ISABEL MARIA PORTO

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

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Orientador: Profa. Dra. Raquel Fernanda Gerlach

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Prof. Dr. José Merzel
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SUMÁRIO

RESUMO

O órgão do esmalte de incisivos de ratos no estágio de maturação da amelogênese (região em que os prismas de esmalte são visíveis e o esmalte é completamente solúvel em ácido) foi removido, e os limites desta remoção (região cirúrgica) foram demarcados por marcas feitas na face labial do esmalte. O órgão do esmalte, da região acima da marca incisal até a crista alveolar, foi danificado utilizando-se uma lima endodôntica e foi feita a ressecção do órgão odontogênico na extremidade basal do incisivo. Os ratos foram divididos em dois grupos: I – quando a marca incisal e II – quando a marca basal apareceu na coroa clínica. Os incisivos experimentais erupcionaram em uma taxa variável e mais lenta que os dentes contralaterais controles. O aspecto histológico do esmalte na região cirúrgica dos ratos do grupo I variou de um esmalte completamente removido pelo descalcificador a resquícios de esmalte com prismas visíveis. Nessa mesma região no grupo II, todo o esmalte estava completamente solúvel em ácido. A microdureza do esmalte na região cirúrgica foi menor nos incisivos experimentais, enquanto o conteúdo mineral (Ca e P) não apresentou diferença em relação ao dente controle. A análise protéica do esmalte dos incisivos, uma semana após a cirurgia, revelou uma quantidade e um padrão de eletroforese similar entre os dentes controle e experimental na região cirúrgica. Na região pós-cirúrgica, o conteúdo protéico do esmalte do dente experimental foi muito menor e mostrou proteínas de baixo peso molecular em relação à região correspondente nos incisivos controles. Os resultados são consistentes com a hipótese de que o ameloblasto no estágio da maturação não interfere no influxo de minerais no esmalte.

ABSTRACT

The enamel organ of rat incisors at the maturation stage of amelogenesis (from the region were enamel rods are visible and the enamel is completely acid-soluble) was removed and the limits of the removal (surgical region) were marked by notches made at the labial face of the enamel. The enamel organ, from the incisal notch up to the alveolar crest, was damaged by an endodontic file and the odontogenic organ at the basal end of the incisor was resected. The rats were divided into two groups: I – when the incisal mark and II when the basal mark appeared at the clinical crown. The experimental incisors erupted at a variable and slower rate than the contralateral controls. The histological aspect of the enamel at the surgical region of rats of group I varied from enamel completely removed by demineralization to remaining of enamel showing rods. In the same region of group II all enamel was completely acid-soluble. Microhardness of the enamel at the surgical region was lower in experimental incisors, while the mineral content (Ca and P) did not differ from the control teeth. Protein analysis of enamel from incisors, one week after surgery, showed an amount and an electrophoresis pattern similar in experimental and control teeth at the surgical region. In the post-surgical region, the protein content of enamel of experimental teeth was much lower and showed more proteins of lower molecular weight than the correspondent region of the control incisors. The results are consistent with the hypothesis that the ameloblast at the maturation stage do not interfere with the influx of minerals into the enamel.

1. INTRODUÇÃO

A formação do esmalte dentário, denominada amelogênese, é dividida basicamente em dois estágios. Primariamente, uma matriz de esmalte é secretada por células epiteliais altamente especializadas denominadas ameloblastos, que estão localizados na camada mais interna do órgão do esmalte. Esta matriz é altamente hidratada, parcialmente mineralizada (contendo cristais de hidroxiapatita, que crescem em comprimento, neste estágio) e contém grande quantidade de proteínas. Dentre estas proteínas, as proteínas mais abundantes são as amelogeninas que constituem 90% do conteúdo orgânico do esmalte no estágio secretório (1).

Após toda a espessura do esmalte ter sido secretada, inicia-se um outro estágio da amelogênese denominado maturação, no qual os cristais de hidroxiapatita crescem em espessura às custas de um intenso influxo de sais de fosfato de cálcio e da perda de água e proteínas. A proteólise ocorre como conseqüência da ação de enzimas proteolíticas presentes na matriz do esmalte (2).

