied in the present work and with previous reports about the effect of bisodic etidronate on the enamel formation.[26,27,30,31] Different from etidronate, the effects of alendronate on the enamel formation have not been extensively investigated. Alendronate belongs to the subgroup of nitrogen-containing bisphosphonates, with a more potent biological effect. For this reason it is more frequently used in humans than etidronate.

In conclusion, bisodic etidronate does not disturb molecular order of the secreted enamel organic matrix and may induce changes in mature enamel by affecting directly ameloblast secretion. Sodium alendronate can induce quantitative changes in the supramolecular organization of the secretory-stage enamel organic ECM.

Acknowledgement

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Figure Captions

Figure 1. Polarizing and bright field light microcopies of the secretory-stage enamel organic ECM from rats included in Control, Etidronate and Alendronate groups. The analyzer and polarizer are demoted by crossed bars. The arrow at 45º with the polarizer and analyzer indicates position of maximum birefringence. Large bars indicate 100 µm. **A.** Birefringence of an unstained 5-µm-section of the enamel organic ECM from a Control rat upper incisor. **B.** Bright field of section A, after

staining with HE. **C.** Birefringence of unstained 5-µm-section of the enamel organic ECM from an Etidronate rat upper incisor. **D.** Bright field of section C, after staining with HE. **E.** Birefringence of unstained 5-µm-section of the enamel organic ECM from an Alendronate rat upper incisor. **F.** Bright field of section E, after staining with HE. Note that bisodic etidronate, although is associated with severe morphological alterations of the secretory-stage enamel organic ECM due to failure of protein secretion by ameloblasts, is not related to decrease in birefringence of the secreted organic matrix. Interestingly, sodium alendronate, although does not induce morphological alterations of the enamel organic ECM, is associated with an expressive decrease in birefringence of the tissue referred to.

Figure 2. Dispersion birefringence curves of Control, Etidronate and Alendronate rats. Optical retardations (nm) of unstained 5 µm thick sections of the enamel organic ECM from rat upper incisors, as a function of the refractive indices (n) of the following imbibing media: water (n=1.333), 80% aqueous glycerin (n=1.435), 100% glycerin (n=1.461), Entellan® resin (n=1.495) and Caedax® Synthetic Canada Balsam (n=1.56). Measurements of optical retardations were made with Brace-Köhler compensator and polychromatic light. Each point in the curve is the mean of 10 measurements. Note that the highest and the smallest (intrinsic birefringence) retardations for the series of refractive indices correspond respectively to n=1.435 (80% aqueous glycerin) and n= 1.495 (Entellan® resin). Alendronate rat shows weaker form and intrinsic birefringence.

Figure 3. Mature enamel cross-sectional microhardness values of Control, Etidronate and Alendronate groups. Different letters express statistical difference ($a \neq b$, $p<0.01$; $a' \neq b'$, $p<0.01$; ANOVA followed by Dunnett multiple comparison test). Etidronate group shows a statistical significant decrease in enamel microhardness at 15 and 30 µm from DEJ. Similarly, Alendronate group shows a significant reduction in enamel microhardness at 15 and 30 µm from DEJ. This result is in agreement with the weaker birefringence observed in Alendronate group

(Fig. 1, Fig. 2). However, we can not conclude that these findings are clinically relevant.

Figure 4. Scanning electron micrograph of upper incisor teeth from rats included in Control, Etidronate and Alendronate groups. Control (A, B) and Alendronate (E, F) animals exhibited similar enamel surface topography. Bisodic etidronate promoted enamel hypoplasia characterized by large enamel free-zones (C, D), which may be a result from failure in protein secretion by ameloblasts.

Tables

Table 1. Optical retardations (nm) of unstained 5-µm-sections from the secretory-stage enamel organic ECM of Control, Etidronate and Alendronate groups.

Groups	n	Optical Retardation (nm) (Mean ± SD)
Control	9	12,61[a] ± 2,48
Etidronate	11	11,46[a] ± 2,01
Alendronate	10	8,69[b] ± 1,34

Different letters express statistical difference; p=0.0008 (ANOVA followed by Dunnett multiple comparison test); SD=Standard Deviation. n=number of animals analyzed. Note that sodium alendronate induced statistical significant decrease in optical retardation of birefringence brightness.

