UNIVERSIDADE ESTADUAL DE CAMPINAS

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EFEITO DO FLUOR E DE ALGUNS METAIS NA ATIVIDADE DE PROTEINASES DA MATRIZ DO ESMALTE *IN VITRO*

Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas para obtenção do título de Doutor em Biologia Celular e Estrutural na área de Biologia Celular.

Orientador: Prof. Dr. Sérgio Roberto Peres Line

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À minha mãe,
porque nada lhe parece difícil.

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SUMÁRIO

O esmalte dental é um tecido epitelial altamente mineralizado, que recobre os dentes dos vertebrados. Em contraste com os tecidos mineralizados conjuntivos, os cristais no esmalte não são depositados sobre uma matriz rica em colágeno, mas crescem concomitantemente à remoção da maioria das proteínas da matriz do esmalte, as quais são gradualmente degradadas durante a mineralização. Existem múltiplas evidências para um papel importante das proteases presentes na matriz do esmalte nesta degradação. A inibição da atividade de proteases presentes no esmalte poderia comprometer a perfeita mineralização do esmalte. Neste trabalho testou-se *in vitro* o efeito de flúor e de alguns metais na atividade de proteases presentes na matriz do esmalte. Três ensaios foram utilizados: um ensaio de atividade enzimática usando um substrato colorimétrico (azocaseína), um ensaio de degradação de proteínas estruturais do esmalte e zimografia. Os resultados mostraram que o flúor (625 uM to 10 mM) não exibe efeito inibitório sobre a atividade das proteases, o que é um dado relevante para a discussão dos possíveis mecanismos patogênicos da fluorose dentária. Os resultados da inibição com alguns metais revelaram que zinco, cádmio e chumbo têm um efeito inibitório em concentrações de até 110 uM. Estes últimos resultados são interessantes à medida em que se sabe que estes metais são incorporados ao esmalte durante o desenvolvimento em concentrações que refletem a exposição durante a formação dos tecidos dentais, e, ainda, porque existe uma associação ainda não explicada entre aumento de cárie e exposição a metais pesados, particularmente cádmio e chumbo.

ABSTRACT

Dental enamel is a highly mineralized epithelial tissue found on mammalian teeth. In contrast to the connective mineralized tissues, enamel crystallites are not deposited onto a collagenous matrix, but grow concomitantly with the removal of most of the enamel matrix proteins, which are gradually degraded during mineralization. There are many evidences indicating that enamel resident proteinases play an important role in the degradation of proteins. Inhibition of enamel matrix proteinases could impair normal enamel mineralization. In this work, we investigated the effect of fluoride and some metals on the activity of enamel matrix proteinases *in vitro* using a colorimetric assay (azocasein), an enamel protein degradation assay and zymography. The results showed that fluoride (625 uM to 10 mM) does not have an inhibitory effect on proteinase activity. This is a relevant finding concerning possible pathogenic mechanisms underlying dental fluorosis. The results of the inhibition by some metals revealed that zinc, cadmium and lead cause inhibition at concentrations as low as 110 uM. These results are interesting due to the fact that dental tissues are known to harbor these metals in concentrations related to the exposure at the time of dental tissues formation, and, furthermore, because there is an unexplained link between increased caries prevalence and exposure to heavy metals, particularly cadmium and lead.

INTRODUÇÃO

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

As amelogeninas são um grupo heterogêneo de proteínas que constitui 90% do total de proteínas da matriz no estágio secretório (Termine et al., 1980). As diferenças entre as amelogeninas ocorrem por 3 razões principais: 1) as cópias do gene que codifica este grupo de proteínas são diferentes. Uma se localizada no cromossomo X e outra, no cromossomo Y, sendo esta última mais curta. 2) Existem vários mRNAs de amelogeninas que são produzidos por splicing alternativo. 3) O processamento proteolítico das formas de maior massa molecular. Dentre as propriedades físicas das amelogeninas está o fato de serem altamente hidrofóbicas, com exceção de uma porção hidrofílica na região carboxi-terminal, a qual é removida por proteólise. É através desta região hidrofílica que as amelogeninas interagem com cristais de hidroxiapatita, e é interessante que esta região também é necessária para a inibição do crescimento de cristais em soluções contendo amelogeninas (Aoba et al., 1987). Em solução, as amelogeninas formam agrados, as chamadas "nanosferas". As implicações biológicas destas características das

amelogeninas poderiam estar relacionadas com a habilidade das amelogeninas de maior massa molecular, organizadas em nanosferas, de interagir com os cristais extremamente finos dos estágios precoces da formação do esmalte e protegê-los de fusões prematuras (Fincham et al., 1999). A localização da amelogenina contendo a região carboxi-terminal também é distinta da localização das demais amelogeninas geradas por proteólise, sendo a marcação da porção carboxi-terminal restrita aos 40 μms superficias de esmalte em desenvolvimento (Uchida et al., 1991). Localizações distintas para a amelogenina de maior massa molecular e seus produtos de degradação sugerem funções distintas.

O papel fundamental das amelogeninas na mineralização do esmalte pode ainda ser verificado: 1) pelo fato do gene da amelogenina estar alterado nos casos de Amelogênese Imperfeita ligada ao cromossomo X (Lagerström-Fermer et al., 1995), doença em que há formação de esmalte pouco mineralizado e bastante desorganizado; 2) por resultados de um ensaio que usa uma ribozima sintética para degradar especificamente amelogenina, o qual demonstrou que os animais em que a ribozima foi injetada não desenvolveram dentes com esmalte perfeitamente mineralizado (Lyngstadaas et al., 1995). Além das amelogeninas, a fase orgânica da matriz contém outras proteínas estruturais e várias proteinases (Overall & Limeback, 1988).

As proteases da matriz do esmalte já identificadas pertencem às famílias de serino-proteinases e de metaloproteinases (Carter et al., 1989; DenBesten & Heffernan, 1989; Moradian-Oldak et al., 1994; Coletta, 1996; Fukae et al., 1998). Tanabe et al. (1992) isolaram serino-proteases (as quais chamaram amelogeninases) de 76 e 78 kDa da porção externa da matriz do esmalte secretório de porco (fase secretória), e demonstraram que estas enzimas são capazes de clivar a porção C-terminal da amelogenina de 25 kDa. Além disso, foi demonstrado que estas amelogeninases também

clivam enamelinas, além das amelogeninas (Tanabe et al., 1994). Estes mesmos autores demonstraram que amelogeninases de 30 e 34 kDa, capazes de clivar a porção N-terminal da amelogenina de 20 kDa, estão presentes na porção interna do esmalte (Tanabe et al., 1992). Recentemente a serino-protease de 30 e 34 kDa foi clonada a partir da varredura de bibliotecas de cDNA de órgão do esmalte com primers degenerados. Esta protease, que no tecido é encontrada com as duas formas (30 e 34 kDa), é atualmente conhecida como EMSP-1 (Simmer et al., 1998). A outra protease do esmalte mais bem caracterizada é uma metaloproteinase que se apresenta em zimogramas com as formas de 41 e 45 kDa, conhecida como enamelisina e classificada como MMP-20 (Bartlett al., 1996; Bartlett et al., 1998; Bartlett & Simmer, 1999). Fragmentos correspondentes ao domínio catalítico da MMP-20 também já foram descritos com massas moleculares de 21 e 25 kDa (DenBesten et al., 1998). A autólise descrita em ensaios utilizando a MMP-20 recombinante produzida em bactérias também indica que sua autodegradação pode ser intensa (Li et al., 1999; Ryu et al., 1999).

As amelogeninas normalmente são hidrolizadas e removidas da matriz de modo mais lento no estágio secretório e de modo bastante intenso no estágio de transição/maturação precoce da formação do esmalte (Smith et al., 1996; Smith, 1998). A remoção das amelogeninas durante as maturação parece um passo crítico para a mineralização do esmalte e parece dependente de proteases (Bartlett & Simmer, 1999). Primeiramente, porque até hoje não se demonstrou, consistentemente, a atividade fagocitária de ameloblastos (Smith, 1998). Em segundo lugar, porque a digestão de proteínas estruturais íntegras *in vitro* pelas proteases (particularmente a MMP-20) produz os mesmos produtos de clivagem já isolados a partir da matriz do esmalte (Ryu et al., 1999). Finalmente, porque quando se acompanha a degradação de proteínas presentes

em extratos de matriz de esmalte, percebe-se a degradação gradual das formas de maior massa molecular, exceto em situações em que a matriz é fervida (Smith & Chen, 1998). Estes achados tornam a inibição das proteases presentes na matriz do esmalte uma hipótese atrativa para explicar defeitos do esmalte com características histológicas de mineralização deficiente e achados bioquímicos de acúmulo de proteínas.

Um defeito deste tipo é encontrado em casos de fluorose dentária (Weatherell et al., 1985). A fluorose dentária está diretamente relacionada a várias formas de exposição a flúor durante a formação do esmalte e se caracteriza por uma camada externa de esmalte normalmente mineralizada e uma camada subjacente hipomineralizada (Aoba, 1997). Vários autores descreveram um retardo na remoção das proteínas da matriz durante a formação do esmalte fluorótico (Larsen et al., 1985; Richards et al., 1986; DenBesten, 1986; Tostes, 1988). Esta alteração, que se torna mais evidente no estágio de maturação, poderia decorrer do aumento na secreção protéica, diminuição da quantidade de proteases ou da atividade destas proteases (DenBesten, 1989; DenBesten & Thariani, 1992). Contudo, nenhuma destas teorias foi comprovada (Aoba, 1997).

Se por um lado se sugeriu por mais de uma década que o flúor pudesse inibir as proteases dos esmalte, sem que houvesse qualquer comprovação *in vitro* desta inibição, por outro lado pouca atenção foi dada ao possível efeito de outros elementos passíveis de incorporação ao esmalte, com é o caso de metais pesados, e que notoriamente são capazes de inibir proteases (Hojima et al., 1994; Auld, 1995; Souza et al., 2000).

Tendo em vista o papel potencialmente importante das proteases na amelogênese e o fato de flúor e outros elementos serem incorporados ao esmalte durante a sua formação (Lappalainen et al., 1979; Anttila & Anttila., 1987; Grünke et al., 1996; Daculsi et al., 1997), torna-se fundamental verificar o efeito de flúor e de alguns

metais presentes no esmalte sobre a atividade das proteases do esmalte. O objetivo deste trabalho foi avaliar *in vitro* a atividade de proteases do esmalte na presença de diferentes concentrações de flúor e de alguns metais.

O primeiro manuscrito, uma carta ao Editor, trata de um aspecto da padronização de testes com metais: a capacidade tampão das soluções. É necessário observar que os efeitos podem ser decorrentes de alterações no pH das soluções contendo metais.

O segundo artigo mostra que o flúor não causa alterações na atividade das proteases do esmalte nos ensaios *in vitro* realizados. Estes resultados depõem, pelo menos parcialmente, contra a hipótese tão notória da inibição de proteases como mecanismo patogênico da fluorose dental.

O terceiro manuscrito mostra que os metais cádmio, zinco e chumbo são capazes de inibir as proteases do esmalte *in vitro*. Embora não se possam ainda saber quais as consequências destes achados, é interessante observar que estudos epidemiológicos mostram aumentos da prevalência de cárie em regiões contaminadas por chumbo (Curzon & Bibby, 1970; Anderson et al., 1976; Gil et al., 1996). Além disso, estudos em animais demonstraram um aumento de 40% na prevalência de cárie em um modelo usando ratos expostos a chumbo (Watson et al., 1997), sem que a causa para este aumento na suscetibilidade à cárie pudesse ser explicada.

O manuscrito incluído no apêndice mostra um outro efeito do chumbo sobre os tecidos dentais: o retardo na erupção em um modelo de crescimento desimpedido de incisivos em ratos.

ARTIGOS

Letter to the Editor

Mechanisms of cell activation by metal ions: Influence of pH

We read with great interest the article by Wagner et al.[1] describing the effects of heavy metal ions on cultured human vascular endothelial cells (HUVECs). Because cobalt, nickel, chromium, and zinc are components of many materials used as surgical implants, the authors analyzed the influence of these metal ions on HUVECs at concentrations ranging from 1 m*M* to 0.1 n*M*. $NiCl_2$ and $CoCl_2$ were shown to upregulate the expression of adhesion molecules (E-selectin and ICAM-1) and cytokines mRNA (IL-6 and IL-8). In contrast, a 5-h incubation with $NiCl_2$ and $CoCl_2$ did not alter cell morphology and did not seem to have a cytotoxic effect, whereas $CrCl_3$ and $ZnCl_2$ strongly damaged the HUVECs. Using different drugs that inhibit specific sites of cellular signal transduction, these authors analyzed the signal transduction pathways induced by the exposure to the heavy metals and concluded that mechanisms of gene activation similar to those induced by proinflammatory mediators take place.

A series of experiments regarding the effect of metal ions on matrix metalloproteinases is being conducted in our laboratory. In our experience, a crucial point to consider in experiments with metal salts is the pH of the solution. Acidic metal salts can interfere with buffered solutions (50 m*M* Tris-HCl). Following this reasoning, we wondered if the effects of the metal solutions on HUVECs described by Wagner et al.[1] may have been elicited in part by changes in culture medium pH. We prepared Dulbecco's modified eagle AU1 medium (GIBCO), adjusted the pH to 7.4 according to the manufacturer's recommendations, and prepared solutions of 1 m*M* $CrCl_3$, 1 m*M* $ZnCl_2$, 1 m*M* $CoCl_2$, and 1 m*M* $NiCl_2$. Six solutions of each metal salt and six control solutions were prepared and pH was determined blindly. Mean and standard deviation of metal salts and control are shown in Table I. The Kruskal–Wallis nonparametric ANOVA test showed that variation between medians was significant and Dunn's multiple comparison test showed that significant differences were found comparing control with $CrCl_3$, control with $NiCl_2$, and $CrCl_3$ with $ZnCl_2$. Calcula- AU2 tions were made using the GraphPad Instat software.

As shown in Table I, metal salts at 1 m*M* significantly lowered the pH of Dulbecco's modified eagle medium. Even more important, the 0.33-pH variation between control and $CrCl_3$ should not be considered a minor change. This variation means acidosis in physiologic terms, and clinically, such pH variation in plasma is very dangerous. Nonetheless, pH fluctuations of this magnitude are not likely to take place in plasma, but may be important in the extracellular milieu surrounding an implant. Acidosis accompanies inflammation, and metals leaking from implants may further enhance the local inflammatory response by lowering the pH. Recent reports have shown that acidosis correlates with alterations in the expression of adhesion molecules,[2,3] particularly intercellular adhesion molecule.[4] Thus, we think that acidosis may have played some role in the cell activation effect of metals found by Wagner et al.[1] Besides, as stated by Wagner et al.[1] themselves, the mechanisms of cell activation shown are similar to those induced by proinflammatory mediators.

TABLE I
pH of Metal Salt Solutions ($N = 6$ for Each Group)

	Control	$CrCl_3$	$NiCl_2$	$CoCl_2$	$ZnCl_2$
Mean	7.45	7.12*	7.24**	7.29	7.32***
SD	0.03	0.01	0.02	0.01	0.05

*$p < 0.001$ $CrCl_3$ vs. control.
**$p < 0.01$ $NiCl_2$ vs. control.
***$p < 0.05$ $CrCl_2$ vs. $ZnCl_2$.