Porém ao contrário da secreção, não se sabe claramente se os ameloblastos têm alguma função no estágio de maturação do esmalte, seja pelo controle do influxo de cálcio na matriz e/ou pela produção e transporte de proteases.

Uma serinoprotease que participa da degradação final das proteínas na maturação do esmalte, conhecida como calicreína-4 foi identificada em ameloblastos no estágio de maturação (3). Sabe-se também que os polipeptídeos, decorrentes da proteólise, deixam a matriz de esmalte por entre os ameloblastos (via paracelular) (4).

Porém outros estudos relacionam amelogênese imperfeita com mutação da metaloprotease de matriz 20 (MMP-20) (5) ou com seu substrato, a amelogenina (6-9). Isso pode ser um indicativo de que os ameloblastos não tenham um papel tão importante na maturação.

Como ocorre o influxo de cálcio também é pouco conhecido. Nos ameloblastos foram identificadas algumas proteínas transportadoras de cálcio, porém em níveis muito baixos (10). Por exemplo, a calbindina $_{28kDa}$ durante a maturação, sofre uma regulação negativa, ficando em níveis baixos, contradizendo a concepção de que esta realize o

transporte transcelular do cálcio (11). Os ameloblastos na maturação sofrem alterações morfológicas cíclicas em sua extremidade basal, e isto é correlacionado ao transporte de cálcio. Nos ameloblastos de borda pregueada, o transporte é feito por via transcelular e nos de borda lisa por via paracelular (12). Porém, em estudo realizado com Ca^{45}, nenhum cálcio marcado foi detectado nos ameloblastos (13).

Em experimentos de erupção dental, em que foi feita a remoção parcial do órgão do esmalte e do periodonto relacionado ao esmalte na zona de maturação de incisivos de ratos, associada à ressecção do órgão odontogênico, foi observado algum tempo após a cirurgia, que o esmalte era removido pelo descalcificador, indicando algum grau de mineralização na ausência de parte dos ameloblastos pós-secretórios (14). Isso talvez seja outro indicativo de que os ameloblastos não atuam ou tenham um papel secundário na maturação do esmalte.

O objetivo deste estudo é, portanto, usar o modelo experimental descrito por Merzel *et al* (2004) para verificar o grau de mineralização do esmalte na ausência dos ameloblastos.

O primeiro artigo verifica os aspectos morfológicos (histologia convencional e análise visual), bioquímicos (quantificação e eletroforese de proteínas e análise inorgânica – EDX) e físicos (microdureza) do esmalte formado durante a fase de maturação em animais nos quais os ameloblastos e demais células do órgão do esmalte foram removidos cirurgicamente.

O segundo artigo padroniza uma técnica de extração de proteínas da matriz do esmalte, comparando a eficiência três métodos de extração (Ácido Acético 0,5M, Ácido Tricloroacético 12% e Uréia 6M) nas diversas fases de desenvolvimento do esmalte dental de incisivos inferiores de ratos (final da secreção, início da maturação, estágio intermediário da maturação e final da maturação).

2. CAPÍTULO

ENAMEL MINERALIZATION AFTER PARTIAL REMOVAL OF POST-SECRETORY AMELOBLASTS IN THE RAT LOWER INCISORS

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Running title: Enamel mineralization in the absence of ameloblasts