Figures

Figure 1

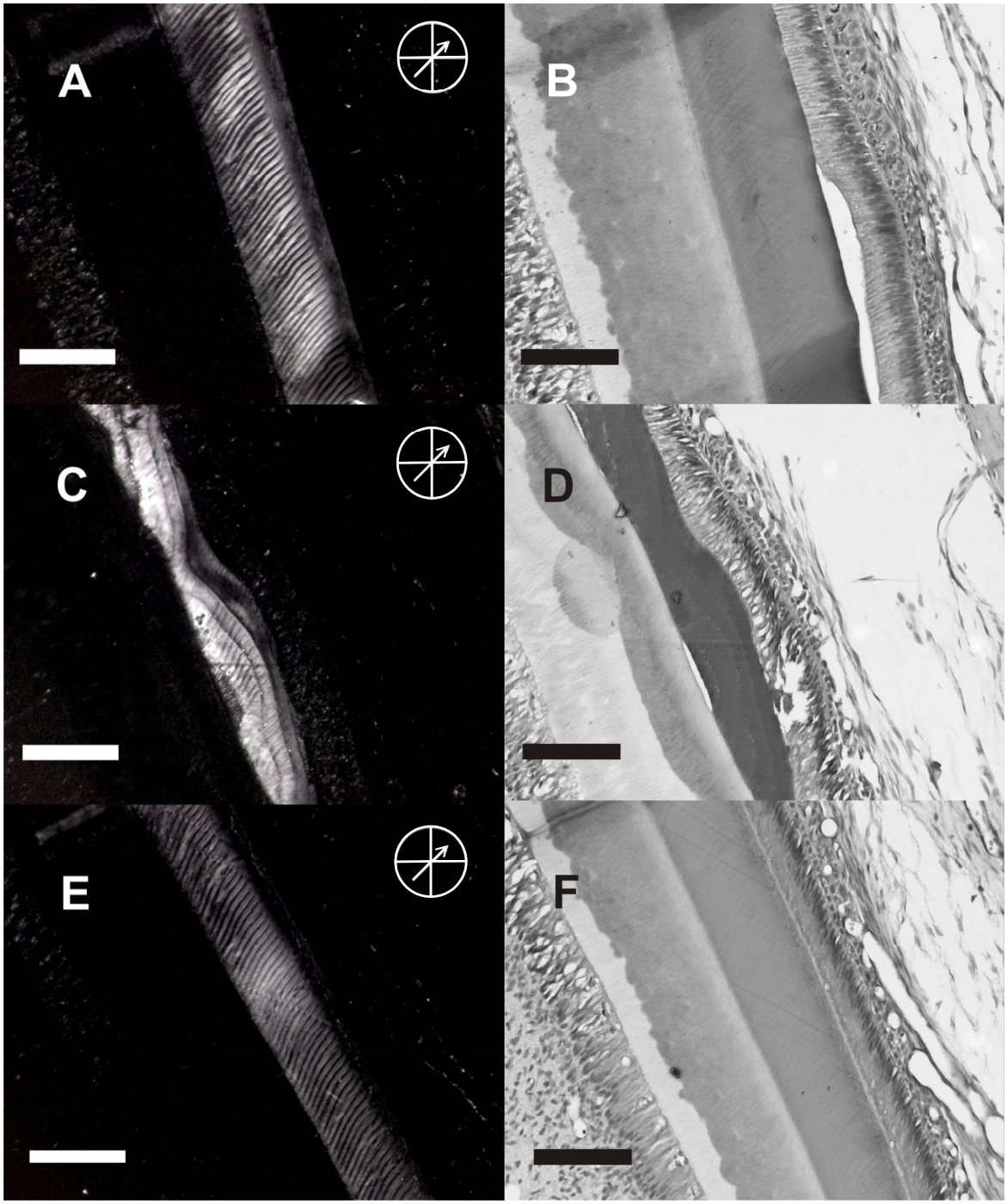


Figure 2

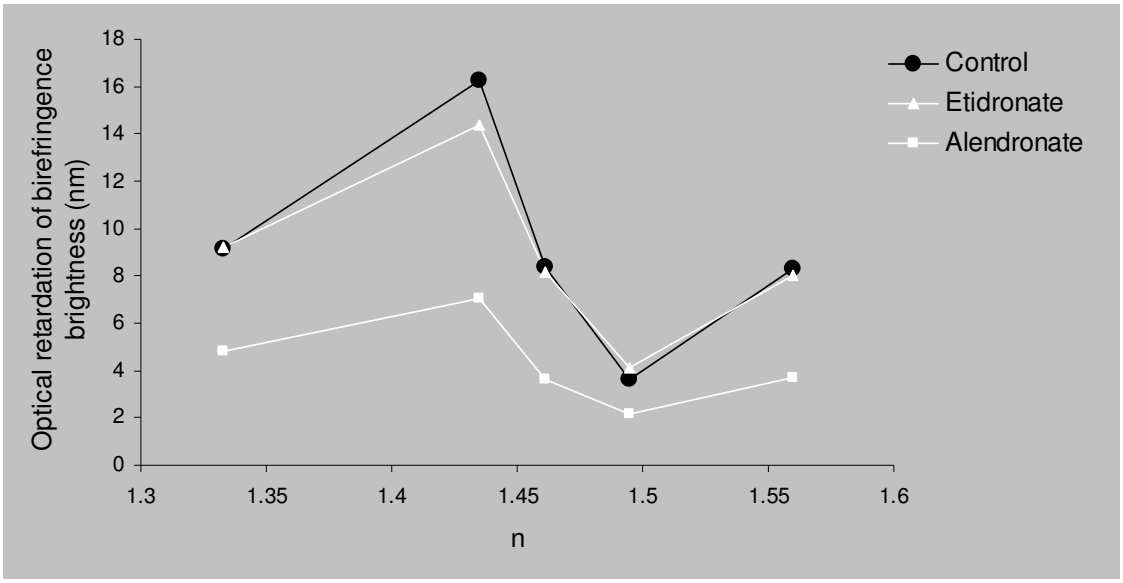


Figure 3

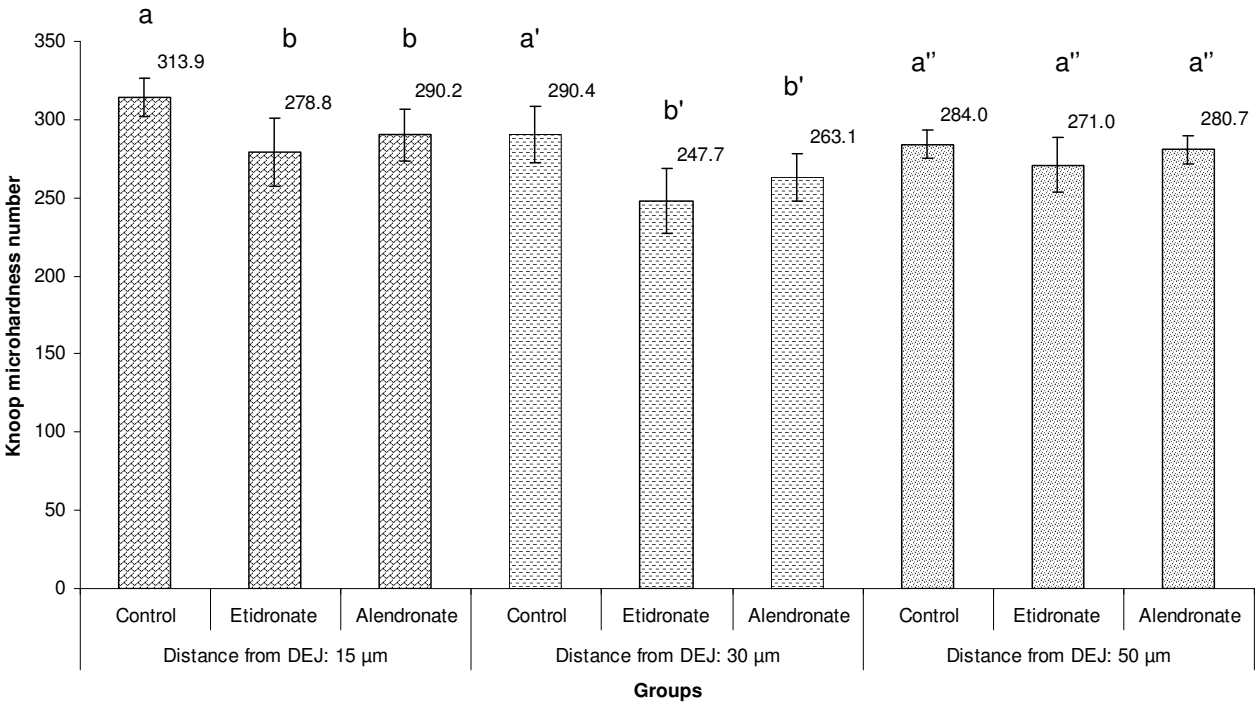
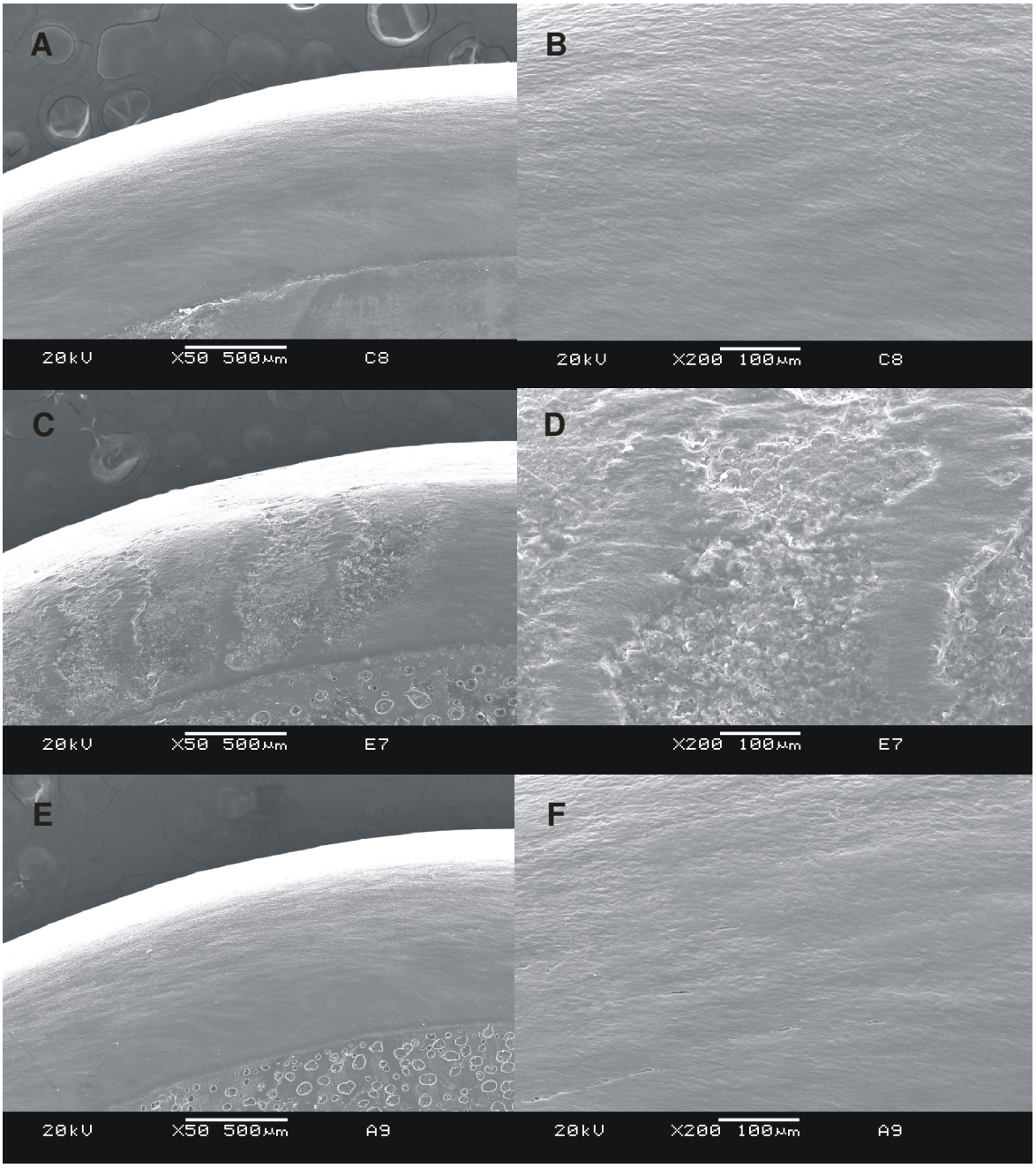


Figure 4


A
20kV X50 500µm C8
B
20kV X200 100µm C8
C
20kV X50 500µm E7
D
X200 100µm E7
E
20kV X50 500µm A9
F
20kV X200 100µm A9

CAPÍTULO 3

Effect of metallo and serine proteinases' inhibitors ex vivo on the birefringence of the secretory-stage enamel organic extracellular matrix.

Artigo a ser submetido à publicação na revista ***European Journal of Oral Sciences***.