References

1. Wagner M, Klein CL, van Kooten TG, Kirkpatrick CJ. Mechanisms of cell activation by heavy metal ions. J Biomed Mater Res 1998;42:443–452.
2. Wesson DE, Simoni J, Green DF. Reduced extracellular pH increases endothelin-1 secretion by human renal microvascular endothelial cells. J Clin Invest 1998;101:578–583.
3. Serrano CV Jr, Fraticelli A, Paniccia R, Teti A, Noble B, Corda S, Faraggiana T, Ziegelstein RC, Zweier JL, Capogrossi MC. pH dependence of neutrophil-endothelial cell adhesion and adhesion molecule expression. Am J Physiol 1996;271:C962–C970.
4. Zund G, Uezono S, Stahl GL, Dzus AL, McGowan FX, Hickey PR, Colgan SP. Hypoxia enhances induction of endothelial ICAM-1: Role for metabolic acidosis and proteasomes. Am J Physiol 1997;273:C1571–C1580.

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Fluoride effect on the activity of enamel matrix proteinases *in vitro*

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Dental fluorosis is common in individuals exposed to different sources of fluoride during tooth development. The mechanism causing this enamel defect is still unknown. Enamel matrix proteinases play a central role in the maturation of dental enamel, and inhibition of these enzymes by fluoride has been one explanation for dental fluorosis. We have investigated the effect of fluoride on the activity of enamel matrix proteinases using a colorimetric assay, casein zymography, and an enamel protein degradation assay. Fluoride (625 μM to 10 mM) inhibited neither the enzymatic activity of the crude matrix extract nor the activity of individual enamel enzymes separated by SDS-PAGE. The proposition that fluoride could directly inhibit enzymes was not confirmed in this study.

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A dramatic decline in dental caries prevalence has been documented worldwide in the last decades as a result of the widespread use of fluoride in various forms (1). However, as a consequence of widespread fluoride ingestion from different sources, the prevalence of dental fluorosis has increased to astonishingly high levels (2), and public health concern has increasingly been directed toward this problem. Despite knowledge regarding timing of exposure and doses of fluoride that cause fluorosis (3), evidence for the mechanism that causes this enamel defect is still lacking. Fluorotic enamel is histologically characterized as a subsurface-hypomineralized lesion covered by a well-mineralized outer enamel surface, which in severe cases breaks apart shortly after eruption (4). The enamel defect has been associated with an arrest in enamel maturation stage characterized by delayed breakdown of amelogenins (5), which may become entrapped in the fluorotic enamel depending on the severity of the insult. Among the many hypotheses that have been proposed for the mechanism by which excess fluoride affects degradation and removal of enamel matrix proteins, three are considered by most researchers (6, 7): 1) fluoride might directly affect ameloblasts (8); 2) proteins may be more tightly bound to fluoridated hydroxyapatite and, thus, proteinolysis might be more difficult (9); 3) fluoride might inhibit enamel proteinases (10).

In recent years, many enamel proteinases have been characterized (11–13). According to Ryu *et al.* (12), enamel proteases play two important roles during enamel formation. First, they process secreted enamel matrix proteins into stable products that are believed to be active during amelogenesis, and secondly they allow the enamel layer to achieve a high degree of mineralization through the complete degradation of the enamel matrix proteins. Despite the important advances in this area, no conclusive results were obtained as to whether fluoride directly inhibits enamel proteinases. The aim of this study was to determine if fluoride has a direct effect on the activity of enamel proteinases.

Material and methods

Enamel matrix sample

Upper and lower incisors of Wistar rats weighing 300 g were used in this study. Mandibles and teeth were kept on ice until the enamel matrix was scraped from the teeth. Prior to scraping, teeth had been carefully cleared of soft tissue and wiped with a moist gauze to remove cell debris. Maturation stage matrix was not separated from secretory stage

matrix. The enamel matrix was demineralized for 1 h by pooling the matrix scraped from the four teeth of each animal into a 1.5-ml microcentrifuge tube containing 1.0 ml of ice-cold 5% (w/v) trichloroacetic acid (TCA), followed by centrifugation for 5 min to precipitate enamel protein (10). The pellet was rinsed once in 50 μl of 1 M Tris, pH 8.0, for pH neutralization. Since the proteolytic assays described below required different sample preparations, the pellets were separately extracted for colorimetric assay, zymography, and enamel protein degradation assay.

Colorimetric assay

For the total proteolytic activity assay, the enamel matrix of 5 rats was weighed (corresponding to 9 mg of enamel), demineralized, and neutralized as described above. Pellets were then extracted for 1 h in ice-cold 10 mM Tris, pH 8.0, containing 1% Triton X-100 (200 μl of this solution for each pellet obtained from 4 incisors). The supernatants were pooled and brought to a final volume of 1.8 ml. pH was adjusted to 7.2 by the addition of 1 M HCl prior to the beginning of the assay (the amount of 1 M HCl necessary to lower the pH to 7.2 was determined using the Henderson–Hasselbalch equation). In order to estimate protein concentration, a protein precipitation step was necessary after extraction, since buffers containing Triton X-100 or SDS as well as glycerol are known to interfere with the dye-binding assay in phosphoric acid (Bradford assay) for protein determination (14). One-hundred μl from 4 samples extracted with buffer containing Triton X-100 were used for ethanol precipitation (10 × v/v). Pellets were resuspended in 100 μl of 6 M Urea, and 10 μl aliquots from the SDS-extracted samples and 20 μl from the Triton X-100-extracted samples were used for protein determination according to the manufacturer's instructions (Bio-Rad, Richmond, CA, USA). Bovine serum albumin solutions at concentrations ranging from 2 to 16 μg were used as protein standards. Blank and standards contained 10 μl 6 M urea. Estimated protein concentration in the supernatant used was 0.3 μg/μl. Total proteolytic activity of the enamel matrix sample was assayed with a freshly prepared solution of heat-denatured azocasein as substrate (Sigma, St. Louis, MO) (15). Nine reaction tubes were prepared and 200 μl of the Triton X-100 supernatant was added to each tube, which contained 8 mg azocasein in 50 mM Tris, pH 7.2, with 1 mM $CaCl_2$ and 0.4 mM NaN_3 in a total volume of 1.0 ml. Three assay blanks were prepared by omitting the Triton X-100 supernatant from the reaction mixture. After 24 h incubation at 37°C, the reaction was stopped by the addition of 0.5 ml 10% (w/v) TCA. After centrifugation for 15 min at 30,000 *g*, the release of diazo-amino acids from azocasein in the supernatant was measured at 440 nm according to CHARNEY and TOMARELLI (15). One unit of endopeptidase activity was the amount of enzyme required to produce an increase of absorbance of 1.0 within 1 h under the assay-conditions. To observe the influence of NaF on enzyme activity, NaF was added to reactions at final concentrations of 5 mM and 10 mM. This assay was made in triplicate. The mean proteolytic activity value of each group was composed from the three values found in the triplicates. Data are presented as means ± SEM. Data were analyzed using ANOVA.

Zymography

Ten % polyacrylamide slab gels containing 0.05% casein or 0.05% gelatin were used. The stacking gel was polymerized using a comb that resulted in one 0.8-cm-wide lane to which the molecular weight standard was applied, and an 11.5-cm-wide lane (continuous lane) to which the sample was applied. Gelatin-containing gels were loaded with twice the amount of protein loaded on casein-containing gels. Samples were prepared in the following way: After neutralization, each pellet was extracted for 30 min in 200 μl non-reducing sample buffer (62.5 mM Tris, 15% glycerol, 1% SDS and 0.05% bromophenol blue, pH 6.8), centrifuged, and the supernatant was applied to the gel. SDS-extracted samples contained 1 μg protein/μl of solution. An aliquot (132 μl) was applied to the continuous lane of the casein-containing gel. An aliquot of 264 μl was applied to the continuous lane of the gelatin-containing gel. Low-molecular-weight protein marker (Amersham Pharmacia Biotech, Piscataway, NJ, USA) was applied to the separate lane on each gel. The proteins were separated by electrophoresis as described by LAEMMLI (16) at 4°C, and SDS was removed by incubating gels for 1 h in two baths of 2.5% Triton X-100. The continuous lane of each gel was cut into strips that were incubated for 40 h at 37°C with shaking in 40-ml tubes. Tris buffer (50 mM Tris/1 mM $CaCl_2$, pH 7.4) was used for incubation, and proteinase inhibitors or different concentrations of NaF were added to this solution. The strips were then stained with 0.05% Coomassie Brilliant Blue in 50% (v/v) methanol and 5% (v/v) acetic acid and destained with 50% (v/v) methanol and 10% (v/v) acetic acid. These procedures were performed three times on separate occasions. NaF concentrations varied from 10 mM to 625 μM. Proteinase inhibitors were purchased from Sigma. Phenylmethylsulphonyl fluoride (PMSF) was used

at 2 mM, N-ethylmaleimide (NEM) at 1 mM, ethylenediamine tetraacetic acid (EDTA) at 10 mM, and 1,10-phenanthroline at 5 mM. PMSF is a serine proteinase inhibitor, NEM is a cysteine proteinase inhibitor, and EDTA and 1,10-phenanthroline are inhibitors of metalloproteinases, but EDTA can also inhibit serine proteinases when they need calcium for structure stabilization (17). The same volume of methanol present in inhibitor solutions was added to the solution in which control strips were incubated.

Fluoride concentrations

Fluoride was measured to determine if free fluoride concentrations were within the expected limits in the incubation solutions of zymograms (fluoride concentrations varied from 625 μM to 10 mM in zymography solutions containing 1 mM Ca). The measurements were carried out using an Orion fluoride ion electrode (model 96/09) and an Orion digital ion-analyzer (model EA-940) (Orion Research, Boston, MA, USA). After incubation of the zymography strips in the solutions, these same solutions were mixed with equal volumes of TISAB (total ionic strength adjusting buffer: 1.0 M acetate buffer, pH 5.0, 1.0 M NaCl and 0.4% 1,2-cyclohexanediaminetetraacetic) and analyzed. All solutions of the triplicate assays were analyzed, so that three readings were available for each concentration.

In vitro enamel protein degradation

In a further attempt to examine the effect of fluoride on the degradation of enamel proteins, an *in vitro* degradation assay was carried out. Enamel matrix derived from 2 rats was prepared as described in the first section of Material and methods. After neutralization, pellets were well homogenized by hand in a glass homogenizer for 1–3 min in 1.0 ml ice-cold 50 mM Tris, pH 7.2, containing 1 mM $CaCl_2$ and 1% Triton X-100, in order to completely dissolve aggregates. Aliquots of 100 μl were pipetted into 1.5-ml microcentrifuge tubes that were incubated at 37°C with shaking for different time periods. One tube contained protease inhibitors and was incubated for 48 h (proteolysis control). The inhibitors were 1,10-phenanthroline, PMSF, EDTA and NEM, all used at 5 mM final concentration. Methanol was added to all other reactions to achieve the same volume present in the inhibitor tube. Three tubes contained 10 mM NaF and were incubated for 12, 24 and 48 h, respectively, and this was also done for three control reactions. Aliquots (100 μl) were separated for ethanol precipitation and protein estimation as described above (samples contained 0.7 μg protein/μl). After each incubation time, samples were mixed with 50 μl reducing sample buffer (250 mM Tris, 60% glycerol, 4% SDS and 0.2% bromophenol blue, pH 6.8, containing 4% β-mercaptoethanol) and heated to 100°C for 5 min. Samples were stored at –20°C until electrophoresis. Seventy μl of each sample and 10 μl of low molecular weight protein marker (Amersham Pharmacia Biotech) were applied to separate lanes of a 15% polyacrylamide gel that was stained with Coomassie Brilliant Blue. These procedures were performed three times on separate occasions.

Results

Under the conditions used in the colorimetric assay, there were no differences in total proteolytic activity between groups ($p=0.61$; $F(2;6)=0.535$) (Fig. 1). Since a crude protein extract containing different enamel enzymes was used in the colorimetric assay, slight alterations in enzyme activity would go unnoticed. To observe if alterations in the activity of individual enzymes were present, we prepared zymograms containing casein and gelatin as substrates and used NaF solutions ranging from 625 μM to 10 mM for incubation. Zymograms revealed no alteration in the negative band pattern of the incubations with different concentrations of NaF (Figs. 2, 3). Incubation of strips with inhibitors revealed that most enzymes present in the crude extract used in this study seemed to be metalloproteinases (Fig. 2, lanes 4 and 5; Fig. 3, lanes 4 and 5), but some serine proteinase activity was also detected (Fig. 2, lane 2; Fig. 3, lane 2).

The hypothesis that fluoride could indirectly inhibit enamel enzymes through complexation with the calcium in the solution (shown to be essential for the activity of enamel enzymes) prompted us to analyze the free fluoride content of the solutions used. Fluoride analysis revealed that the solutions contained the expected concentrations of free

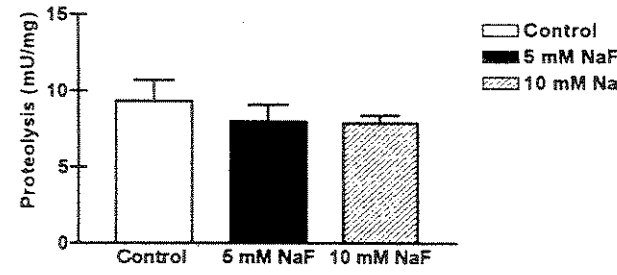

Fig. 1. Total proteolytic activity of enamel matrix incubated with NaF. One unit (U) of peptidase activity was defined as the amount of enzyme that increased the absorbance by 1.0 per h. Data are reported as means and SEM. One-way analysis of variance (ANOVA) revealed no difference between the groups.

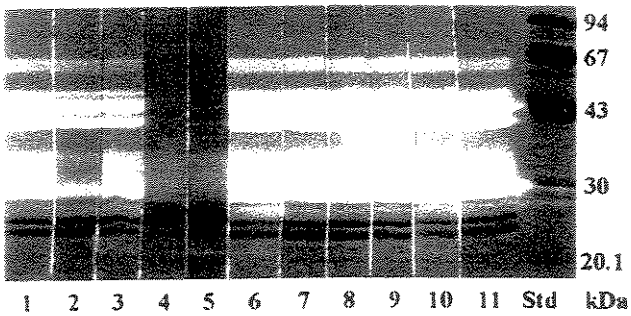

Fig. 2. Strips of 10% polyacrylamide gel containing 0.05% casein and the electrophoresed enamel extract were incubated with different concentrations of NaF or protease inhibitors in assay buffer (50 mM Tris/1 mM $CaCl_2$, pH 7.4). Lanes: 1 and 11 – control; 2 – 2 mM PMSF; 3 – 1 mM NEM; 4 – 10 mM EDTA; 5 – 5 mM 1,10-phenanthroline; 6 – 10 mM NaF; 7 – 5 mM NaF; 8 – 2.5 mM NaF; 9 – 1.25 mM NaF; 10 – 0.625 mM NaF; Std – molecular mass protein markers.