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Abstract: The enamel organ of rat incisors at the maturation stage of amelogenesis was removed and the limits of the removal (surgical region) were marked by notches made at the labial face of the enamel. The enamel organ, from the incisal notch up to the alveolar crest, was damaged by an endodontic file and the odontogenic organ at the basal end of the incisor was resected. The rats were divided into two groups: I – when the incisal mark and II - when the basal mark appeared at the clinical crown. The experimental incisors erupted at a variable and slower rate than the contralateral controls. The histological aspect of the enamel at the surgical region of rats of group I varied from enamel completely removed by demineralization to the presence of remains of enamel showing rods. In the same region of group II all enamel was completely acid-soluble. Microhardness of the enamel at the surgical region was lower in experimental incisors, while the mineral content (Ca and P) did not differ from the control teeth. Protein analysis of enamel from incisors, one week after surgery, showed an amount and an electrophoresis pattern similar in experimental and control teeth at the surgical region. In the post-surgical region, the protein content of enamel of experimental teeth was much lower and showed more proteins of lower molecular weight than the correspondent region of the control incisors. The results are consistent with the hypothesis that the ameloblasts at the maturation stage do not interfere with the influx of minerals into the enamel.

Key words: mineralization, enamel, ameloblasts.

Introduction:

Amelogenesis is a process usually divided into two phases: secretion in which an organic and partially mineralized matrix is secreted and maturation where this immature matrix loses water and organic material giving place to a highly mineralized tissue.

Enamel maturation is not yet well understood. The bulk of the maturation process is related to post-secretory ameloblasts, however their role in this process is obscure yet.

Calcium is mostly incorporated during maturation, where 86% of total calcium is acquired (1). Post-secretory ameloblasts undergo a cyclic morphological transition between cells with ruffled and smooth apical borders (2-6). Ameloblasts show features consistent with absorptive activity in maturation phase (7). However, there are indications that this ameloblasts might secrete degrading enzymes, but do not resorb enamel matrix (7). Ameloblasts double their mitochondrial content during maturation (8-9), similar to the expression of calcium store and calcium transport proteins (10). All main components of endoplasmic reticulum related to calcium storage: Ca-ATPase pump, luminal buffer proteins, calcium-release channel, are present in rat in post-secretory ameloblasts (10).

In ruffled ameloblasts the epithelium is tight and the calcium takes a transcellular route. In smooth ameloblasts, there is a breach of the impermeability, allowing calcium to take a paracellular route (10). After ^{45}Ca injection it was shown that calcium phosphate is deposited directly into the enamel matrix and no labeled calcium was detected in the ameloblasts (11).

High-affinity calcium binding protein, calbindin $_{28Kda}$ is extremely down regulated during maturation contradicting the concept that calbindins act as transcellular calcium ferries during maturation phase (12).

Some studies correlated the ameloblasts to the loss of organic content. It was suggested that secretory ameloblasts would be responsible for enzyme elaboration related to the breakdown of extracelular organic content (13). Studies using the uptake of [^{3}H] proline and $^{35}SO_4$ show that the decrease of enamel labeled proteins in the enamel occurs when the matrix is still related to the secretory ameloblasts (14-16). Enamelysin (MMP-20),

which is expressed during the secretory to late secretory and transition stage of enamel development (17), is secreted into enamel matrix by secretory ameloblasts (18). A serinoproteinase, kalicrein-4, that plays a role in the final degradation of enamel proteins was identified in post-secretory ameloblasts (19).

In experiments where the dental follicle and the enamel organ, at the level of the maturation zone of rat incisors, were partially removed, it was observed, as the tooth erupted, that all enamel was removed by demineralization, indicating some degree of maturation in the absence of most of the post- secretory ameloblasts (20). In these experiments the connective tissue of the dental follicle was totally regenerated within a few days, while the epithelial tissues of the enamel organ were not due the resection of the odontogenic organ at the tooth base. Besides the loss of pigmentation no macroscopic alterations could be seen as the experimental region of the incisor emerged into the oral cavity. The purpose of this study was to verify if dental enamel matrix mineralizes in the absence of ameloblasts.