Short Communication

Effect of metallo and serine proteinases' inhibitors *ex vivo* on the birefringence of the secretory-stage enamel organic extracellular matrix

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Running title:

Birefringence in the secretory-stage enamel organic ECM

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Effect of metallo and serine proteinases' inhibitors *ex vivo* on the birefringence of the secretory-stage enamel organic extracellular matrix

Eur J Oral Sci

Abstract

The establishment of an ordered enamel organic extracellular matrix (ECM) seems to be a crucial step for the proper formation of mineral phase. Polarizing microscopy demonstrates that the ordered supramolecular structure of the secretory-stage enamel organic ECM exhibits a strong birefringence. We have previously reported that this birefringence is extremely vulnerable to fixation process. Samples that are not immediately fixed may lack birefringence brightness. To test the hypothesis that this rapid loss of birefringence may be caused by the activity of enamel proteinases, we analyzed the effects of metallo and serine proteinases' inhibitors *ex vivo* on the birefringence of the secretory-stage enamel organic ECM. Proper incubation with 1,10-phenanthroline (inhibitor of metalloproteinases, like enamelysin) and PMSF (inhibitor of serine proteinases, like kallikrein-4) has clearly prevented large decrease in the birefringence of the abovementioned tissue. It is concluded that rapid loss of birefringence in not immediately fixed secretory-stage enamel organic ECM is caused by the activity of enamel proteinases. In addition, the present study indicates that Klk4 activity may be dependent of a previous Mmp20 catalytic action on amelogenin molecules.

Key words: enamel, birefringence, enamel proteinases, PMSF, Phenanthroline.

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Introduction

Enamel covers dental crown and this is the reason why it is an important theme for both fields of health and aesthetics. The tissue referred here is the most mineralized in the vertebrate body and contains the largest known biologically formed hydroxyapatite crystals (1). Its formation occurs extracellularly, in a microenvironment created and temporarily maintained by specialized cells called ameloblasts (2). This microenvironment is composed mainly by structural proteins that assemble to form a supramolecular framework which controls orientation and morphology of the abovementioned inorganic crystals during their growth (3). While the orchestrated growth of these crystals proceeds in length, width and thickness, the protein framework is degraded with the help of proteolytic enzymes and removed by the enamel producing cells (4). As three-dimensional growth of hydroxyapatite crystals is completed and almost total organic material is removed, enamel formation is finished. Knowledge on this highly complex process is the basis for better prosthetic materials discovery and laboratorial enamel regeneration.

There are a reasonable number of studies on the induction of apatite crystals growth *in vitro* with the purpose of future enamel production in laboratory (5-16). One of these experiments shows apatite crystals growth in a framework composed of birefringent amelogenins' microribbons (16). This finding is in accordance with our recent reports on the birefringence of the secretory-stage enamel organic extracellular matrix (ECM) from rats and mice (17,18). We have analyzed the structured enamel organic ECM with all its components in non-stained ordinary sections. This is important to mention because besides amelogenin, little is known about the precise function of other organic elements expressed during the early phases of enamel development. In this sense, deficiency in each component of the enamel organic ECM may be associated with alterations in the birefringence of that tissue. Therefore, as researches on laboratorial enamel production proceed and

new enamel proteins' interactions are *in vitro* achieved, birefringence analysis may be useful for indicating quality of the *in vitro* constructed organic frameworks.

Here we focus on enamel biosynthesis. It has previously been reported that birefringence of the enamel organic ECM is extremely vulnerable to fixation process (17). Rat and mouse incisors should be rapidly extracted and immediately immersed in fixative solution. Hemimandibles and hemimaxillae of those animals should be perfused for proper preservation of the enamel organic matrix. The rapid loss of birefringence may be caused by the activity of enamel proteases, which are present during all stages of amelogenesis. To test this hypothesis, in the present study we analyzed the effects of metallo and serine proteinases' inhibitors *ex vivo* on the birefringence of the secretory-stage enamel organic ECM. The results presented and discussed in this short communication allow inference of an interrelationship between the most important enzymatic components of the enamel organic ECM: matrix metalloproteinase-20 (Mmp20, enamelysin), which is known to be expressed during the secretory and transition stages of enamel formation (19-21), and kallikrein-4 (Klk4, EMSP1), which is expressed during transition through maturation stages (21-23). The former cleaves amelogenin within the hydrophilic C-terminal area, allowing polymerization of hydrophobic amelogenins' nanospheres which regulate enamel crystals' axial growth. The second extensively cleaves amelogenins within the hydrophobic N-terminal area, contributing to proteins removal and so allowing enamel crystals thickening.

Material and methods

Ethical approval for the present study was granted by Ethical Committee for Animal Research of the State University of Campinas - UNICAMP, Brazil.

Male Wistar rats weighing ≈ 200 g were used in the present study. After animals sacrifice by cervical displacement, distal 10 mm fragments of upper

incisors were immediately removed and immersed for 15 h under continuous shaking, at 37º C, in one of the following solutions:

1. 10 mM Tris pH 8.0, 150 mM NaCl (***Negative Control Group, n=6***).
2. 2% paraformaldehyde, 0.5% glutaraldehyde in 0.2 M phosphate-buffered saline (PBS), pH 7.2 (***Positive Control Group, n=6***).
3. 10 mM Tris pH 8.0, 150 mM NaCl, 20 mM 1,10-phenanthroline (***Phenanthroline Group, n=7***).
4. 10 mM Tris pH 8.0, 150 mM NaCl, 20 mM phenylmethylsulphonyl fluoride (PMSF) (***PMSF Group, n=7***).

After the abovementioned period of 15 h, distal fragments from all groups, inclusive those from Positive Control Group, were transferred and kept for 24 h inside a medium containing 2% paraformaldehyde, 0.5% glutaraldehyde in 0.2 M phosphate-buffered saline (PBS), pH 7.2 for proper fixation. They were then decalcified by immersion in 5% nitric acid, 4% formaldehyde under continuous shaking for 9 h. After dehydration and clarification, decalcified samples were embedded in paraffin, and 5-µm-thick longitudinal sections were obtained. The sections were treated with xylene for removal of the paraffin, and hydrated.

Unstained sections of each distal fragment were analyzed in order to determine optical retardation (nm) of the area that showed the highest birefringence brightness in secretory-stage enamel. Fifteen sections from each tooth were immersed in 80% glycerin for 30 min and 10 measurements were performed by an observer blind to the studied groups. A mean value was obtained for each upper incisor. A Leica DM LP microscope (Leica Microsystems) equipped with polarizing filters, a Brace-Köhler compensator (Wild Leitz, Wetzlar, Germany), and polychromatic light, was used. Measurements were submitted to statistical analysis in order to compare the abovementioned four groups (ANOVA followed by Tukey test). All the sections investigated with polarizing microscopy were also stained by HE and analyzed with bright field light microscopy.