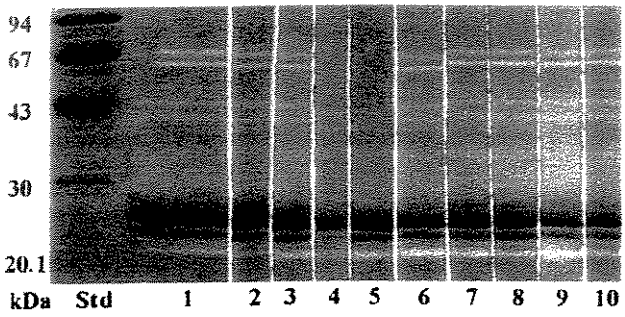

Fig. 3. Strips of 10% polyacrylamide gel containing 0.05% gelatin and the electrophoresed enamel extract were incubated with different concentrations of NaF or protease inhibitors in assay buffer (50 mM Tris/1 mM $CaCl_2$, pH 7.4). Lanes: Std – molecular mass protein markers; 1 – control; 2 – 2 mM PMSF; 3 – 1 mM NEM; 4 – 10 mM EDTA; 5 – 5 mM 1,10-phenanthroline; 6 – 10 mM NaF; 7 – 5 mM NaF; 8 – 2.5 mM NaF; 9 – 1.25 mM NaF; 10 – 0.625 mM NaF.

fluoride (results not shown). Although fluoride may interact with calcium under physiologic conditions, in this study fluoride remained free, perhaps as a consequence of the use of Tris. It is worth remembering that fluoride was in far excess in some of the solutions.

We also obtained enamel matrix homogenates taken from secretory and maturation stages to follow the degradation of proteins present in this extract over 48 h. After incubation for 12, 24 or 48 h, aliquots were separated on a 15% polyacrylamide gel stained with Coomassie blue. Homogenates containing 10 mM NaF showed the same degradation pattern as the control homogenates (Fig. 4). The proteolysis control (with protease inhibitors) showed proteins similar to the control and fluoride-exposed homogenates at time 12 h, indicating that enzymes present in the homogenates were degrading the proteins. In this system, high concentrations of fluoride did not affect the degradation of enamel proteins present in the homogenates.

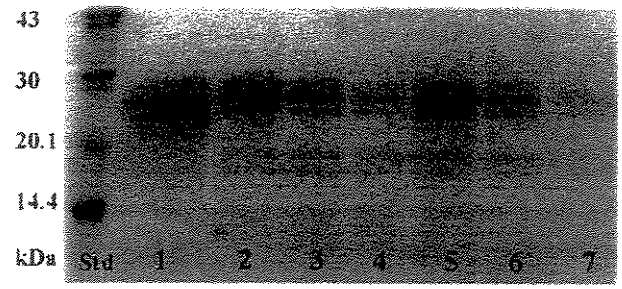

Fig. 4. Polyacrylamide gel (15%) stained with Coomassie blue. Enamel matrix homogenates taken from secretory and maturation stages and incubated *in vitro* 0–48 h at 37°C in 50 mM Tris/1 mM $CaCl_2$, pH 7.2, containing 10 mM NaF. Lanes: Std – molecular mass protein markers; 1 – proteolysis control containing 5 mM PMSF, 5 mM NEM, 5 mM EDTA and 5 mM 1,10-phenanthroline; 2 – control, 12-h incubation; 3 – 10 mM NaF, 12-h incubation; 4 – control, 24-h incubation; 5 – 10 mM NaF, 24-h incubation; 6 – control, 48-h incubation; 7 – 10 mM NaF, 48-h incubation.

Discussion

When a substance is supposed to affect enzymatic activity, the usual way to answer this question is to observe the effect of this substance in a controlled environment, i.e., an environment in which the activity of enzymes can be measured (18). When the specific target for inhibition is not known, the first step is to assay as many proteases as possible. If the substance indeed shows an inhibitory effect, isolation of the specific enzyme and the search for the inhibition mechanism are the next steps.

The enamel defect caused by fluorosis has been associated with an arrest in enamel maturation stage characterized by delayed breakdown of amelogenins (5). One hypothesis proposed for the mechanism by which excess fluoride affects degradation and removal of enamel matrix proteins is that fluoride might inhibit enamel proteinases (10). To our knowledge, this hypothesis has never been tested in a controlled environment. In this study, the direct effect of fluoride on the activity of enamel enzymes present in a crude enamel extract was tested using three different assays. Whereas zymography gives a rather qualitative result indicating the possible proteases we are dealing with, the azocasein substrate is a typical quantitative assay employed to test possible inhibitors, and it has not earlier been used to test the effect of fluoride on the activity of enamel proteases. Since casein and gelatin are not natural substrates for enamel proteases, we also prepared enamel matrix homogenates taken from secretory and maturation stages to follow the degradation of proteins by the very proteases also present in the crude extracts. By this system, a more discrete effect of fluoride on enzymes might be detected if the enzymes were highly specific. When proteases were inhibited by

PMSF, NEM, 1,10-phenanthroline and EDTA, proteolysis did not occur. Within the limitations of the procedures used in this study, fluoride does not appear to have a direct effect on the activity of enamel proteases.

Many researchers refer to the work of DENBESTEN (5) and DENBESTEN & HEFFERNAN (10) as reasonable evidence of a direct inhibitory effect of fluoride on enamel proteinases. It should be noted that zymography is not a quantitative technique when the starting amount of enzyme is unknown, as in the case of loading protein from equal weights of enamel. The results of zymography can be misleading under such conditions. Nonetheless, fluoride could have an effect on proteinase expression (19), and under such circumstances enamel proteinase activity would be altered.

No attempt was made to separate the enamel matrix according to different developing stages, since our first goal was to show as many enzymes as possible in the same assay to characterize the influence of fluoride on them under the same conditions. Using decalcification in 5% TCA followed by extraction in non-reducing sample buffer (10), we obtained an extract that resulted in many negative bands after zymography. The extraction procedure using sample buffer (with SDS) was more effective than the extraction with buffer containing Triton X-100 when the amount of estimated protein present in the samples is taken into account (1 $\mu g/\mu l$ versus 0.3 $\mu g/\mu l$, respectively), although when the same amount of protein from both extraction procedures was applied to zymograms, the same proteolytic bands were visible (not shown). We obtained a broad range of enzymes present in the developing enamel that are supposed to be zymogens and active forms of enzymes. SDS and Triton X-100 are not normally used for the extraction of proteins present in extracellular matrix, since detergents are indicated to dissolve cell membranes. The efficiency of these detergents in the extraction of enamel matrix proteinases might be explained by the dissolution of the protein aggregates that may cover most of the enzymes in the enamel matrix, and it is possible that these aggregates are hardly dissociated by salt precipitation alone. The use of cold TCA, a denaturing agent, probably also contributed to the dissociation of the protein aggregates in addition to demineralizing the enamel matrix. On the other hand, some effect of the acid solution on the proteolytic activity cannot be ruled out. Although acidic environments are known to decrease the activity of some enzymes, SMITH *et al.* (20) observed that acetic acid pre-treatment affected enamel proteinase activity in zymograms by increasing the overall activity for proteinases of many molecular weights, sharpening the appearance of proteolytic bands in general and altering the proteolytic band profile. Finally, we used Triton X-100 to extract samples to be used in colorimetric and enamel protein degradation *in vitro* because it is a nonionic detergent, and in these assays enzyme activity should not be hindered by alteration in protein conformation. Nonetheless, some proteolytic activity may be diminished or lost using the decalcification and extraction methods described here.

The bands visible on gelatin zymograms were fewer and less intense, but their molecular masses seemed to correspond to the major bands visible on casein zymograms. We used inhibitors to determine the type of proteases assayed in the zymograms. No cysteine proteinases were observed. Serine proteinase activity was present, since inhibition of casein degradation by PMSF was observed in bands with relative molecular masses of 32–36 kDa and 23 kDa. A serine proteinase which carried amelogeninase activity was described with apparent molecular masses originally assigned at 34 and 37 kDa, but was later shown to resolve into two bands of 30 and 34 kDa (21). More recently, the 34-kDa porcine serine proteinase was isolated and characterized at both the protein and DNA level (11). From the inhibition profiles obtained by incubation with EDTA and 1,10-phenanthroline, it appears that most of the other proteinases present in our crude extract were metalloproteinases. An enamel matrix metalloproteinase recently characterized is MMP-20, or enamelysin. This enzyme has been cloned from pig and human species (12, 13). Relative molecular mass bands from recombinant enamelysin in gelatin and casein zymograms vary from 41 to 46 kDa, and proteolytic activity was shown to be more intense against casein (13). Bands with relative molecular masses around 43 kDa were visible both in our casein and gelatin zymograms, and caseinolytic activity was more intense. Our objective in this study was not to characterize the proteases present in our extract (for which goal Western blotting with antibodies directed at the specific enzymes would be needed), but rather to explore the possibility that some of the proteinases described so far may have been recovered through the procedures employed in this study. In any case, one cannot be sure to assay every enamel protease because this system is too complex to be reproduced *in vitro*.

The classification of proteinases assayed in this study as serine or metalloproteinases further indicates the importance of using zymography. As stated by HANEMAAIJER *et al.* (22), in contrast to serine proteinases with a similar function to MMPs, MMP enzyme activities are very difficult to assay due to their unique substrate recognition

characteristics, and the currently most widespread method for measuring MMP-activity is zymography.

In this report, three methods were employed to test the direct effect of fluoride on the activity of enamel proteinases. The results showed no inhibitory effect. These results are valid for the effect of free fluoride, since binding of fluoride to calcium did not take place under the conditions of these assays. The importance of these findings resides in the exclusion of the hypothesis that fluoride has any direct effect on enamel proteolytic enzymes. In the light of these findings, hypotheses that take into account alterations in crystal surface energy in the presence of fluoride gain more support (23). The recent characterization of enamel proteolytic enzymes at the molecular level may help to establish if alterations in fluorotic enamel result from altered enamel proteinase activity.

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References

1. Leverett DH. Fluoride and the changing prevalence of dental caries. *Science* 1982; **216**: 26–30.
2. Woolfolk MW, Faja BW, Bagramian RA. Relation of sources of systemic fluoride to prevalence of dental fluorosis. *J Public Health Dent* 1989; **50**: 18–23.
3. DenBesten PK, Thariani H. Biological mechanisms of fluorosis and level and timing of systemic exposure to fluoride with respect to fluorosis. *J Dent Res* 1992; **71**: 1238–1243.
4. Fejerskov O, Yanagisawa T, Tohda H, Larsen MJ, Josephsen KAJ, Mosha H. Posteruptive changes in human dental fluorosis – a histological and ultrastructural study. *Proc Finn Dent Soc* 1991; **87**: 607–615.
5. DenBesten PK. Effects of fluoride on protein secretion and removal during enamel development in the rat. *J Dent Res* 1986; **65**: 1272–1277.
6. Limeback H. Enamel formation and the effect of fluoride. *Community Dent Oral Epidemiol* 1994; **22**: 144–147.
7. Aoba T. The effect of fluoride on apatite structure and growth. *Crit Rev Oral Biol Med* 1997; **8**: 136–153.
8. DenBesten PK, Crenshaw MA, Wilson MH. Changes in the fluoride-induced modulation of maturation stage ameloblasts of rats. *J Dent Res* 1985; **64**: 1365–1370.
9. Tanabe T, Aoba T, Moreno EC, Fukae M. Effect of fluoride in the apatite lattice on adsorption of enamel proteins onto calcium apatites. *J Dent Res* 1988; **67**: 536–542.
10. DenBesten PK, Heffernan LM. Enamel proteases in secretory and maturation enamel of rats ingesting 0 and 100 ppm fluoride in drinking water. *Adv Dent Res* 1989; **3**: 199–202.
11. Simmer JP, Fukae M, Tanabe T, Yamakoshi Y, Uchida T, Xue J, Margolis HC, Hu C-C, Barlett JD. Purification, characterization and cloning of enamel matrix serine proteinase 1. *J Dent Res* 1998; **77**: 377–386.
12. Ryu OH, Fincham AG, Hu C-C, Zhang C, Qian Q, Barlett JD, Simmer JP. Characterization of recombinant pig enamelysin activity and cleavage of recombinant pig and mouse amelogenins. *J Dent Res* 1999; **78**: 743–750.
13. Fukae M, Tanabe T, Uchida T, Lee S-K, Ryu O-H, Murakami C, Wakida K, Simmer JP, Yamada Y, Barlett JD. Enamelysin (Matrix Metalloproteinase-20): localization in the developing tooth and effects of pH and calcium on amelogenin hydrolysis. *J Dent Res* 1998; **77**: 1580–1588.
14. Stoscheck CM. Quantification of protein. In: Deutsch MP, ed. *Methods in Enzymology: Guide to protein purification*. Vol. 182. San Diego: Academic Press, 1990: 50–68.
15. Charney J, Tomarelli RM. A colorimetric method for the determination of proteolytic activity of duodenal juice. *J Biol Chem* 1947; **171**: 501–505.
16. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970: **227**: 680–685.
17. Salvesen G, Nagase H. Inhibition of proteolytic enzymes. In: Beynon RJ, Bond JS, eds. *Proteolytic enzymes: a practical approach*. Oxford: Oxford University Press, 1989: 83–102.
18. Souza AP, Gerlach RF, Line SRP. Inhibition of human gingival gelatinases (MMP-2 and MMP-9) by metal salts. *Dent Mater* 1999; **15**: in press.
19. Smith CE, Nanci A, DenBesten PK. Effects of chronic fluoride exposure on morphometric parameters defining the stages of amelogenesis and ameloblast modulation in rat incisors. *Anat Rec* 1993: **237**: 243–258.
20. Smith CE, Issid M, Margolis HC, Moreno EC. Developing changes in the pH of enamel fluid and its effects on matrix-resident proteinases. *Adv Dent Res* 1996: **10**: 159–169.
21. Tanabe T, Fukae M, Uchida T, Shimizu M. The localization and characterization of proteinases for the initial cleavage of porcine amelogenin. *Calcif Tissue Int* 1992: **51**: 213–217.
22. Hanemmaaijer R, Visser H, Konttinen YT, Koolwijk P, Verheijen JH. A novel and simple immunocapture assay for determination of gelatinase-B (MMP-9) activities in biological fluids: saliva from patients with Sjögren's syndrome contain increased latent and active gelatinase-B levels. *Matrix Biol* 1998: **17**: 657–665.
23. Robinson C, Brookes SJ, Shore RC, Kirkham J. The developing enamel matrix: nature and function. *Eur J Oral Sci* 1998: **106**: 282–291.

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Effect of lead, cadmium and zinc on the activity of enamel matrix proteinases *in vitro*

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Environmental contamination with heavy metals leads to their uptake by the body. Dental tissues are known to harbor metals in concentrations related to the exposure at the time of dentin or enamel formation. Several enzymes involved in the metabolism of extracellular matrix components have been shown to be inhibited by excess metal ions. Enamel matrix proteinases seem to play a central role in the maturation of dental enamel, and inhibition of these enzymes by metals could interfere with amelogenesis. We have investigated the effect of lead, cadmium and zinc on the activity of enamel matrix proteinases using a colorimetric assay, casein and gelatin zymography, and an assay of enamel protein degradation *in vitro*. All three metals inhibited the proteolysis, as shown by the three methods employed, in concentrations as low as 110 μM. This study showed that contaminant metals can inhibit proteinolysis when a crude enamel extract containing enamel proteases is tested *in vitro*. Therefore, the presence of heavy metals in enamel matrix could interfere with amelogenesis due to alteration in normal proteolysis.

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Heavy metals are ubiquitous and are taken up into the body by food, drinking water, and polluted air (1). The primary accumulation site for metals is bone (2). Dental tissues are known to harbor metals in concentrations related to the exposure at the time of dentin or enamel formation (3), and they are considered reliable biomarkers for determination of exposure to metals, particularly lead (4). Besides contributing to the assessment of heavy metal exposure in children (5–7), the metal content of teeth has raised the interest of many epidemiologists during the last decades as to whether children living in highly contaminated areas have increased susceptibility to dental caries (8–10). Recently, a consistent link between lead exposure at the time of dental formation and increased caries prevalence has been shown (11), but the reason for this association remains unknown.