Materials and methods

Animals with an average weight of 250g were used. The surgical procedures were described by Merzel *et* al (21). Briefly, under anesthesia with ketamine (90 mg/kg) and xylasine (8 mg/kg), the lower border of the left hemimandible was exposed. Using a small osteotome, starting from the insertion of anterior belly of the digastric muscle up to the level of the mesial root of the second lower molar, 4 mm of bone as removed, exposing the labial surface of the incisor. The enamel was scrubbed with a piece of gauze to remove the enamel-related periodontium (dental follicle) and the enamel organ covering it. An excavator was used to remove the same tissues at lateral surface. Then a no. 15 endodontic file was introduced into the incisal part of the labial periodontal space until its tip appeared trough the gingival margin, in order to damage the soft tissues covering the diastema related enamel. Two notches were made to mark the incisal-basal extension of the exposed enamel (Fig 1a). For root resection, the elevation at the lateral surface of the ramus of the left hemimandible indicative of the odontogenic base of the lower incisor was exposed

through a skin incision following a plane between the angle of the mouth and external auditory meatus and a second incision of the anterior half of the masseter above the facial nerve. The bone was perforated with a no. 1 round bur, removed with a dental excavator together with the odontogenic organ, and the cavity thoroughly curetted. The wound was sutured and the animals were kept under controlled conditions of lighting (12-hr light, 12-hr dark) and temperature (25-30ºC), with food and water ad libitum. During the first week after surgery, solid food was offered either ground or as regular commercial pellets and thereafter only as pellets. The protocol for this study was approved by University's Ethical Committee for Animal Research (CEEA – IB – UNICAMP, Protocol number: 556-1). The contralateral incisor was used as control.

The rats were divided into two groups: in the first one (Group I), the rats were killed when the enamel incisal mark done during surgery appeared in the clinical crown (Fig 1b) In the other (Group II), the rats were killed when the basal mark was seen in the clinical crown. The hemimandibles were dissected out, all external soft tissues removed and immersed in 10% buffered formol (pH 7.2-7.4) for 48hr. The right contralateral incisors were used as control. After extraction of teeth, notches were made in the contralateral incisors, at the same level of the ones of the experimental group.

Histology

Experimental and contralateral hemimandibles of eight rats, killed 1, 7, 14 and 21 days after surgery (2 rats in each day), and three rats from group I and other three from group II, were radiographed after fixation. Next, the pieces were demineralized with 5% nitric acid in 10% formol, and, oriented by the radiographs, divided into three segments: pre-surgical (anterior to incisal mark), surgical (between marks) and post-surgical region (posterior to the basal mark) (Fig 1A and B). Parallel segments were prepared from the contralateral mandibles. The pieces were embedded in paraplast and semiserial cross sections of each region were cut at a thickness of 5 μm and stained with haematoxylin and eosin.

Microhardness

The lower incisors of 15 rats of each group were used for enamel cross-sectional microhardness testing. The teeth were extracted, fixed as described above and embedded in acrylic resin with the mesial face towards the bottom of the block. Each block was ground until the appearance of the full length of the pulp and polished. Under a 10X eyepiece, the blocks were oriented with the long axis of the indenter (Model FMA-ARS, Future Tech Corp., Tokyo, Japan) parallel to the dentin-enamel junction. In each region (pre-surgical, surgical, post-surgical), five indentations, apart 100 μm each other, were made on the outer aprismatic enamel. Load used was 25g and time 5s.

Scanning electron microsocope (SEM) and Energy dispersive X-ray (EDX)

The incisors used for microhardness analysis were used for EDX analysis. The blocks were polished again, dehydrated at 37°C for 2 days and covered with a carbon film. Energy dispersive X-ray spectrometry (EDX) (Noran Instruments, Middleton-USA) was carried out in combination with SEM (Jeol JSM-5600LV, Tokyo-Japan). Analysis was performed in 6 points for each region, being 3 points in outer aprismatic enamel and 3 points in prismatic enamel, separated by 200 μm from one another, for 100 s.

Protein Analysis

To scrape and collected the enamel from the surgical region of rats of group I and II was very difficult. This only could be done in the incisor surgical and post-surgical regions of rats killed one week after surgery. The proteins were extracted using trichloroacetic acid (TCA) method. To the lyophilized samples it was added 195 μl of TCA, 1 μl of Phenylmethyl-Sulfonyl Fluoride (PMSF), 1 μl of N–Ethyl Maleimide (NEM), 1 μl of 1,10- Phenanthroline and one hour later, 2 μl of sodium deoxycolate (20 mg/ml) (22). The samples were stored overnight at 0°C, and then centrifuged at 2500g and 4ºC for 45 min. The pellet was redissolved in 197 μl of 6M urea together with 1 μl of each of the protease inhibitors mentioned above.