Dispersion birefringence curves were obtained after determining optical retardations of the area that showed the highest birefringence brightness, as a function of each refractive index (n) of the following imbibing media: water, 80% aqueous glycerin, 100% glycerin, Entellan® resin (Merck, Darmstadt, Germany) and Caedax® Synthetic Canada Balsam (Merck, Darmstadt, Germany). These fluids were used in the sequence they are quoted. Their correspondent refractive indices are reported in Fig. 3. Sections were immersed in the imbibing media for 30 min before measuring optical retardations, which were determined in nanometres (nm). A Leica DM LP microscope (Leica Microsystems) equipped with polarizing filters, Brace-Köhler compensator (Wild Leitz, Wetzlar, Germany) and polychromatic light was used.

Results and Discussion

As expected, Positive Control Group showed the highest birefringence brightness in the present study (Fig. 1C, D; Fig. 2; Fig. 3) because immediately immersion of dental fragments in 2% paraformaldehyde, 0.5% glutaraldehyde provides good structural preservation and effective enzyme inactivation (17,24). The secretory-stage enamel organic ECM from Negative Control samples became extensively degraded and almost lacked birefringence brightness (Fig. 1A, B; Fig. 2; Fig. 3), since the solution of that group did not contain any enzymatic inhibitor. Although Phenanthroline and PMSF groups exhibited a statistically significant reduction of ≈ 21% in optical retardation values in comparison to Positive Control group, they showed 3.5 fold higher optical retardation values when compared to Negative Control group (Fig. 1; Fig. 2, $p<0.01$, ANOVA followed by Tukey test; Fig. 3). Negative Control samples showed very lower dispersion birefringence curve in comparison with Positive Control, Phenanthroline and PMSF samples (Fig. 3). It is clear that phenanthroline and PMSF have exerted a protective action, avoiding an expressive decrease in the birefringence brightness of their groups. 1,10-

phenanthroline and PMSF are specific inhibitors of metalloproteinases and serine proteinases, respectively (25,26). The main enamel matrix proteinases are Mmp20, a metalloproteinase that is primarily expressed in the secretory stage of amelogenesis, and Klk4, a serine proteinase that is expressed in transition through maturation stages of enamel formation (4, 19-23).

Interestingly, 1,10-phenanthroline preserved the birefringence of the secretory-stage enamel organic ECM. This finding gives additional support for the idea that amelogenins' cleavage within the C-terminal area by Mmp20 (27) is essential for proper removal of proteins during transition through maturation stages of enamel development (28). Our results indicate that Mmp20 activity seems to be necessary for posterior extensive amelogenins' cleavage within the N-terminal area by Klk4 (29). Here, PMSF (specific inhibitor of serine proteinases, like Klk4) preserved birefringence of the secretory-stage enamel organic ECM, since Klk4 acts during transition and maturation stages. There are previous reports that show the presence of slightly Klk4 activity in the inner enamel matrix (22) and the existence of Klk4 pro-enzyme in the outer enamel layer (30) during the secretory stage. There are also evidences that secretory ameloblasts express a little amount of Klk4 mRNA and that odontoblasts may secrete Klk4 via dentinal tubules into the secretory-stage enamel matrix of dentin-enamel junction (31). From the results presented here and in our previous report (18), it is reasonable to assume that initial amelogenins' C-terminal removal by Mmp20 generates a more birefringent enamel organic matrix in the secretory stage and is necessary for subsequent amelogenins' N-terminal degradation by Klk4, which in turns largely decreases birefringence of the enamel organic matrix in transition through maturation stages. Kallikrein-4 activity dependent of enamelysin is here confirmed with the birefringence preservation by 1,10-phenanthroline (specific inhibitor of metalloproteinases, like Mmp20). We believe that complementary actions of Mmp20 and Klk4 in the secretory stage of enamel development are not observed in immediately fixed samples because amelogenesis is a continuous process and the result of those actions is seen during transition and maturation stages. In the

present investigation, we have stopped eruption process of upper incisor and allowed or inhibited the activity of those enzymes *ex vivo* during the secretory-stage.

In summary, this is the first report on the effect of metallo and serine proteinases' inhibitors *ex vivo* on the birefringence of the secretory-stage enamel organic extracellular matrix. It is clear from this study that the rapid loss of birefringence in not immediately fixed secretory-stage enamel organic ECM is caused by the activity of enamel proteinases. Moreover, the present work indicates that Klk4 activity may be dependent of a previous Mmp20 catalytic action on amelogenin molecules.

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Figure legends

Figure 1. Polarizing and bright field light microcopies of the secretory-stage enamel organic ECM from distal fragments of incisors included in Negative Control, Positive Control, Phenanthroline and PMSF groups. The analyzer and polarizer are demoted by crossed bars. The arrow at 45º with the polarizer and analyzer

indicates position of maximum birefringence. **A.** Birefringence of an unstained 5-µm-section of the enamel organic ECM from a Negative Control upper incisor fragment. **B.** Bright field of section A, after staining with HE. **C.** Birefringence of unstained 5-µm-section of the enamel organic ECM from a Positive Control upper incisor fragment. **D.** Bright field of section C, after staining with HE. **E.** Birefringence of unstained 5-µm-section of the enamel organic ECM from a Phenanthroline upper incisor fragment. **F.** Bright field of section E, after staining with HE. **G.** Birefringence of unstained 5-µm-section of the enamel organic ECM from a PMSF upper incisor fragment. **H.** Bright field of section G, after staining with HE. Note extensive degradation of the enamel organic ECM of Negative Control sample. Secretory-stage enamel organic matrices from samples included in Positive Control, Phenanthroline and PMSF groups showed similar morphological aspects. Interestingly, Phenanthroline and PMSF acted preserving the birefringence of the secretory-stage enamel organic ECM.