In contrast to the connective mineralized tissues, enamel crystallites are not deposited onto a collagenous matrix, but grow concomitantly with the removal of most of the enamel matrix proteins (12). The first enamel crystallites appear as long, thin ribbons extending from the amelodentinal junction towards the surface of the enamel (13). Once the secretory stage is completed, the matrix proteins are largely degraded, resorbed and replaced by fluid (14). The fluid is finally substituted with mineral, increasing the crystallites in width and thickness (15). Enamel proteinases have been traditionally accepted as responsible for the degradation of the organic matrix to lower molecular-weight proteins that leave the matrix (16). Abnormal post-secretory processing of matrix proteins results in hypomaturation and hypocalcified amelogenesis imperfecta (17). Increasing evidence suggests that enamel protein cleavage products perform different functions within the enamel matrix, regulating enamel biomineralization, a concept that further highlights the importance of the proteolytic process that generates those cleavage products (16). As stated by Smith (18), although limited biochemical/molecular details are known about enamel proteinases at this time, there is enough information

available to predict that these enzymes likely play a central role in the enamel maturation process.

Excess metal ions inhibited the activity of several proteolytic enzymes (19–21). The hypothesis that fluoride could inhibit enamel enzymes has yet been considered by many researchers (22–25), but fluoride fails to have an inhibitory effect *in vitro* (26). Nevertheless, no attention has yet been given to the possibility that trace elements present in enamel could interfere with the activity of enamel proteinases and, thus, negatively affect amelogenesis. The aim of this study was to evaluate if lead, cadmium and zinc, the most prevalent metals described to be incorporated into enamel, have a direct effect on the activity of enamel proteinases *in vitro*.

Material and methods

Enamel matrix extract containing proteinases

Incisors of 28 Wistar rats weighing 300 g were used in this study as source of the crude extract containing proteinases. Mandibles and teeth were kept on ice until enamel matrix was scraped from the teeth, which had been cleared of soft tissue prior to scraping. Matrix from both secretory and maturation stages of the 4 incisors of each rat was pooled together, demineralized for 1 h in a 1.5 ml microcentrifuge tube containing 1 ml of ice-cold 5% (w/v) trichloroacetic acid (TCA), followed by centrifugation for 5 min to precipitate enamel protein. The pellet was rinsed once in 50 μl of 1 M Tris-HCl, pH 8.0. Since the proteolytic assays described below required different preparations of the crude extract, separate extractions for each of them were carried out. Protein concentrations were determined by the Bradford assay according to the manufacturer's instructions (Bio-Rad, Richmond, CA, USA). A detailed description of the procedures is given in an earlier work (26).

Colorimetric assay

For the colorimetric assay, pellets derived from 12 rats were extracted for 1 h in ice-cold 1% Triton-X-100 in 10 mM Tris-HCl, pH 8.0. The final pH of the solution was adjusted to 7.2. The amount of extract containing 100 μg of protein was used for each reaction, which consisted of a 0.8% azocasein solution (Sigma, St. Louis, MO, USA) freshly prepared in 50 mM Tris-HCl, pH 7.2, containing 10 mM $CaCl_2$, 1 μM $ZnCl_2$ and 0.4 mM/l NaN_3, in a total volume of 0.5 ml. Blanks were prepared by omitting enamel matrix extract from the reaction mixture. After a 48-h incubation at 37°C under continuous agitation, the reaction was stopped by the addition of 0.2 ml 10% (w/v) TCA. After centrifugation for 5 min at 30,000 × *g*, the release of diazo-amino acids from azocasein in the supernatant was measured at 440 nm according to Charney & Tomarelli (27). One unit of endopeptidase activity was the amount of enzyme required to produce an increase of absorbance of 1.0 within 1 h under the assay conditions. To observe the influence of lead, cadmium and zinc on enzyme activity, salts were added to reactions at final concentration of 300 μM. Phenylmethylsulphonyl fluoride (PMSF) was used at 2 mM and 1,10-phenanthroline was used at 5 mM. Reactions were prepared in triplicate. The mean proteolytic activity value of each group was composed from the three values found in the triplicates. Data are presented as means ± SD, and were analyzed using ANOVA.

Zymography

Twelve-% polyacrylamide slab gels (16 cm × 14 cm × 1 cm) containing 0.05% casein or 0.05% gelatin were used. The stacking gel was polymerized using a comb that resulted in one 0.8-cm wide lane to which the molecular weight standard was applied, and a 11.5-cm wide lane (continuous lane) to which the enamel matrix sample was applied. Enamel matrix was extracted for 30 min with SDS non-reducing sample buffer (62.5 mM Tris, 15% glycerol, 1% SDS and 0.05% bromophenol blue, pH 6.8), centrifuged and the supernatant was applied to the gel. The continuous lane of the gelatin-containing gel was loaded with 200 μg of protein, whereas 100 μg of protein were applied to the continuous lane of the casein-containing gel. Low-molecular-weight protein marker (20 μl) (Amersham Pharmacia Biotech, Piscataway, NJ, USA) was applied to the separate lane on each gel. The proteins were separated by electrophoresis using a discontinuous buffer system (28) at 16 mA/gel constant current at 4°C (running zymograms at low temperature is imperative to avoid degradation of the substrate during the separation, which results in a "smeary" appearance instead of distinct bands). After electrophoresis, SDS was removed by incubating gels for 1 h in two baths of 2.5% Triton X-100. The continuous lane of each gel was cut into strips that were incubated for 40 h at 37°C with shaking in 40-ml tubes. Tris-$CaCl_2$ buffer (50 mM Tris-HCl, pH 7.2, containing, 10 mM $CaCl_2$, and 1 μM $ZnCl_2$) was used for incubation, and proteinase inhibitors or different concentrations of metal salts were added to this solution. After incubation, strips were stained for 4 h with 0.05% Coomassie Brilliant Blue in

50% methanol and 5% acetic acid and destained with 50% methanol and 10% acetic acid. These procedures were performed three times on separate occasions.

Inhibitor solutions

PMSF was used at 2 mM and 5 mM and 1,10-phenanthroline was used at 5 mM. These inhibitors were freshly prepared as 200 mM and 500 mM stock solutions in methanol, respectively. Methanol was added to all other reactions to achieve the same volume present in the inhibitor reactions.

Metal solutions

The metal salts used were $Pb(CH_3CO_2)_2.3H_2O$, $CdCl_2.2^{1/2}$ H_2O and $ZnSO_4.7H_2O$. Metal concentrations in solutions ranged from 110 μM to 3 mM. Solutions were freshly prepared prior to the beginning of each assay by dissolving the salts in buffer (Tris-HCl 50 mM, pH 7.2). It is worth remembering that metal salts usually lower the pH of solutions, thus care must be taken to avoid interference with enzyme activity due to alterations in solution pH.

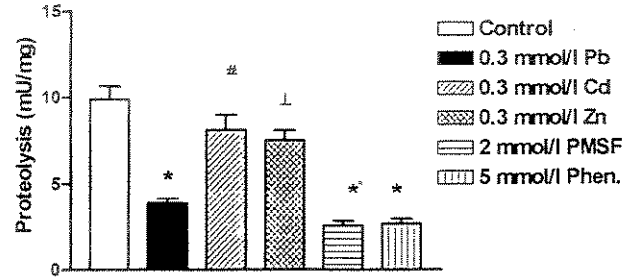
Enamel protein degradation *in vitro*

In a further attempt to examine the degradation of enamel proteins, an *in vitro* degradation assay was carried out. Enamel matrix derived from 4 rats was prepared as described in the first section of the Material and methods section. After neutralization, pellets were well homogenized by hand in a glass homogenizer for 1–3 min in 1.0 ml ice-cold 50 mM Tris-HCl, containing 10 mM $CaCl_2$, 1 μM $ZnCl_2$ and 1% Triton-X-100. Aliquots of 100 μl (30 μg of protein) were used for each reaction, that also contained 0.4 mM NaN_3 and the different metal salts or inhibitors in a final volume of 115 μl. Proteolysis control reactions were incubated for 40 h and contained a cocktail of inhibitors (5 mM PMSF, 5 mM phenanthroline, 12 mM EDTA). Metal salts were added to reactions at 300 μM. Incubations were made at 37°C with shaking for 20 h or 40 h. After incubation, samples were mixed with 55 μl of a double strength reducing sample buffer (125 mM Tris, 30% glycerol, 2% SDS, 80 mM dithiothreitol, and 0.1% bromophenol blue, pH 6.8), and heated to 100°C for 5 min. Samples were stored at −20°C until electrophoresis. Sixty μl of each sample were applied to separate lanes of a 15% polyacrylamide gel, which was run and stained with Coomassie Brilliant Blue. Ten μl of low molecular weight protein marker were used (Amersham Pharmacia Biotech). These procedures were performed 3 times on separate occasions.

Results

Results of proteolysis in solution using a colorimetric assay are shown in Fig. 1. Solutions containing Pb, Cd and Zn at 0.3 mM final concentrations showed significantly lower proteolysis values than controls ($P<0.001$, $P<0.05$ and $P<0.01$, respectively). In addition, solutions containing 2 mM PMSF or 5 mM phenanthroline exhibited proteolysis values similar to those found in solutions containing 0.3 mM Pb.

Results from zymograms using casein and gelatin as substrate are shown in Figs 2 and 3. Controls are seen in lanes 1 and 13 in both these figures. Less intense negative bands were observed in strips incubated with different concentrations of lead,



* $P<0.001$ versus control; ⊥ $P<0.01$ versus control; # $P<0.05$ versus control. ANOVA followed by Tukey-Kramer multiple comparison test

Fig. 1. Total proteolytic activity of enamel matrix incubated with Pb, Cd and Zn at 0.3 mM, PMSF at 2 mM and phenanthroline at 5 mM. One unit (U) of peptidase activity was defined as the amount of enzyme that increased the absorbance by 1.0 per h. Data are reported as means and SD. One way analysis of variance (ANOVA) revealed significant differences when all groups were compared to control.

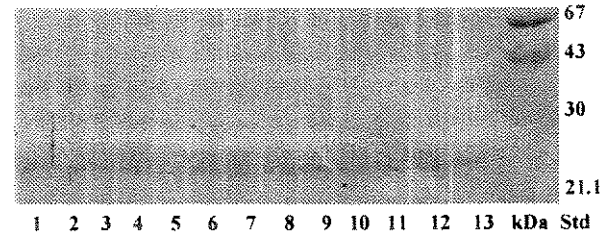


Fig. 2. Strips of 12% polyacrylamide gel containing 0.05% casein and the electrophoresed enamel extract were incubated with different concentrations of Cd, Pb and Zn salts in assay buffer (50 mM Tris-HCl, 10 mM $CaCl_2$, 1 μM $ZnCl_2$, pH 7.2) for 40 h. 1 and 13 – Control, 2 – 0.33 mM $CdCl_2$, 3 – 1 mM $CdCl_2$, 4 – 3 mM $CdCl_2$, 5 – 0.11 mM $Pb(CH_3CO_2)_2$, 6 – 0.33 mM $Pb(CH_3CO_2)_2$, 7 – 1mM $Pb(CH_3CO_2)_2$, 8 – 0.11 mM $ZnSO_4$, 9 – 0.33 mM $ZnSO_4$, 10 – 1 mM $ZnSO_4$, 11 – 2 mM/l PMSF, 12 – 5 mM phenanthroline, Std – Low Molecular Weight Protein Marker.

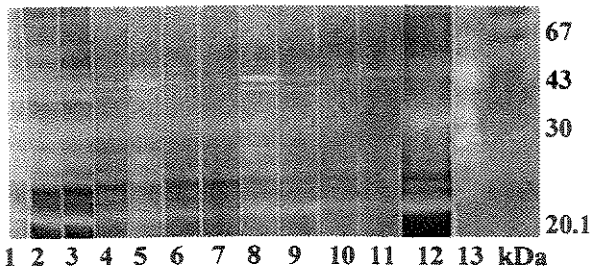


Fig. 3. Strips of 12% polyacrylamide gel containing 0.05% gelatin and the electrophoresed enamel extract were incubated with different concentrations of Cd, Pb and Zn salts in assay buffer (50 mM Tris-HCl, 10 mM $CaCl_2$, 1 μM $ZnCl_2$, pH 7.2) for 40 h. 1 and 13 – Control, 2 – 0.11 mM $CdCl_2$, 3 – 0.33 mM $CdCl_2$, 4 – 1 mM $CdCl_2$, 5 – 0.11 mM $Pb(CH_3CO_2)_2$, 6 – 0.33 mM $Pb(CH_3CO_2)_2$, 7 – 1 mM $Pb(CH_3CO_2)_2$, 8 – 0.11 mM $ZnSO_4$, 9 – 0.33 mM $ZnSO_4$, 10 – 1 mM $ZnSO_4$, 11 – 2 mM PMSF, 12 – 5 mM phenanthroline, Std – Low Molecular Weight Protein Marker.

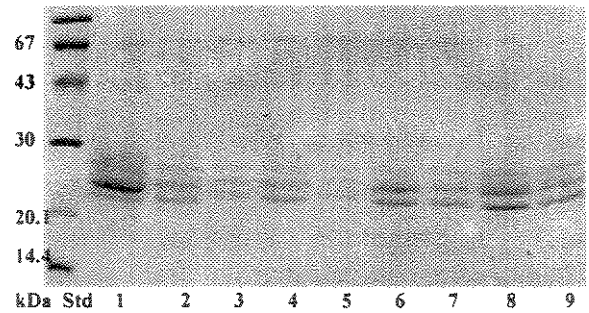


Fig. 4. Polyacrylamide gel (15%) stained with Coomassie Blue. Enamel matrix homogenates were taken from secretory and maturation stages and incubated *in vitro* for 20 h and 40 h at 37°C in buffer solutions containing Cd, Pb and Zn or protease-inhibitors. Std – Low Molecular Weight Protein Marker, 1 – Inhibitors cocktail/ 40 h, 2 – Control/ 20 h, 3 – Control/ 40 h, 4 – 0.3 mM $CdCl_2$/ 20 h, 5 – 0.3 mM $CdCl_2$/ 40 h, 6 – 0.3 mM $Pb(CH_3CO_2)_2$/ 20 h, 7 – 0.3 mM $Pb(CH_3CO_2)_2$/ 40 h, 8 – 0.3 mM $ZnSO_4$/ 20 h, 9 – 0.3 mM $ZnSO_4$/ 40 h.

cadmium and zinc. The inhibitory effect on proteolytic activity was recognized as decreased brightness of the bands, and is easily visualized by observing the effect of PMSF and phenanthroline (lanes 11 and 12, respectively, Figs 2, 3). Judging by the naked eye, the inhibitory effect was more remarkable with 1 mM Zn (Fig. 2, lane 10, Fig 3, lane 10). Furthermore, Zn appeared to exhibit a greater effect on the inhibition of serine proteinases, whereas all three metals seemed to affect metalloproteinases (considering MMPs the bands inhibited by phenanthroline). The inhibition of proteases by phenanthroline was not complete in Fig. 3, strip 12. Some precipitated material was observed in the Tris-$CaCl_2$ buffer after addition of 1 mM $Pb(CH_3CO_2)_2$. However, we believe that the inhibition of enzyme activity was not due to precipitation of Ca, since it was present in large excess. Besides, a significant inhibition of enzyme activity occurred with the lower concentrations of Pb, without the formation of precipitated material. Some changes in band pattern and intensity were observed in Fig. 3. These distortions, however, did not interfere with the interpretation of results. Metals and proteinase inhibitors seemed to interfere with Coomassie staining of gelatin-containing gels, causing some differences in staining intensity. Small differences in band pattern between lanes 12 and 13 occurred due to uneven migration of bands in the gel borders (smiling) during electrophoretic running.