After extractions, protein content was determined by Bradford's method (Bradford BioRad Reagent) using microplates. The amount of protein determined for each

sample was multiplied by the volume of the extraction solution giving the total amount of protein.

For qualitative evaluation, 25 µg of protein from two samples of each group and region were used for separation by electrophoresis using Laemmli's discontinuous buffer system SDS-PAGE carried out in 15% slab gels (23). Samples were heated at 100ºC for 10 min prior to application and electrophoresis was performed at 25 mA. Completed the runs, the gels were fixed and stained with silver nitrate (24).

Statistical analysis

For the comparison between experimental and control group t-test was used and F test followed by t-test was used for comparison between experimental groups. The level of significance was determined as $p < 0.05$

Results

Histology

In all animals the experimental incisor erupted at a slower rate than the control incisor. The incisal notch emerged in the clinical crown from 20 to 42 days (group I) and the basal notch from 24 to 124 days (group II). In group I the base of the tooth was at level or slightly in front of the mesial face of the first lower molar (Fig 1B). In group II the base of the incisors was close to the alveolar crest.

One day after surgery, the surgical region showed the absence of the alveolar bone and the dental follicle. At the incisal part of the region all enamel was completely removed by demineralization (Fig 2A) while towards the basal end enamel matrix was seen (not shown). A week later the dental follicle and part of the alveolar bone were regenerated and in the enamel space (acid-soluble enamel) at the middle of the region, some disorganized enamel matrix, similar the one normally seen in the transition between the partially and completely acid-soluble enamel was present (Fig 2B). Towards the basal end the enamel matrix was organized (not shown). Three weeks after surgery, some organized enamel matrix was still present at the basal end of the region (Fig 2C and D). In rats of

group I, the aspect of enamel at the basal part of the region varied. In one of rats, killed 20 days after surgery, a large amount of well-preserved enamel matrix was present (Fig 2E), while in other, killed 30 days after surgery, all the enamel was completely removed by acid (Fig 2F)

In areas where the enamel was in direct contact with the connective tissue, there was formation of cementum occured (not shown). The pre and post-surgical regions showed a morphological pattern similar to the contralateral incisor during the first week. At later time intervals, mainly in rats of group I, the post-surgical region of experimental incisors showed completely soluble enamel, while in the controls an organized enamel matrix was present (not shown).

Microhardness

A significant decrease ($p < 0.001$) of the microhardness values was observed in the surgical region (group I and II) when compared with the control group. In the pre and post-surgical regions, there were not statistically significant differences between the experimental (groups I and II) and control groups. Between experimental groups, only in the pre-surgical region the microhardness of group II was significantly higher than group I (Table 1). Immature enamel of lower control incisors, at the level of the first molar, showed knoop values of 45.58 ± 15.89 %, significantly different from all values shown in table 1 ($p < 0,001$).

Energy dispersive X-ray (EDX)

The results indicated that there were no differences between experimental and control groups neither within experimental groups nor between regions (Table 2 and Fig 3). Immature enamel of the control incisor at the level of first molar showed the following values: 57.62 ± 4.89 % of calcium weight and 25.58 ± 1.28% of phosphorus weight. These values are significantly different from all values given in table 2 ($p < 0.05$).

Protein analysis

In the surgical region, the protein content of the experimental group was not different from the one of the control group. In post-surgical region, the experimental group showed a significant decrease in the amount of protein compared to control group ($p < 0.05$). Within the experimental group, surgical e post-surgical regions were not statistically different (Table 3).