Figure 2. Optical retardations (nm) of unstained 5-µm-sections from the secretory-stage enamel organic ECM of Negative Control, Positive Control, Phenanthroline and PMSF groups. Different letters express statistical difference. $a \neq b$, $a \neq c$, $p<0.01$; $b \neq c$, $p<0.05$ (ANOVA followed by Tukey test). Note that Phenanthroline and PMSF were able to maintain high levels of optical retardation of birefringence brightness.

Figure 3. Dispersion birefringence curves of Negative Control, Positive Control, Phenanthroline and PMSF incisor fragments. Optical retardations (nm) of unstained 5 µm thick sections of the enamel organic ECM from upper rat incisor fragments, as a function of the refractive indices (n) of the following imbibing media: water (n=1.333), 80% aqueous glycerin (n=1.435), 100% glycerin (n=1.461), Entellan® resin (n=1.495) and Caedax® Synthetic Canada Balsam (n=1.56). Measurements of optical retardations were made with Brace-Köhler compensator and polychromatic light. Each point in the curve is the mean of 10

measurements. Note that the highest and the smallest (intrinsic birefringence) retardations for the series of refractive indices correspond respectively to n=1.435 (80% aqueous glycerin) and n= 1.495 (Entellan® resin). Phenanthroline and PMSF incisor fragments clearly shows higher form and intrinsic birefringences when compared to Negative Control sample.