Degradation of enamel proteins *in vitro* showed a distinct pattern of degradation after 20-h and 40-h incubation of enamel matrix homogenates with Pb and Zn at 300 μM (Fig. 4). Enamel protein degradation in homogenates containing 300 μM Cd was similar to that found in control homogenates. The proteolysis control, incubated with inhibitors, indicates that enzymes present in the homogenates were responsible for the degradation of the proteins separated in the gel.

Discussion

The results showed altered proteolysis in the presence of Zn, Pb and Cd at μmolar levels under the *in vitro* conditions tested. The use of three different methods to assess proteinase activity further indicates the consistency of these results. We have also tested $CoCl_2$, $AlSO_4$, $AlSO_4$+NaF, $SnCl_2$, $FeSO_4$, and NaF at 5 mM in solutions for the zymography assay, since this was the most sensitive method employed, but these salts produced no inhibition of proteolysis (data not shown). NaF results were of particular interest because of the possible linkage of proteolysis inhibition with the pathogenesis of fluorosis (26).

In the azocasein assay, 0.3 mM Pb produced a decrease in proteolysis comparable to that obtained by 2 mM PMSF and 5 mM phenanthroline. The concentration of proteolytic inhibitors (PMSF and phenanthroline) used was set according to the results from zymographic assays. Initial concentration of inhibitors used (0.5 mM) failed to inhibit proteolytic bands present in zymograms, and only at 5 mM phenanthroline produced a reproducible inhibition pattern. These inhibitors are not stable in aqueous solutions over long periods, which might account for the higher levels needed and for the failure to inhibit metalloproteinases completely (Fig. 3, strip 12).

Zymography results showed a more or less pronounced inhibition of proteases by metals depending on the substrate, a result also confirmed when more zymograms were analyzed (but only the

result of one containing each substrate is presented). Whereas Zn produced the higher inhibition with both substrates, Pb produced inhibitory profiles comparable to Zn only when casein was the substrate, and Cd hardly produced any inhibition, even at 3 mM. Zn appeared to have a more intense inhibitory effect on serine proteinases. Finally, Zn and Pb were able to disturb the degradation of the enamel matrix homogenates, and, after a 40-h incubation, more intense bands corresponding to amelogenins were still seen.

The results from our different assays support each other in a general sense. The apparent discrepancy between the azocasein results (Pb causing the highest inhibition) and the zymogram results (Zn causing the highest inhibition) may be caused by several factors. First, in the azocasein assay and in the enamel homogenates, both substrate and enzymes are in solution, whereas in zymography substrates and enzymes are in different phases. Second, not necessarily all proteases present in the extract can be seen in zymograms (proteases without affinity for the substrates used or cleaving the substrate molecules in fragments too large to diffuse out will not be detected) and, conversely, not all proteolytic bands observed in the zymograms will be active (or have the same activity) in solution. Third, heavy metals are known to catalyze the oxidation of proteins containing sulfhydryls in the presence of oxygen; for this reason, buffers used to extract tissues usually contain EDTA to chelate contaminating metals (29). Histidines, and to a lesser extent other amino acids, have affinity for metals, and this is the principle underlying the use of metal chelating affinity columns to separate proteins that have been modified by the addition of a small sequence of histidines (30). Thus, the inhibitory effect on proteolysis showed here cannot be credited solely to the interaction of metals with the enzymes, since this effect can be caused by interaction of metals with the substrates as well.

The banding pattern observed in our zymograms closely matches the one found in casein zymograms of porcine enamel matrix extracted with alkaline buffer by FUKAE & TANABE (31). There are many reasons why zymogram patterns exhibit a great variation in the literature. The most obvious reasons are animal species, matrix developmental stage, incubation time, buffer composition, and extraction procedure (we used a crude extract, without long purification steps). When considering extraction procedures, activation of proteinases, specially metalloproteinases, might be caused by pH fluctuations, denaturation, or cleavage by other proteases present in the extract (32). We consider that the many bands observed in our extract are the higher molecular forms (some of them probably zymogens and active forms) and degradation products of proteolytic enzymes present in enamel. The pH change due to the use of TCA and the use of detergents might account for variations in band pattern (33). The fact that we mixed the matrix from all stages can probably be responsible for some increase in the number of bands and activity due to degradation of higher molecular forms and/or activation by cleavage of propeptides. This reasoning is supported by the fact that the two best characterized proteases present within enamel matrix, enamelysin and EMSP-1, require the removal of a propeptide for activation, and these two proteases exhibit a distinct pattern of expression (34). Enamelysin is found in the earliest stage of enamel formation (35, 36) while EMSP-1 is found in the later stages (37, 38). Besides, although it is generally accepted that metalloproteinases are activated during separation in SDS-PAGE (34), this is not necessarily true, since APMA activation has been shown to be capable of further increasing proteolytic activity (39). Furthermore, different types of enamel proteinases may degrade (and/or activate) themselves. Recent studies showed that the 21 and 25 kDa forms of a bovine enamel matrix metalloproteinase represent two forms of the catalytic domain of enamelysin (40), and further evidence of autocatalytic degradation is provided by studies on the recombinant enamelysin, whose higher molecular form is purified together with its degradation products (35, 41, 42).

Buffer composition might be another cause for different results. We used incubation for a prolonged time (40 h) in a buffer considered adequate for optimal metalloproteinase activity (50 mM Tris-HCl, containing 10 mM $CaCl_2$, 1 μM $ZnCl_2$), since this group of enzymes was shown to be present as many bands using inhibitors. Although the reported optimal pH of EMSP-1 is 5.7 (34), the serine proteinase with the apparent molecular weight of EMSP-1 was still active in our zymograms. The use of lower concentrations of Ca^{2+} is a matter of dispute in some recent papers (43, 44), and it may be responsible for many of the discrepancies in zymogram results. In this respect, we think that *in vitro* conditions must be optimized to detect any possible proteolytic activity. The use of 1 μM Zn is not always reported, since this concentration is sometimes achieved by small contamination, but the absolute lack of Zn in solutions for *in vitro* incubation might have a negative effect when one is looking for all possible proteolytic bands.

Several enzymes involved in the metabolism of extracellular matrix components have earlier been shown to be inhibited by metal ions. Cu and Cd ions were shown to inhibit 50% of the activity of

procollagen C- and N-proteinases, respectively, in concentrations as low as 14 μM and 40 μM, whereas much higher concentrations of these ions were needed to inhibit the crude extract containing the enzymes (19). Galactosylhydroxylysyl glucosyltransferase is inhibited Zn, Cu, Cd, and Ni in concentrations from 50 to 250 μM (20). Gelatinases A and B were shown to be inhibited by Zn and Cu in concentrations around 100 μM (21). The mechanism of enzyme inactivation by excess metal has been associated with conformational changes that inactivate the catalytic function of enzymes, and such inhibition could be very important to regulatory and/or toxicological processes of zinc enzymes (45).

Heavy metals compete with calcium for specific binding sites (46). In the incubation solutions used in this study, Ca was maintained in far excess in all solutions (10 mM), but it is difficult to estimate the actual heavy metal activities as well as the actual Ca concentration. Since the metals used interact with biological systems, even atomic absorption spectrometry readings of the solutions would not indicate the true values of free ions. Thus, we decided to work with the most usual method: to add soluble salts to the experimental solutions, and report the total metal ion concentration that produced a given effect (47).

Interestingly, Zn and Pb are elements described to be present in mature human enamel in the highest concentrations among trace elements (ppm levels), only comparable to the levels recovered for fluoride and strontium (48). Zinc concentrations have been reported varying from around 115 μg/g in whole enamel (49) to around 1000 μg/g in the enamel surface (50). Lead concentrations vary from around 2 μg/g in whole enamel (49) to around 270 μg/g in the enamel surface (51). Reports often reveal conflicting data due to the analytical technique used, type and surface of the tooth, and geographic place of sampling (51). It is very difficult at this time to make any extrapolation to the conditions present in the forming enamel, but a concerted synergistic effect of several different metals must not be forgotten.

According to the results presented here, an alternative perspective can be surmised. The presence of heavy metals in enamel matrix could impair amelogenesis due to alteration in normal proteolysis. Distinct patterns of proteinases expression have been reported, enamelysin being expressed in the earliest (35, 36) and EMSP-1 in the latest stages of enamel formation (37, 38). Since bands having the electrophoretic profile of these proteinases were inhibited by the metals used in this study, it is likely that these metals can interfere in both secretory and maturation stages of enamel development. There are few works that investigated the formation of dental tissues in the presence of metals. Eisenmann & Yaeger (52) used 134 different salts to inject approximately 500 rats and analyzed dentin and enamel of incisors by microradiography after 1 wk. They showed that many ions have the ability to interfere with dentin and enamel formation, but 1 wk is a too short a time period to make any valid statements for enamel. Appleton (53) described a "lead line" in dentin after a single intravenous injection of lead acetate, but again animals were sacrificed 1 wk after injection. Mercado & Bibby (54) added various trace elements to the drinking water of rats during 50 d and showed that changes in incisor pigmentation was produced by 5 ppm of Cd, 25 ppm of Cr, 5–75 ppm of F, 50 ppm of Sr and 50 ppm of Y. These authors did not test Pb and Zn.

In this report the inhibitory effect of zinc, lead and cadmium on the activity of enamel proteinases was demonstrated *in vitro*. More studies on amelogenesis in animals exposed to metals are necessary and more sensitive methods of assessing alterations in enamel are recommended. Furthermore, availability of purified and characterized enamel proteinases will make it possible to study the interaction of metals with these enzymes in better detail. Finally, determination of trace amounts of metals present in the enamel fluid is imperative to elucidate the impact of these results on amelogenesis.

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References

1. Cicuttini FM, Fairley CK, McNeil JJ, Taylor ER. The public health problem of environmental lead contamination. *Med J Aust* 1994; **160**: 173–174.
2. Gulson BL. Tooth analyses of sources and intensity of lead exposure in children. *Environ Health Perspect* 1996; **104**: 306–312.
3. Attramadal A, Jonsen J. The content of lead, cadmium, zinc and cupper in deciduous and permanent human teeth. *Acta Odontol Scand* 1976; **34**: 127–131.
4. Rabinowitz MB, Leviton A, Bellinger D. Relationships between serial blood lead levels and exfoliated tooth dentin lead levels: models of tooth lead kinetics. *Calcif Tissue Int* 1993; **53**: 338–341.
5. Frank RM, Sargentini-Maier ML, Turlot JC, Leroy MJF. Comparison of lead levels in human permanent teeth from Strasbourg, Mexico City, and rural zones of Alsace. *J Dent Res* 1990; **69**: 90–93.
6. Cleymaet R, Retief DH, Quartier E, Slop D, Coomans D, Michotte Y. A comparative study of the lead and cadmium content of surface enamel of Belgian and Kenyan children. *Sci Total Environ* 1991; **104**: 175–189.
7. Tvinnereim HM, Eide R, Riise T, Wesenberg GR, Fosse G, Steinnes E. Lead in primary teeth from Norway:

changes in lead levels from the 1970s to the 1990s. *Sci Total Environ* 1997; **207**: 165–177.
8. Curzon MEJ, Bibby BG. Effect of heavy metals on dental caries and tooth eruption. *J Dent Child* 1970; **37**: 463–465.
9. Anderson RJ, Davies BE, James PMC. Dental caries prevalence in a heavy metal contaminated area of the West of England. *Brit Dent J* 1976; **141**: 311–314.
10. Gil F, Facio A, Villanueva E, Pérez ML, Tojo R, Gil A. The association of tooth lead content with dental health factors. *Sci Total Environ* 1996; **192**: 183–191.
11. Watson GE, Davis BA, Raubertas RF, Pearson SK, Bowen WH. Influence of maternal lead ingestion on caries in rat pups. *Nature Med* 1997; **3**: 1024–1025.
12. Eisenmann DR. Amelogenesis. In: Ten Cate AR, ed. *Oral Histology: development, structure and function.* 5th edition. St. Louis: Mosby-Year Book, 1998; 197–217.
13. Kerebel B, Daculsi G, Kerebel LM. Ultrastructural studies of enamel crustallites. *J Dent Res* 1979; **58B**: 884–850.
14. Robinson C, Briggs HD, Aktinson PJ, Weatherell JA. Matrix and mineral changes in developing enamel. *J Dent Res* 1979; **58**: 871–880.
15. Shore RC, Robinson C, Kirkham J, Brookes SJ. Structure of developing enamel. In: Robinson C, Kirkham J, Shore RC, eds. *Dental Enamel – Formation to Destruction.* Boca Raton, FL: CRC press, 1995: 135–150.
16. Bartlett JD, Simmer JP. Proteinases in developing dental enamel. *Crit Rev Oral Biol Med* 1999; **10**: 425–441.
17. Wright JT, Hall KI, Yamakuchi M. The enamel proteins in human amelogenesis imperfecta. *Arch Oral Biol* 1997; **42**: 149–159.
18. Smith CE. Cellular and chemical events during enamel maturation. *Crit Rev Oral Biol Med* 1998; **9**: 128–161.
19. Hojima Y, Behta B, Romanic AM, Prockop DJ. Cadmium ions inhibit procollagen C-proteinase and cupric ions inhibit procollagen N-proteinase. *Matrix Biol* 1994; **14**: 113–120.
20. Myllyla R, Anttinen H, Kivirikko KI. Metal activation of galactosylhydroxylysyl glycosyltransferase, an intracellular enzyme of collagen biosynthesis. *Eur J Biochem* 1979; **10**: 261–269.
21. Souza AP, Gerlach RF, Line SRP. Inhibition of human gingival gelatinases (MMP-2 and 9) by metal salts. *Dent Mater 2000*; **16**: 103–108.
22. Denbesten PK, Thariani H. Biological mechanisms of fluorosis and level and timing of systemic exposure to fluoride with respect to fluorosis. *J Dent Res* 1992; **71**: 1238–1243.
23. Limeback H. Enamel formation and the effect of fluoride. *Community Dent Oral Epidemiol* 1994; **22**: 144–147.
24. Bawden JW, Crenshaw MA, Wright JT, Legeros RZ. Considerations of possible biologic mechanisms of fluorosis. *J Dent Res* 1995; **74**: 1349–1352.
25. Aoba, T. The effect of fluoride on apatite structure and growth. *Crit Rev Oral Biol Med* 1997; **8**: 136–153.
26. Gerlach RF, Souza AP, Cury JA, Line SRP. Fluoride effect on the activity of enamel matrix proteinases *in vitro*. *Eur J Oral Sci* 2000; **108**: 49–54.
27. Charney J, Tomarelli RM. A colorimetric method for the determination of proteolytic activity of duodenal juice. *J Biol Chem* 1947; **171**: 501–505.
28. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; **227**: 680–685.
29. Gray WR. Disulfide bonds between cysteine-residues. In: Creighton TE, ed. *Protein Structure: a practical approach.* 2nd edition. New York: Oxford University Press, 1997: 179.
30. Rosenberg IM. *Protein analysis and purification: benchtop techniques.* 1st edition. New York: Springer-Verlag, 1996: 319–320.
31. Fukae M, Tanabe T. Degradation of enamel proteins in porcine secretory enamel. *Connect Tissue Res* 1998; **39**: 123–129.
32. Nagase H. Activation mechanisms of matrix metalloproteinases. *Biol Chem* 1997; **378**: 151–160.
33. Smith CE, Issid M, Margolis HC, Moreno EC. Developmental changes in the pH of enamel fluid and its effect on matrix-resident proteinases. *Adv Dent Res* 1989; **10**: 159–169.
34. Bartlett JD, Simmer JP. Proteinases in developing enamel. *Crit Rev Oral Biol Med* 1999; **10**: 425–441.
35. Bartlett JD, Ryu OH, Xue J, Simmer JP, Margolis HC. Enamelysin mRNA displays a developmetally defined pattern of expression and encodes a protein which degrades amelogenin. *Connect Tissue Res* 1998; **39**: 405–413.
36. Fukae M, Tanabe T, Uchida T, Lee S-K, Ryu OH, Muramaki C, Wakida K, Simmer JP, Yamada Y, Bartlett JD. Enamelysin (MMP-20): localization in the developing tooth and effects of pH and calcium an amelogenin hydrolysis. *J Dent Res* 1998; 77: 1580–1588.
37. Scully JL, Bartlett JD, Chaparian MG, Fukae M, Uchida T, Xue J, Hu C-C, Simmer JP. Enamel matrix serine proteinase 1: stage-specific expression and molecular modeling. *Connect Tissue Res* 1998; **39**: 415–426.
38. Simmer JP, Fukae M, Tanabe T, Yamakoshi Y, Uchida T, Xue J, Margolis HC, Hu C-C, Barlett JD. Purification, characterization and cloning of enamel matrix serine proteinase 1. *J Dent Res* 1998; **77**: 377–386.
39. Galazka G, Windsor LJ, Birkedal-Hansen H, Engler JA. APMA activation of stromelysin-1 involves protein interactions in addition to those with cysteine-75 in the propeptide. *Biochemistry* 1996; **35**: 11221–11227.
40. Denbesten PK, Punzi JS, Li W. Purification and sequencing of a 21 kDa and 25 kDa bovine enamel metalloproteinase-20. *Eur J Oral Sci* 1998; **106**: 345–349.
41. Li W, Machule D, Gao C, Denbesten PK. Activation of recombinant bovine matrix metalloproteinase-20 and its hydrolysis of two amelogenin oligopeptides. *Eur J Oral Sci* 1999; **107**: 352–359.
42. Ryu OH, Fincham AG, Hu C-C, Zhang C, Qian Q, Barlett JD, Simmer JP. Characterization of recombinant pig enamelysin activity and cleavage of recombinant pig and mouse amelogenins. *J Dent Res* 1999; **78**: 743–750.
43. Smith CE, Chen W-Y. Degradative changes in whole enamel homogenates incubated *in vitro* in the presence of low calcium ion concentrations. *Connect Tissue Res* 1998; **39**: 379–391.
44. Brookes SJ, Kirkham J, Shore RC, Bonass WA, Robinson C. Enzyme compartmentalization during biphasic enamel matrix processing. *Connect Tissue Res* 1998; **39**: 393–403.
45. Auld DS. Removal and replacement of metal ions in metallopeptidases. *Meth Enzymol* 1995; **248**: 228–242.
46. Schirrmacher K, Wiemann M, Bingmann D, Büsselberg D. Effects of lead, mercury, and methyl mercury on gap junctions and [Ca2+]i in bone cells. *Calcif Tissue Int* 1998; **63**: 134–139.
47. Simons TJB. Lead-calcium interactions in cellular lead toxicity. *Neurotoxicology* 1993; **14**: 77–86.
48. Daculsi G, Bouler J-M, Legeros RZ. Adaptative crystal formation in normal and pathological calcifications in synthetic calcium phophate and related biomaterials. *Int Rev Cytol* 1997; **172**: 129–191.
49. Grünke K, Stärk H-J, Wennrich R, Franck U. Determination of traces of heavy metals (Mn, Cu, Zn, Cd and Pb) in microsamples of teeth material by ETV-ICP-MS. *Fresenius J Anal Chem* 1996; **354**: 633–635.