In the surgical region, the band patterns of enamel proteins showed no differences between experimental and control groups. On both groups most proteins had a molecular mass between 30 and 15 kDa. In the post-surgical region, the molecular mass of most proteins of the experimental group was smaller than 30 kDa while in the control group most proteins were around 30 kDa and a clear band had a molecular mass between 30 and 40 kDa (Fig 4).

Discussion

The reason to associate the exposure of enamel with root transection (20) was to avoid the cessation of the eruptive movement, as occurred in 25% of the incisors when exposure was the single procedure (21).

The region chosen for enamel exposure by removing he alveolar bone, dental follicle and enamel organ, corresponds, in the 250g rats used, to the maturation stage of amelogenesis from the region where the enamel rods become visible (a line projecting the mesial root, between, the 2nd and 1st molar) up the region where the enamel is completely acid-soluble (a line projecting the mesial face of the 1st molar) (25).

The slowdown of the eruption rate of the experimental incisors was variable and this probably reflected in the aspect of the enamel at the surgical region of animals of group I. Some showed parts of well-organized enamel matrix with visible rods, others only disorganized enamel matrix, and other enamel completely soluble in acid.

The analysis of the mineral content showed that the amount of calcium and phosphorus at the surgical region was not significantly different from the control or from the other two regions. On the other hand, the microhardness of the enamel region of both

groups was significantly lower at the surgical region in both groups. The three regions of the control incisors had similar values for microhardness and mineral content. As they were sampled parallel the regions of the experimental tooth, all were localized at the diastema region, therefore, at the end of the maturation stage. However such values are significantly higher than the one measured at the beginning of the maturation stage.

There was no correlation between microhardness and mineral content of the enamel at the surgical regions (Fig 5). Thus microhardness does not reflect directly the amount of mineral present into the enamel as was shown by Kodaka *et al* (1992)

The amount and the pattern of proteins were similar in the surgical region of both experimental and control incisors, indicating that their degradation and removal were occurring at the same rate. Probably the enzymes necessary for degradation could also be secreted by ameloblasts at the secretory stage or by ameloblasts at the very beginning of the maturation stage present at the post-surgical region. As shown by quantitative and qualitative results, the production of such enzymes is probably accelerated in the post-surgical region of the experimental teeth. However, these data are from rats one week after surgery. We have seen that the surgical region of rats from group I may have a variable amount of enamel organic matrix.

The ameloblasts at the maturation stage change repetitively from ruffled-ended to smooth-ended cells and the enamel covered by these cells changes in pH from midly acidic, when related to the former type to near-physiologic when related to the latter type. The putative function of these cells is regulating the movement of calcium and others ions to allow surface crystal growth (1). The herewith results are indicating that the absence of most of the ameloblasts at the maturation stage does not interfere in the influx of minerals. However, the absence of these cells seems to interfere in the organization and growth of mineral crystals, which is reflected in the lower microhardness shown by the enamel present at the surgical region.