Figures

Figure 1

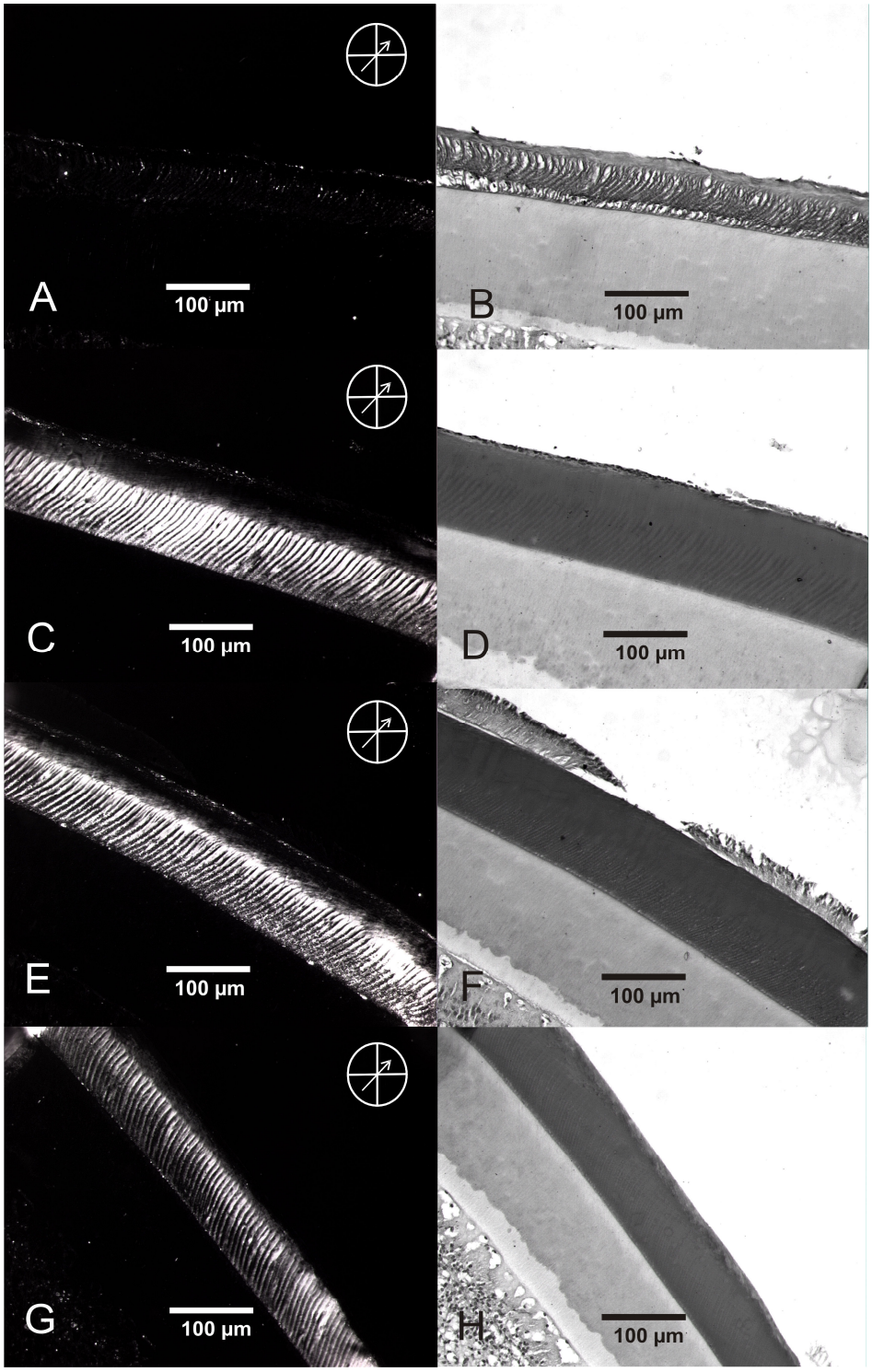


Figure 2

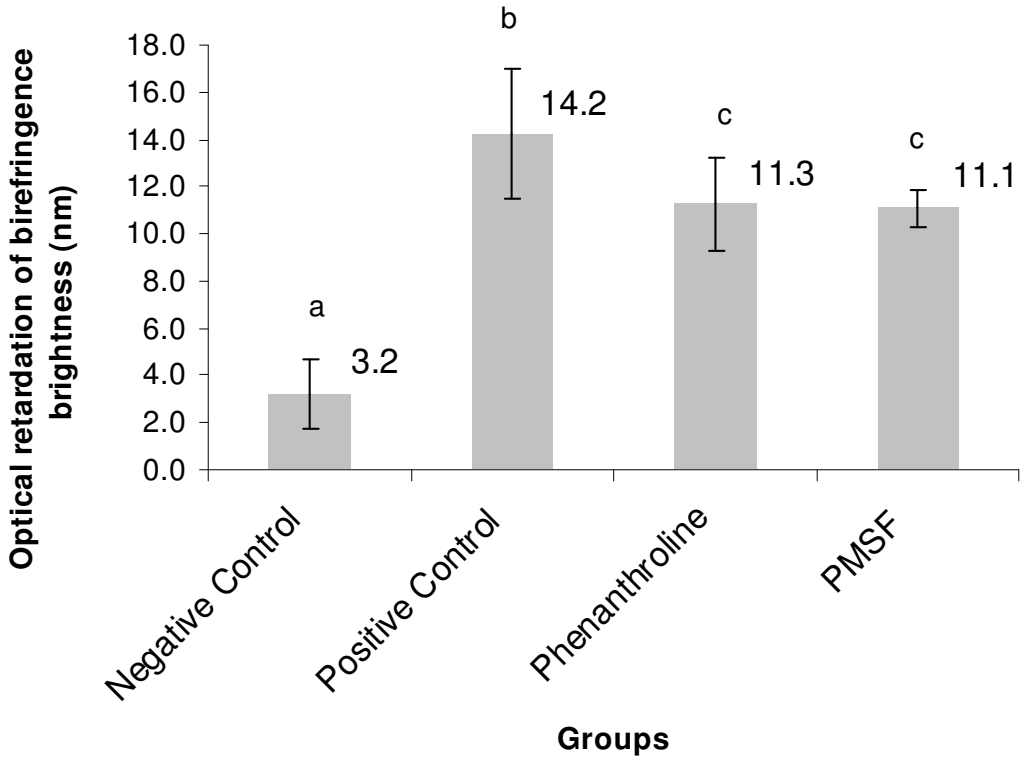
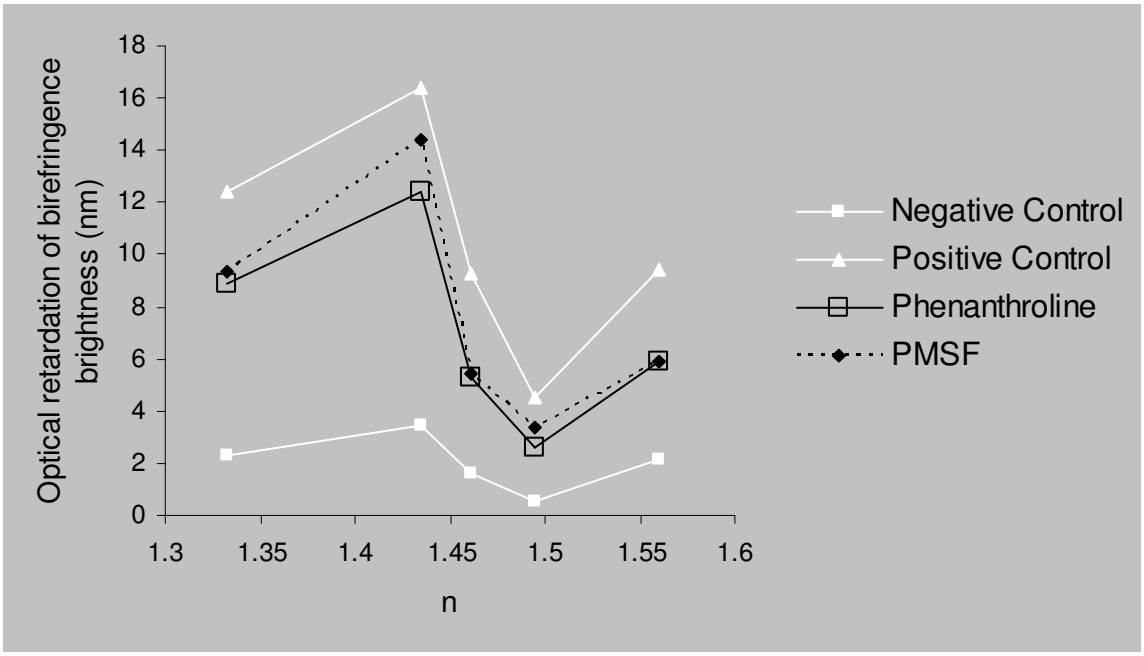


Figure 3



CONCLUSÃO

Os resultados apresentados e discutidos nos três capítulos deste trabalho de tese dão suporte às seguintes conclusões:

1. A birrefringência da matriz orgânica do esmalte no estágio de secreção da amelogênese depende da organização supramolecular das amelogeninas e é influenciada pela atividade proteolítica da MMP-20.

2. O etidronato dissódico não altera a ordem molecular da matriz orgânica do esmalte secretada, mas pode induzir defeitos severos no esmalte maduro através de interferência direta sobre a atividade secretora dos ameloblastos.

3. O tratamento com alendronato de sódio causa diminuição da birrefringência da matriz orgânica do esmalte secretório e está associado a uma redução da microdureza do esmalte maduro.

4. Inibidores de serina proteinases (como Klk4) e metaloproteinases (como Mmp20) preservam a birrefringência da matriz orgânica do esmalte secretório *ex vivo*.