50. ANTTILA A., ANTTILA A. Trace-element content in the enamel surface and in whole enamel of deciduous incisors by proton-induced X-ray emission of children from rural and urban finnish areas. *Arch Oral Biol* 1987; **32**: 713–717.
51. LAPPALAINEN R, KNUUTTILA M. The distribution and accumulation of Cd, Zn, Pb, Cu, Co, Ni, Mn and K in human teeth from five different geological areas of Finland. *Arch Oral Biol* 1979; **24**: 363–368.
52. EISENMANN DR, YAEGER JA. Alterations in the formation of rat dentine and enamel induced by various ions. *Arch Oral Biol* 1969; **14**: 1045–1064.
53. APPLETON J. The effect of lead acetate on dentine formation in the rat. *Arch Oral Biol* 1991; **36**: 377–382.
54. MERCADO RC, BIBBY BG. Trace element effects on enamel pigmentation, enamel growth and molar morphology in rats. *Arch Oral Biol* 1973; **18**: 629–635.

CONCLUSÕES

Os resultados destes estudos permitem concluir que:

1. Flúor não apresenta um efeito inibitório *in vitro* na atividade de proteases presentes na matriz do esmalte dental segundo os métodos utilizados nestes estudos.
2. Entre os metais testados, chumbo, cádmio e zinco apresentaram um efeito inibitório *in vitro* na atividade de proteases presentes na matriz do esmalte em concentraçoes próximas de 100 uM.
3. Nos ensaios enzimáticos com o substrato azocaseína, chumbo foi o metal que apresentou o maior efeito inibitório, comparável aos inibidores enzimáticos comerciais utilizados.
4. Os ensaios *in vitro* utilizando um extrato bruto de matriz do esmalte são importantes para confirmar possibilidades sugeridas como mecanismos patogênicos de defeitos do esmalte, e merecem atenção antes que estas possibilidades sejam aceitas pela comunidade científica.

REFERÊNCIAS BIBLIOGRÁFICAS

ANDERSON, R.J., DAVIES, B.E., JAMES, P.M.C. Dental caries prevalence in a heavy metal contaminated area of the West of England. **Br Dent J**, v.141, n.10. p.311-314, 1976.

ANTTILA, A., ANTTILA, A. Trace-element content in the enamel surface and in whole enamel of deciduous incisors by proton-induced X-ray emission of children from rural and urban finnish areas. **Arch Oral Biol**, v.32, n.10, p.713-717, 1987.

AOBA, T. The effect of fluoride on apatite structure and growth. **Crit Rev Oral Biol Med**, v.8, n.2, p.136-153, 1997.

AOBA, T., FUKAE, M., TANABE, T., et al. Selective adsorption of porcine amelogenins onto hydroxapatite and their inhibitory activity on hydroxapatite growth in supersaturated solutions. **Calcif Tissue Int**, v.41, n.5, p.281-289, 1987.

AOBA, T., TANABE, T., MORENO, E.C. Function of amelogenins in porcine enamel mineralization during secretory stage of amelogenesis. **Adv Dent Res**, v.1, n.2, p.252-260, 1987.

AULD, D.S. Removal and replacement of metal ions in metallopeptidases. In: BARRET, A.J. (Ed.) Proteolytic enzymes: aspartic and metallopeptidases. **Meth Enzymol**, v.248, p.228-242, 1995.

BARTLETT, J.D., SIMMER, J.P. Proteinases in developing dental enamel. **Crit Rev Oral Biol Med**, v.10, n.4, p.425-441, 1999.

BARTLETT, J.D., SIMMER, J.P., XUE, J., et al. Molecular cloning and mRNA tissue distribution of a novel metalloproteinase isolated from porcine enamel organ. **Gene**, v.183, n.1-2, p.123-128, 1996.

BARTLETT, J.D., XUE, J., SIMMER, J.P., MARGOLIS, H.C. Enamelysin mRNA displays

a developmentally defined pattern of expression and encodes a protein which degrades amelogenin. **Connect Tissue Res**, v.39, n.1-3, p.405-413, 1998.

BRONCKERS, A.L.J.J., BERVOETS, T.J.M., LYARUU, D.M., WÖLTGENS, J.H.M. Degradation of Hamster amelogenins during secretory stage enamel formation in organ culture. **Matrix Biol**, v.14, n7, p.533-541, 1995.

CARTER, J., SMILLIE, A.C., SHEPHERD, M.G. Purification and properties of a protease from developing porcine dental enamel. **Archs Oral Biol**, v34, n.3, p.195-202, 1989.

COLLETA, R.D. **Análise das metalo-proteases no germe dental de ratos**. Piracicaba, 1996. Tese (Mestrado em Biologia e Patologia Buco-Dental) – Universidade Estadual de Campinas.

CURZON, M.E.J., BIBBY, B.G. Effect of heavy metals on dental caries and tooth eruption. **ASDC J Dent Child**, v.37, n.6, p.463-465, 1970.

DACULSI, G., BOULER, J.-M., LEGEROS, R.Z. Adaptative crystal formation in normal and pathological calcifications in synthetic calcium phophate and related biomaterials. **Int Rev Cytol**, v.172, p.129-191, 1997.

DENBESTEN, P.K. Effects of fluoride on protein secretion and removal during enamel development in the rat. **J Dent Res**, v.65, n.10, p.1272-1277, 1986.

DENBESTEN, P.K., HEFFERNAN, L.M. Enamel proteases in secretory and maturation enamel of rats ingesting 0 and 100 ppm of fluoride in drinking water. **Adv Dent Res**, v.3, n.2, p.199-202, 1989a.

DENBESTEN, P.K., HEFFERNAN, L.M. Separation by polyacrylamide gel eletrophoresis of multiple proteases in rat and bovine enamel. **Archs Oral Biol**, v.34, n.6, p.399-404, 1989b.

DENBESTEN, P.K., PUNZI, J.S., LI, W. Purification and sequencing of a 21 kDa and 25

kDa bovine enamel metalloproteinase. **Eur J Oral Sci**, v.106, Suppl.1, p.345-349, 1998.

DENBESTEN, P.K., THARIANI, H. Biological mechanism os fluorosis and level and timing of systemic exposure to fluoride with respect to fluorosis. **J Dent Res**, v.71, n.5, p.1238-1243, 1992.

EISENMANN, D.R. Amelogenesis. In: TEN CATE, A.R. (Ed). **Oral Histology: development, structure and function**. 5th edition. Mosby-Year Book, 1998. Cap.10, p. 197-217.

FINCHAM, A.G., MORADIAN-OLDAK, J., SIMMER, J.P. The structural biology of the developing dental enamel matrix. **J Struct Biol**, v.126, p.270-299, 1999.

FUKAE, M., TANABE, T., UCHIDA, T., et al: Enamelysin (matrix metalloproteinase-20): localization in the developing tooth and effects of pH and calcium on amelogenin hydrolysis. **J Dent Res**, v.77, n.8, p.1580-1588, 1998.

GIL, F., FACIO, A., VILLANUEVA, E., PÉREZ, M.L., TOJO, R., GIL, A. The association of tooth lead content with dental health factors. **Sci Total Environ**, v.192, n.2, p.183-191, 1996.

GRÜNKE, K., STÄRK, H.-J., WENNRICH, R., FRANCK, U. Determination of traces of heavy metals (Mn, Cu, Zn, Cd and Pb) in microsamples of teeth material by ETV-ICP-MS. **Fres J Anal Chem**, v.354, n.5, p.633-635, 1996.

HOJIMA, Y., BEHTA. B., ROMANIC. A.M., PROCKOP, D.J. Cadmium ions inhibit procollagen C-proteinase and cupric ions inhibit procollagen N-proteinase. **Matrix Biol**, v.14, n.2, p.113-120, 1994.

LAGERSTRÖM-FERMER, M., NILSSON, M., BÄCKMAN, B., et al. Amelogenin signal peptide mutation: correlation between mutations in the amelogenin gene (AMGX) and manifestations of X-linked Amelogenesis Imperfecta. **Genomics**, n.26, n.1, p.159-162,

1995.

LAPPALAINEN, R., KNUUTTILA, M. The distribution and accumulation of Cd, Zn, Pb, Cu, Co, Ni, Mn and K in human teeth from five different geological areas of Finland. **Arch Oral Biol**, v.24, n.5, p.363-368, 1979.

LARSEN, M.L., RICHARDS, A., FEJERSKOV, O. Development of dental fluorosis according to age at start of fluoride administration. **Caries Res**, v.19, n.6, p.519-527, 1985.

LI, W., MACHULE, D., GAO, C., DENBESTEN, P.K. Activation of recombinant bovine matrix metalloproteinase-20 and its hydrolysis of two amelogenin oligopeptides. **Eur J Oral Sci**, v.107, n.5, p.352-359, 1999.

LYNGSTADAAS, S.P., RISNES, S., SPROAT, B.S., et al. A synthetic, chemically modified ribozyme eliminates amelogenin, the major translation product in developing mouse enamel *in vivo*. **EMBO J**, v.14, n.21, p.552224-5229, 1995.

MORADIAN-OLDAK, J., SIMMER, J.P., ZEICHNER-DAVID, M., FINCHAM, A.G. Specific cleavage of a recombinant amelogenin at the carboxy-terminal region by a proteinase fraction isolated from developing bovine tooth enamel. **Archs Oral Biol**, v.39, n.8, p.647-656, 1994.

OVERALL, C.M., LIMEBACK, H. Identification and characterization of enamel proteinases isolated from developing enamel. **Biochem J**, v.256, n.3, p.965-972, 1988.

RICHARDS, A., KRAGSTRUP, J., FEJERSKOV, O. Dental Fluorosis development in post-secretory enamel. **J Dent Res**, 65, v.12, p.1406-1409, 1986.

ROBINSON, C., BRIGGS, H.D., AKTINSON, P.J., WEATHERELL, J.A. Matrix and mineral changes in developing enamel. **J Dent Res**, v.58, Special Issue B, p.871-880, 1979.

RYU, O.H., FINCHAM, A.G., HU, C.C., et al. Characterization of recombinant pig enamelysin activity and cleavage of recombinant pig and mouse amelogenins. **J Dent Res**, v.78, n.3, p.743-750, 1999.

SHORE, R.C., ROBINSON, C., KIRKHAM, J., BROOKES, S.J. Structure of developing enamel. In: ROBINSON, C., KIRKHAM, J., SHORE, R.C. (Eds). **Dental Enamel – Formation to Destruction**. CRC press, Boca Raton, 1995. Cap. 6, p.135-150.

SIMMER, J.P., FINCHAM, A.G. Molecular mechanisms of dental enamel formation. **Crit Rev Oral Biol Med**, v.6, n.2, p.84-108, 1995.

SIMMER, J.P., FUKAE, M., TANABE, T., et al. Purification, characterization and cloning of enamel matrix serine proteinase 1. **J Dent Res**, v.77, n.2, p.377-386, 1998.

SMITH, C.E., CHEN, W.-Y. Degradative changes in whole enamel homogenates incubated *in vitro* in the presence of low calcium ion concentrations. **Connect Tissue Res**, v.39, n.1-3, p.379-391, 1998.

SMITH, E.C. Cellular and chemical events during enamel maturation. **Crit Rev Oral Biol Med**, v.9, n.2, p.128-161, 1998.

SMITH, E.C., ISSID, M., MARGOLIS, H.C., MORENO, E.C. Developmental changes in the pH of enamel fluid and its effects on matrix-resident proteinases. **Adv Dent Res**, v.10, n.2, p.159-169, 1996.