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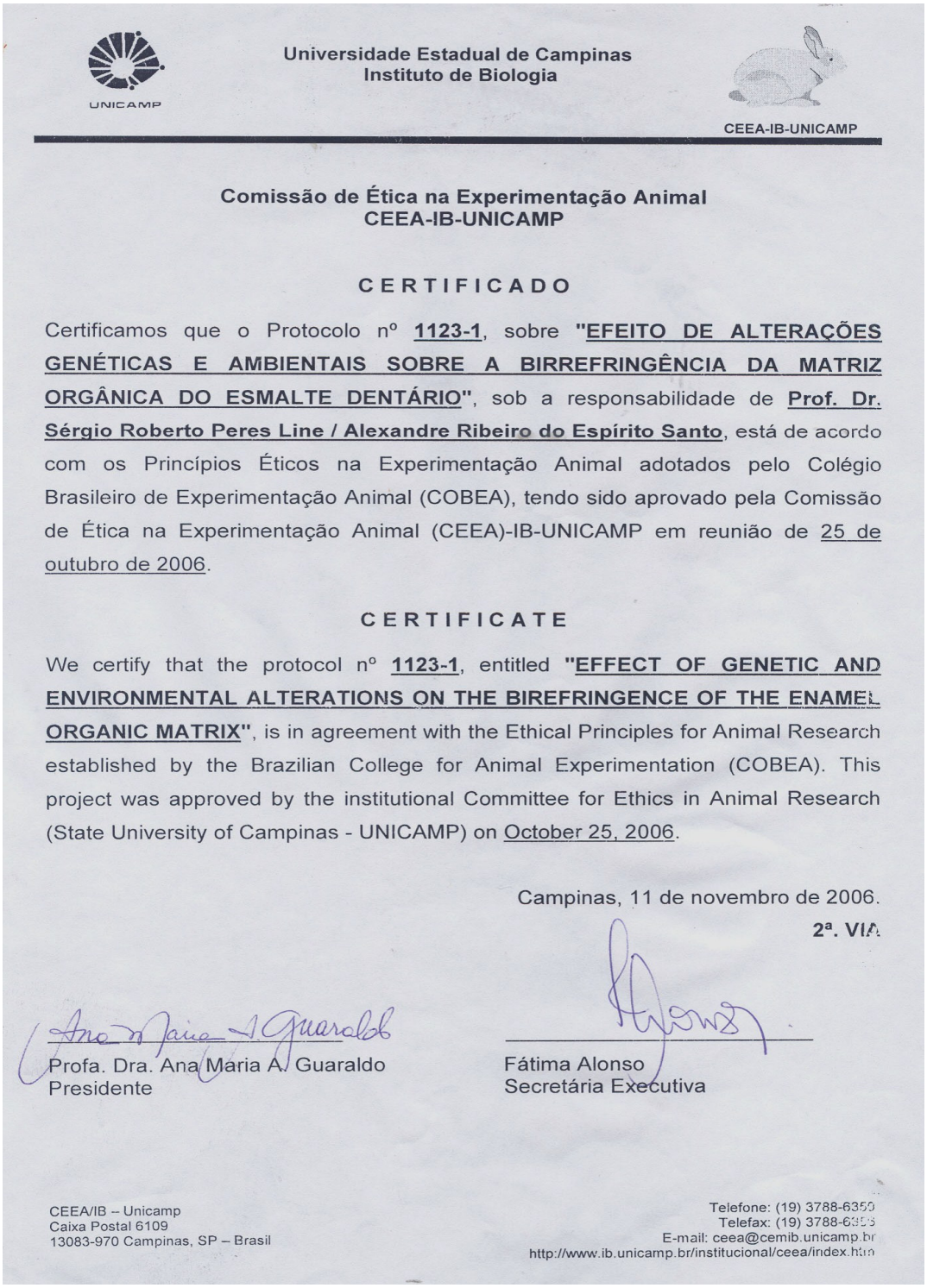
ANEXOS

ANEXO 1



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C E R T I F I C A D O

Certificamos que o Protocolo nº **1123-1**, sobre **"EFEITO DE ALTERAÇÕES GENÉTICAS E AMBIENTAIS SOBRE A BIRREFRINGÊNCIA DA MATRIZ ORGÂNICA DO ESMALTE DENTÁRIO"**, sob a responsabilidade de **Prof. Dr. Sérgio Roberto Peres Line / Alexandre Ribeiro do Espírito Santo**, 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 reunião de 25 de outubro de 2006.

C E R T I F I C A T E

We certify that the protocol nº **1123-1**, entitled **"EFFECT OF GENETIC AND ENVIRONMENTAL ALTERATIONS ON THE BIREFRINGENCE OF THE ENAMEL ORGANIC MATRIX"**, 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 25, 2006.

Campinas, 11 de novembro de 2006.

2ª. VIA

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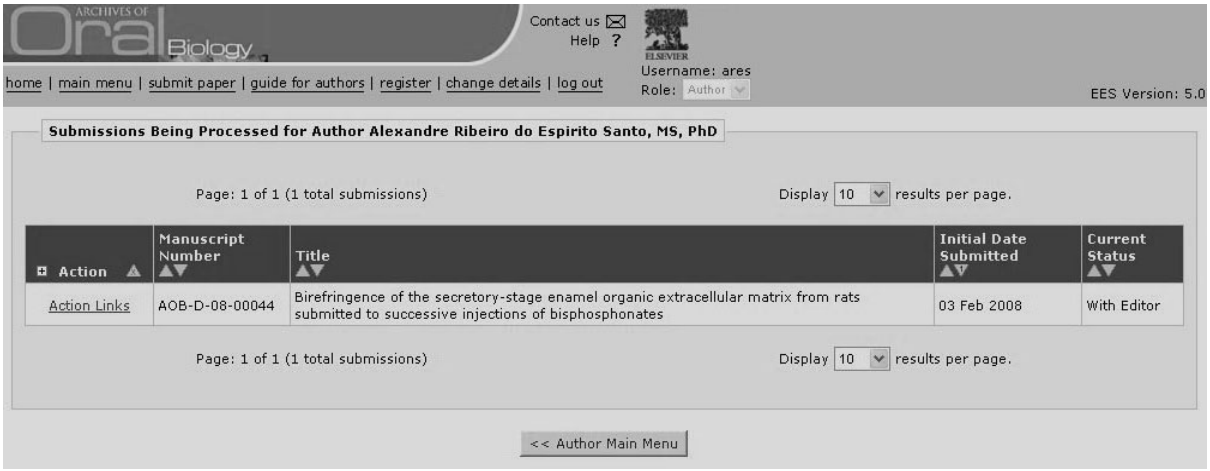
ANEXO 2



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