SOUZA, A.P., GERLACH, R.F., LINE, S.R.P. Inhibition of human gingival gelatinases (MMP-2 and 9) by metal salts. **Dent Mater**, v.16, n2. p.103-108, 2000.

TANABE, T., FUKAE, M., SHIMIZU, M. Degradation of enamelins by proteinases found in porcine secretory enamel *in vitro*. **Arch Oral Biol**, v.39, n.4, p.277-281, 1994.

TANABE, T., FUKAE, M., UCHIDA, T., SHIMIZU, M. The localization and characterization of proteinases for the initial cleavage of porcine amelogenin. **Calcif Tissue Int**, v.51, n.3,

p.213-217, 1992.

TERMINE, J.D., BELCOURT, A.B., CHRISTNER, P.J., et al. Properties of dissociatively extracted foetal tooth matrix proteins. I. Principle molecular species in developing bovine enamel. **J Biol Chem**, v.255, n.20, p.9760-9768, 1980.

TOSTES, D. **Efeito crônico do flúor no esmalte e nos ameloblastos secretores de incisivos de ratos.** Piracicaba, 1988. Tese (Mestrado em Biologia Buco-Dental) – Universidade Estadual de Campinas (in portuguese).

UCHIDA, T., TANABE, T., FUKAE, M., SHIMIZU, M., YAMADA, M., MIAKE, K., KOBAYASHI, S. Immunohistochemical and immunohistochemical studies using antisera against porcine 25 kDa amelogenin, 89 kDa enamelin and the 13-17 kDa nonamelogenins, on immature enamel of the pig and rat. **Histochemistry**, v.96, p. 129-138, 1991.

WATSON, G.E., DAVIS, B.A., RAUBERTAS, R.F., PEARSON, S.K., BOWEN, W.H. Influence of maternal lead ingestion on caries in rat pups. **Nat Med**, v.3, n.9, p.1024-1025, 1997.

WEATHERELL, J., DEUTSCH, D., ROBINSON, C., HALLSWORTH, A.S. Fluoride concentrations in developing enamel. **Nature**, v.256, p.230, 1985.

APÊNDICE

The effect of lead on the eruption rates of incisor teeth in rats

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Running Title: lead effect on eruption rates

Abstract

The effects of the exposure to lead on the eruption rates of continuously erupting rat incisors under normo-, hyper- and hypofunctional conditions were investigated. Left lower incisors of 20 rats were rendered unimpeded (hypofunctional) by cutting them out of occlusion every two days, whereas the right lower incisors of these rats were considered hyperfunctional. Measurments on normally growing teeth (normofunctional) were carried out in a group of 10 rats whose teeth were not cut, but only marked every two days. On day 7 of the experiment, half of the rats from these two groups were given a single intraperitoneal injection of lead acetate (40 mg/kg), whereas the other half received sodium acetate (22 mg/kg). Another group of 15 rats was used to obtain blood samples for blood lead determination after 1h, 10, 20, and 30d of lead administration. Animals were sacrificed on day 32. Hypofunctional incisors from lead-treated rats erupted more slowly than control ones ($p<0.05$). These results show a previously unreported toxic effect of heavy metals.

Keywords: Eruption; Lead; Metals; Incisor; Rat.

1. Introduction

Environmental lead exposure is considered a major public health issue in many countries due to the detrimental effect of lead on the intelligence of children (Cicuttini et al., 1994). An association between exposure to lead and higher caries scores was shown by many researchers (Curzon and Bibby, 1970; Tvinnereim et al., 1997; Watson et al., 1997). Curzon and Bibby (1970) reported some evidence of delayed tooth eruption in a heavy

metal contaminated area. Pearl and Roland (1980) described a case of congenital lead poisoning in which primary dentition was severely delayed.

The continuously erupting incisors of rats are often used to study tooth eruption. This model is particularly suited to studies on eruption because the eruption rate of the incisors can be increased by trimming teeth out of occlusion (see Moxham and Berkovitz, 1995 for a review). This experimental model offers conditions in which slight alterations in the eruption rate can be detected, and substances thought to interfere with tooth eruption have been tested using this model (e.g. Berkovitz, 1972; Chiba et al., 1980; Bum-Murdoch, 1990).

The purpose of the present study was to determine the effects of lead on the eruption rates of continuously erupting rat incisors under hyper-, hypo- and normofunctional conditions. Our working hypothesis was that lead-protein interactions could affect protein turnover, which is very high in the periodontal ligament (Sodek, 1977) and is thought to be increased in unimpeded eruption (Beersten and Everts, 1977; Rippin, 1978).

2. Materials and Methods

2.1. Animals

45 male Wistar rats (starting weight = 175 g) were acquired from CEMIB-UNICAMP, and were randomly assigned to 3 groups. In one group of rats (n=10), the teeth were maintained under normofunctional conditions and every two days the lower incisors were only marked and measured in order to keep a record of normally growing incisors. Another group of 20 rats had their left lower incisors sectioned at the level of the gingival papilla every other day to keep them out of occlusal contact. Thus, the left lower incisors were considered unimpeded or hypofunctional. It is considered that the right lower incisors

that are still in occlusion (impeded) become hyperfuncional by this procedure (Steigman et al., 1989). The last group of 15 rats was used only for blood lead determination after 1h, 10, 20 and 30d of lead administration.

2.2. Eruption rate measurements

A calibrated grid in a microscope eyepiece was used for measurements at 10X magnification. A trained examiner unaware of the group to which each animal belonged measured the distances. Normal, impeded (hyperfunctional) and unimpeded (hypofunctional) eruption rates of the lower incisors were measured every two days at the same time by recording the distance from the gingival margins to: 1- landmarks made on both incisors of rats from the normofunctional group; 2- landmarks made on the left incisors (impeded teeth); 3- the trimmed edge on right incisors (unimpeded). The reference marks and the occlusal edge of right incisors were made with a diamond high-speed rotating instrument after each measurement. For all procedures the animals were lightly anaesthetized with ether. The weight of each animal was recorded every two days. Rats were housed in groups of five, provided with food (Purina pellets) and tap water *ad libitum*. Animals were sacrificed after 32 days of experiment.

2.3.Lead administration

Lead acetate (40 mg/kg) and sodium acetate (22 mg/kg) were intraperitoneally injected on day 7. Five rats from the normofunctional group and 10 rats from the hyper-/hypofunctional groups received lead acetate, whereas the control animals received sodium acetate. Three animals assigned to the group of blood lead determination received sodium acetate, and the remaining 12 animals received lead acetate.

2.4. Blood lead determination

Three animals that received lead acetate and the 3 animals that received sodium acetate

were killed under ether anesthesia 1 h after the injections (day 0). One ml heparinized blood was collected. The remaining animals injected with lead acetate were sacrificed in groups of three animals on days 10, 20, and 30 for blood collection. Lead was determined by atomic absorption spectroscopy (Zeeman Atomic Absorption Spectrometer, Perkin Elmer, Model 4100 ZL, equipped with a graphite furnace) according to the method described by Parsons and Slavin (1993).

2.5. Statistical analysis

Averages of the measures obtained every two days were calculated for each group both for eruption rate and weight gain. Differences in eruption rates and weight gain were analyzed by paired *t*-tests. Comparisons were made between lead-treated and control for the same functional state (normo-, hypo-, or hyperfunctional) for each time interval. To evaluate differences in weight gain, paired *t*-tests were used. The sample size in the current study was sufficient to detect differences in eruption rates between controls and lead treated groups at a significance level of 0.05 with the power of 70%-80%.

3. Results

Rendering teeth unimpeded doubled the eruption rate of incisors prior to administration of lead acetate. After exposure to lead, no alterations in eruption rates of rat incisors were observed under normo- or hyperfunctional conditions. However, reduced eruption rates were recorded at various time periods (day 8, 14, 16, 22, 24 and 28) under hypofunctional conditions ($p < 0.05$) (Fig. 1).

The growth of all animals was followed throughout the 32-day duration of the experiment by weighing each animal every other day and recording the data. A single intraperitoneal injection of lead acetate (40 mg/kg) did not affect weight gain, an important parameter

used to assess possible systemic toxicity that could interfere with eruption.

Figure 2 shows the mean lead levels (μg/dl) in the blood of animals at different times after exposure. In the rats killed immediately after injection of lead acetate, the blood lead levels were highest and a steady decrease in blood lead was verified until the end of the experiment. Lead was not detected in the blood from control animals.

4. Discussion

Eruptive mechanisms are not yet fully understood, and it is currently accepted that eruption is a multifactorial process in which cause and effect are difficult to separate (Ten Cate, 1998). As stated by Moxham and Berkovitz (1995), a change in eruption may be due to a change in the eruptive force, to a change in the resistance to eruption, to a change in the remodeling characteristics of the supportive tissues, or indeed to more than one of these factors. Experimental alteration of the eruption rate of the rat incisor provides a useful model to test the effect of chemicals on dental eruption. Since Bryer (1957) stated that only unimpeded (hypofunctional) teeth expressed the full eruptive movement, erupting 2 to 3 times faster than impeded teeth, most of the experiments carried out to study the effects of diverse substances on eruption were done using unimpeded continuously growing teeth of rodents and lagomorphs.

Our results showed decreased eruption rates of incisors from Pb-treated rats when compared to control animals under hypofunctional conditions. The reason why only the eruption rate of hypofunctional rat incisors was affected by lead exposure is not clear, but some considerations are noteworthy.

The partial or total removal of occlusal forces does not only affect the eruption rate of teeth, but also the formation of dental and periodontal tissues (Steigman et al., 1989),

indicating alterations in protein metabolism, cell secretion and proliferation (Michaeli et al., 1986), which may be directly or indirectly related to the eruptive process. It has been demonstrated before that different chemicals induce diverse responses in eruption rates, and that the effect on impeded or unimpeded eruption rates seems to be so far unpredictable. Lathyrogens were shown to affect only impeded rates (Sarnat and Sciaky, 1965; Berkovitz et al., 1972), whereas cyclophosphamide has been reported to delay only unimpeded eruption (Adatia and Berkovitz, 1981; Burn-Murdoch, 1990). Vitamin C-deficient diets have been reported to affect eruption rates of both impeded and unimpeded teeth (Berkovitz, 1974). The selective effect of cytotoxic agents (cyclophosphamide is one example) on unimpeded rates over impeded rates suggests that hypofunctional teeth have increased cell proliferation rates. Burn-Murdoch (1990) made important considerations about why cyclophosphamide affects only unimpeded eruption: a maximal speed (as in unimpeded eruption) must be achieved for the effects of this drug to be detected.

The primary site of lead accumulation is the bone, and this tissue is not a mere physiological sink; on the contrary, lead is mobilized in response to normal and pathological changes in bone metabolism (Schirrmacher et al., 1998). Thus, the alveolar bone and its possible alterations in unimpeded eruption must be borne in mind. Hass et al. (1967) described the inhibition of bone matrix synthesis in experimental animals during chronic ingestion of inorganic lead. Observations on the harvesian system after chronic low level lead intoxication in dogs suggested decreased bone formation rates at the cellular, tissue and organ levels of bone formation (Anderson and Danylchuk, 1977). Recently, many interactions have been described at the cellular level between lead and osteoblasts (Schirrmacher et al., 1998), and some of them could have a special bearing

on the retarded eruption described here. It has been shown that Pb perturbed epidermal growth factor (EGF) modulation of intracellular calcium metabolism and collagen synthesis in rat osteoblastic cells (Long and Rosen, 1992).

Lead is a cytotoxic agent (Goering, 1993). We have no data on cytotoxicity of lead to periodontal ligament cells. However, lead was shown to have no effect on ameloblasts (Pasetto et al., 1999), and ameloblasts are cells that are particularly sensitive to changes in their environment due to their unique features (Eisenmann, 1998). Thus, it is conceivable that the effects of lead on unimpeded teeth presented in the present study might not be related to the cytotoxic effects on cells detectable at the ultrastructural level. On the other hand, one must consider that the hazardous effects of lead on the organism may not depend solely on its cytotoxicity, but also on its interaction with proteins and enzymes of the extracellular matrix as well.

The tooth is embedded in a collagen-rich extracellular matrix. Thus, eruption movements are necessarily accompanied by extracellular matrix remodeling, although the intensity of remodeling of each matrix component (collagens, proteoglycans and non-collagenous proteins) are still a matter of debate (Van den Bos and Tonino, 1984; Kirkham et al., 1993). Studies on collagen metabolism have pointed to increased collagen turnover in the periodontal ligament of unopposed rat molars (Rippin, 1978; Kanoza et al., 1980) and in unimpeded mouse incisors (Beertsen and Everts, 1977).

A growing body of evidence indicates that enzymes shown to participate in the remodeling of the extracellular matrix are inhibited *in vitro* by many metals and, interestingly, current concepts on tooth eruption also highlight the fact that, regardless of the actual mechanism underlying tooth eruption, matrix proteases seem to play a major role in this process (Gorski et al., 1988). MMP-1 and MMP-3 were detected by immunoblotting in extracts

from canine dental follicles (Gorski and Marks, 1992). MMP-1 was detected by in situ hybridization in rat root resorbing tissue induced by tooth movement (Domon et al, 1999). In addition, it has been proposed that degeneration of the enamel organ provides a timed stimulus for the onset of prefunctional tooth eruption and that this stimulus could be related to the activity of proteolytic enzymes released from the degenerating enamel organ (Gorski and Marks, 1992). Another possible role of MMPs in eruption concerns EGF, a molecule associated with the control of eruption (Thesleff, 1987). Protease action is required to liberate biologically active EGF from membrane-bound precursor (Gorski and Marks, 1992).

Several enzymes involved in the metabolism of extracellular matrix components were shown to be inhibited by excess metal ions (> 5 μmol/l). Cu and Cd ions were shown to inhibit procollagen C- and N-proteinases, respectively (Hojima et al., 1994). Galactosylhydroxylysylglucosyl-transferase, an intracellular enzyme of collagen biosynthesis, is inhibited by excess Zn, Cu, Cd, Ni and Ca (Myllyla et al., 1979). Neutrophil collagenase was shown to be inhibited by Au(I), Cd(II), Cu(II) and Zn(II) (Mallya and Van Wart, 1989), whereas gelatinases A and B were shown to be inhibited by Zn and Cu (Souza et al., 1999). A survey of the effect of several metal ions on the activity of carboxypeptidase A revealed that only Pb(II) inhibited this enzyme with a potency comparable to that of Zn(II) (Larsen and Auld, 1991). The mechanism of enzyme inactivation by excess metal has been associated with conformational changes that inactivate the catalytic function of enzymes, and this inhibition could be very important to regulatory and/or toxicological processes of zinc enzymes (Auld, 1995).

These results may be of great importance in view of the possible effects of the lifetime chronic accumulation of lead on the dental structures. Furthermore, the use of the

unimpeded rat incisor to study eruption rates in metal-exposed animals may make an additional contribution to our understanding of eruption in contaminated areas.

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References

Adatia, A.K., Berkovitz, B.K.B. 1981. The effects of cyclophosphamide on eruption of the continuously growing mandibular incisor of the rat. Arch Oral Biol 26, 607-613.

Anderson, C., Danylchuk, K.D. 1977. The effect of chronic low level lead intoxication on the harvesian remodelling system in dogs. Lab Invest 37, 466-469.

Auld, D.S. 1995. Removal and replacement of metal ions in metallopeptidases. In: Barret, A.J. (Ed.), Proteolytic enzymes: aspartic and metallopeptidases. Methods in Enzymology 248, 228-242.

Beersten, W., Everts, V. 1977. The site of remodelling of collagen in the periodontal ligament of the mouse incisor. Anat Rec 189, 479-498.

Berkovitz, B.K.B., Migdalski, A., Solomon, M. 1972. The effect of the lathyritic agent aminoacetonitrile on the unimpeded eruption rate in normal and root-resected rat lower incisors. Arch Oral Biol 17, 1755-1763.

Berkovitz, B.K.B., 1974. The effect of vitamin C deficient diet on eruption rates for the guinea pig lower incisor. Arch Oral Biol 19, 807-811.

Bryer, L.W. 1957. An experimental evaluation of the physiology of tooth eruption. Int Dent J 7, 432-478.

Burn-Murdoch, R.A. 1990. The effect of cyclophosphamide on the eruption of impeded and resected incisors in rats. Arch Oral Biol 35, 801-806.

Chiba, M., Takizawa, K., Ohshima, S. 1980. Dose-response effects of colchicine and vinblastine on unimpeded eruption rates of the rat mandibular incisor. Arch Oral Biol 25, 115-119.

Cicuttini, F.M., Fairley, C.K., McNeil, J.J., Taylor, E.R. 1994. The public health problem of

environmental lead contamination. Med J Aust 160, 173-174.

Curzon, M.E.J, Bibby, B.G. 1970. Effect of heavy metals on dental caries and tooth eruption. J Dent Child 37, 463-465.

Domon, S., Shimokawa, H., Matsumoto, Y., Yamaguchi, S., Soma, K. 1999. In situ hybridization form matrix metalloproteinase-1 and cathepsin k in rat root resorbing tissue induced by tooth movement. Arch Oral Biol 44, 907-915.

Eisenmann, D.R. Amelogenesis. 1998. In: Ten Cate, A.R. (Ed), Oral Histology: development, structure and function. 5th edition. Mosby-Year Book, pp. 197-217.

Goering, P.L. 1993. Lead-protein interactions as a basis for lead toxicity. Neurotoxicology 14, 45-60.

Gorski, J.P., Marks, S.C., Cahill, D.R., Wise, G.E. 1988. Developmental changes in the extracellular matrix of the dental follicle during tooth eruption. Connect Tissue Res 18, 175-190.

Gorski, J.P., Marks, S.C. 1992. Current concepts of biology of tooth eruption. Crit Rev Oral Biol Med 3, 185-206.

Hass, G.M., Landerholm, W., Hemmens, A. 1967. Inhibition of intercellular matrix synthesis during ingestion of inorganic lead. Am J Pathol 50, 815.

Hojima, Y., Behta, B., Romanic, A.M., Prockop, D.J. 1994. Cadmium ions inhibit procollagen C-proteinase and cupric ions inhibit procollagen N-proteinase. Matrix Biol 14, 113-120.

Kanoza, R.J.J., Kelleher, L., Sodek, J., Melcher, A.H. 1980. A biochemical analysis of the effect of hypofunction on collagen metabolism in the rat molar periodontal ligament. Arch Oral Biol 25, 663-668.

Kirkham, J., Robinson, C., Phull, J.K., Shore, R.C., Moxham, B.J., Berkovitz, B.K.B. 1993.

The effect of rate of eruption on periodontal ligament glycosylaminoglycan content and enamel formation in the rat incisor. Cell Tissue Res 274, 413-419.

Larsen, K.S., Auld, D.S. 1991. Characterization of an inhibitory metal binding site in carboxipeptidase A. Biochemistry 30, 2613-2618.

Long, G.F., Rosen, J.F. 1992. Lead perturbs epidermal growth factor (EGF) modulation of intracellular calcium metabolism and collagen synthesis in clonal rat osteoblasic (ROS 17/2.8) cells. Toxicol Appl Pharmacol 114, 63-70.

Mallya, S.K., Van Wart, H.E. 1989. Mechanism of inhibition of human neutrophil collagenase by gold(I) chrysotherapeutic compounds. J Biol Chem 264, 1594-1601.

Michaeli, Y., Steigman, S., Yitschaky, M., Weinreb, M.Jr. 1986. The response of fibroblast population in the periodontal ligament of rat incisors to altered eruption rates. J Periodont Res 21, 86-94.

Moxham, B.J., Berkovitz, B.K.B. 1995. The periodontal ligament and physiological tooth movements. In: Berkovitz, B.K.B., Moxham, B.J., Newman, H.N. (Eds), The periodontal ligament in health and disease. Mosby, London, pp. 183-214.

Myllyla, R., Anttinen, H., Kivirikko,K.I. 1979. Metal activation of galactosylhudroxylysyl glcosyltransferase, an intracellular enzyme of collagen biosynthesis. Eur J Biochem 10, 261-269.

Parsons, P.J., Slavin, W. 1993. A rapid Zeeman graphite furnace atomic absorption spectrometric method for the determination of lead in blood. Spectrochimica Acta 48B, 925-939.

Pasetto, S., Gerlach, R.F., Novaes, P.D., Romani, E.A.N., Line, S.R.P. 1999. Morphologic features of ameloblasts from rats exposed to lead via the maternal drinking water. Acta Microscopica 8, Suppl.B, 13-14.

Pearl, M., Roland, N.M. 1980. Delayed primary dentition in a case of congenital lead poisoning. J Dent Child 47, 269-271.

Rippin, J.W. 1978. Collagen turnover in the periodontal ligament under normal and altered functional forces. J Periodontal Res 13, 149-154.

Samat, H., Sciaky, I. 1965. Experimental lathyrism in rats; Effects of removing incisal stress. Periodontics 3, 128-134.

Schirrmacher, K, Wiemann, M., Bingmann, D., Büsselberg, D. 1998. Effects of lead, mercury, and methyl mercury on gap junctions and Ca^{2+} in bone cells. Calcif Tissue Int 63: 134-139.

Sodek, J. 1977. A comparison of the rates of synthesis and turnover of collagen and non-collagen protein in adult rat periodontal tissues and skin using a microassay. Arch Oral Biol 22, 655-665.

Souza, A.P., Gerlach, R.F., Line, S.R.P. 2000. Inhibition of human gingival gelatinases (MMP-2 and 9) by metal salts. Dent Mater 16: 103-108.

Steigman, S., Michaeli, Y., Yitzhaki, M., Weinreb, M. 1989. A three-dimensional evaluation of the effects of functional occlusal forces on the morphology of the dental and the periodontal tissues of the rat incisor. J Dent Res 68: 1269-1274.

Ten Cate, A. R. 1998. Physiologic tooth movement: eruption and shedding. In: Ten Cate, A. R. (Ed.), Oral histology: development, structure, and function. Mosby, St. Louis, U.S.A., pp. 289-314.

Thesleff, I. 1987. Does epidermal growth factor control tooth eruption? J Dent Child 54, 321-329.

Tvinnereim, H.M., Eide, R., Riise, T., Wesenberg, G.R., Fosse, G., Steinnes, E. 1997. Lead in primary teeth from Norway: changes in lead levels from the 1970s to the 1990s.

Sci Total Environ 207, 165-177.

Van den Bos, T., Tonino, G.J.M. 1984. Composition and metabolism of the extracellular matrix in the periodontal ligament of impeded and unimpeded rat incisors. Arch Oral Biol 29, 893-897.

Watson, G.E., Davis, B.A., Raubertas, R.F., Pearson, S.K., Bowen, W.H. 1997. Influence of maternal lead ingestion on caries in rat pups. Nature Medicine 3, 1024-1025.

Figure Legends

Fig.1. Eruption rates of rat lower incisors under normo- (upper graph), hypo- (middle graph) and hyperfunctional conditions (lower graph). Data are reported as mean ± SD. A single dose of lead acetate was administered on day 7 (arrow) to the experimental group (●), while the controls (O) received an equivalent dose of sodium acetate. * $p<0.05$ versus control.

Fig.2. Blood lead disappearance curve in rats given a single intraperitoneal injection of 40 mg/kg body wt of lead acetate trihydrate. Each point is mean ± SE of samples taken from 3 rats.

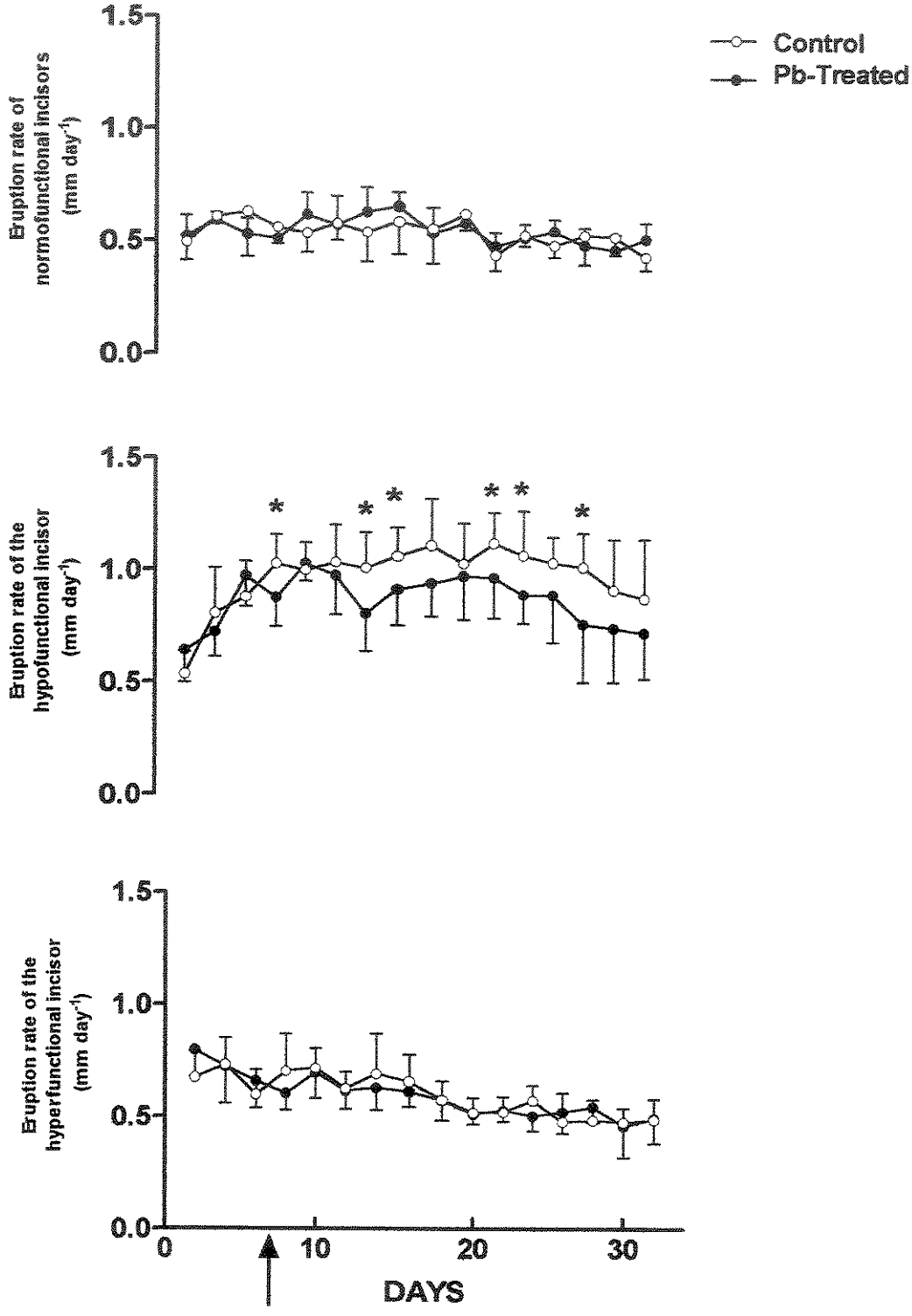
Control
Pb-Treated
Eruption rate of normofunctional incisors (mm day-1)
Eruption rate of the hypofunctional incisor (mm day-1)
Eruption rate of the hyperfunctional incisor (mm day-1)
1.5
1.0
0.5
0.0
*
0
10
20
30
DAYS

Figure 1

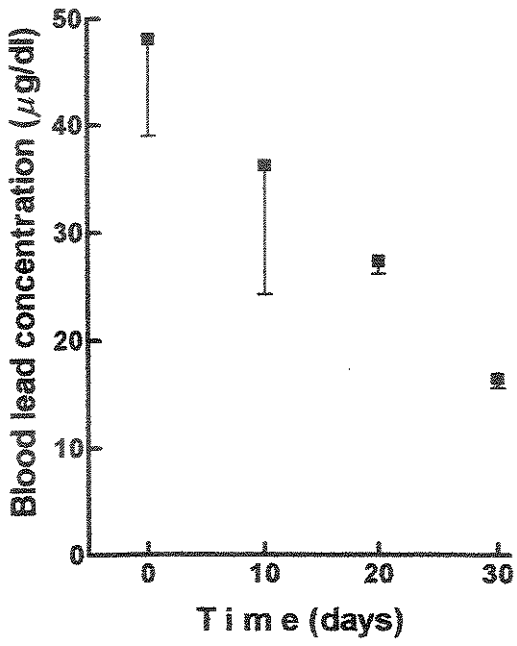
Blood lead concentration (μg/dl)
50
40
30
20
10
0
0
10
20
30
Time (days)

Figure 2

Archives of Oral Biology

REF:
C. 1020

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Your manuscript entitled "The effect of lead on the eruption rates of incisor teeth in rats"

has been accepted for publication and was fowarded to the publisher on 30.6.2000

A reprint order form will be sent with the galley proof.

D. B. FERGUSON
Editor in Chief

Lista de Abreviações:

APMA: (4-aminophenyl) mercuric acetate, um potente ativador de MMPs

EDTA: ácido etileno-diamino-tetraacético

EMSP-1: Serino-protease do esmalte (número) 1

kDa: 1000 daltons

MMP-20: metaloproteinase 20

NEM: N-ethyl-maleimide, inibidor de cysteína-proteinases

PMSF: phenylmethylsulphonylfluoride, inibidor de serina-proteinases

SDS-PAGE: eletroforese em gel de poliacrilamida com SDS (dodecil-sulfato de sódio)

TCA: ácido tricloroacético

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FACULDADE DE ODONTOLOGIA DE PIRACICABA

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SUBJECT: ERRATUM

Dear Dr. Linde;

Unfortunately there is a mistake in one of the legends of the article "Fluoride effect on the activity of enamel matrix proteinases *in vitro*" that appeared in the February issue of the *European Journal of Oral Sciences.* The mistaken text is the legend to Figure 4 and the correct text should be the following:

Fig. 4. Polyacrylamide gel (15%) stained with Coomassie blue. Enamel matrix homogenates taken from secretory and maturation stages and incubated *in vitro* from 0-48 h at 37°C in 50 mM Tris/1 mM $CaCl_2$, pH 7.2, containing 10 mM NaF. Lanes: Std - molecular mass protein marker; 1 - proteolysis control containing 5 mM PMSF, 5 mM NEM, 5 mM EDTA and 5 mM 1,10-phenanthroline; 2 - control, 12 h incubation; 3 - control, 24 h incubation; 4 - control, 48 h incubation; 5 - 10 mM NaF, 12 h incubation; 6 - 10 mM NaF, 24 h incubation; 7 - 10 mM NaF, 48 h incubation.

We would like to apologize for the inconvenience and we thank in advance for your comprehension.

Sincerely,

Sérgio R. P. Line
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