Figure 1

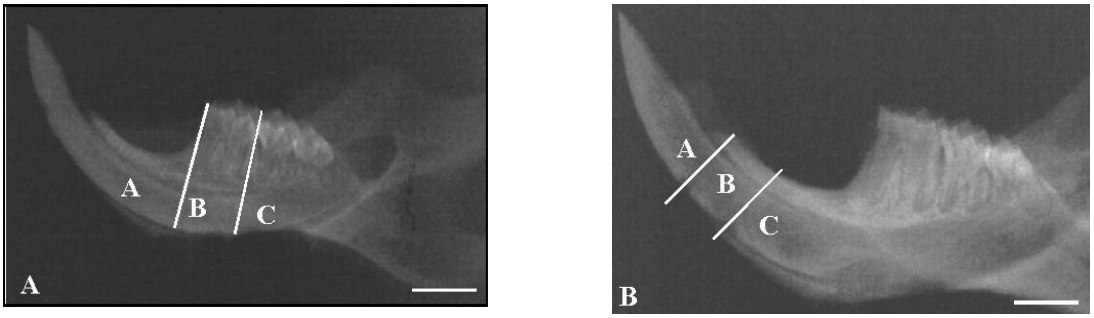
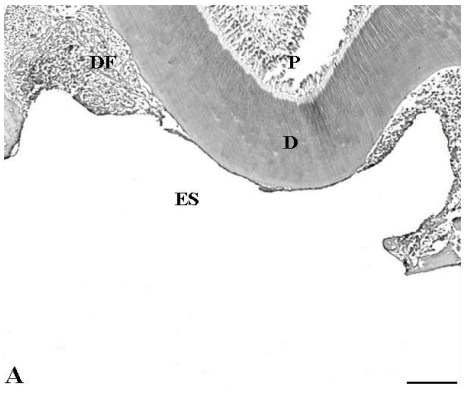
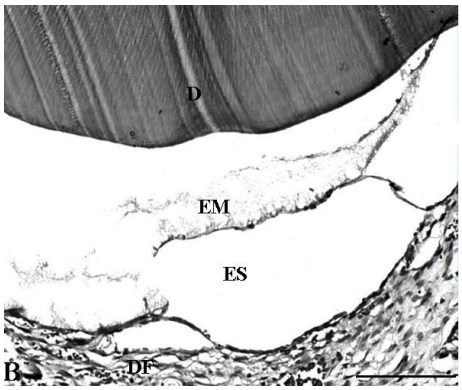
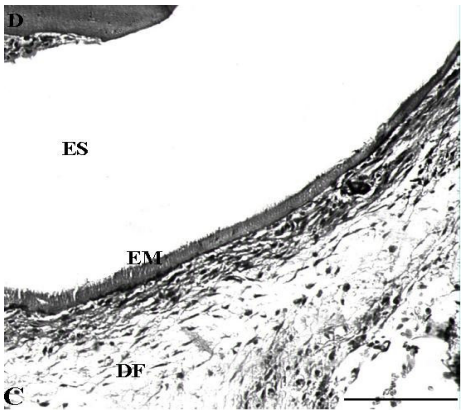
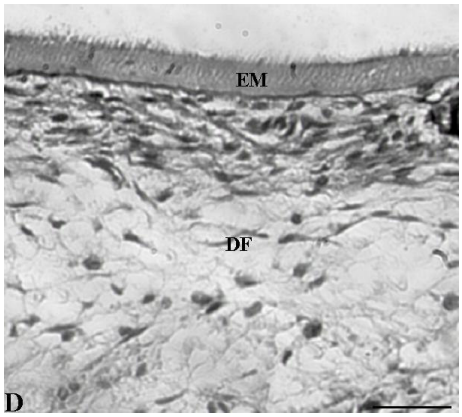


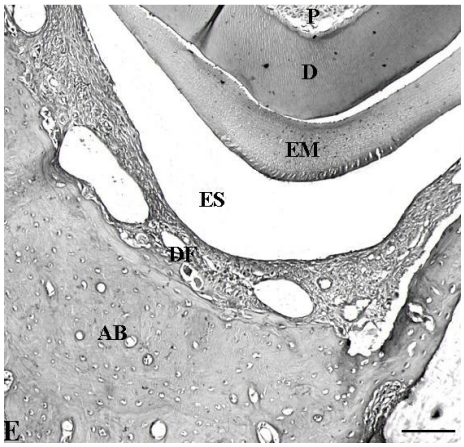
Figure 2











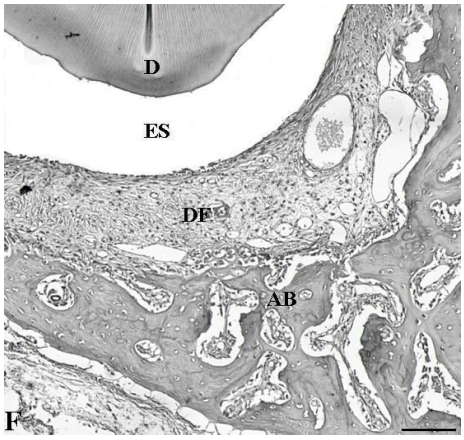


Figure 3

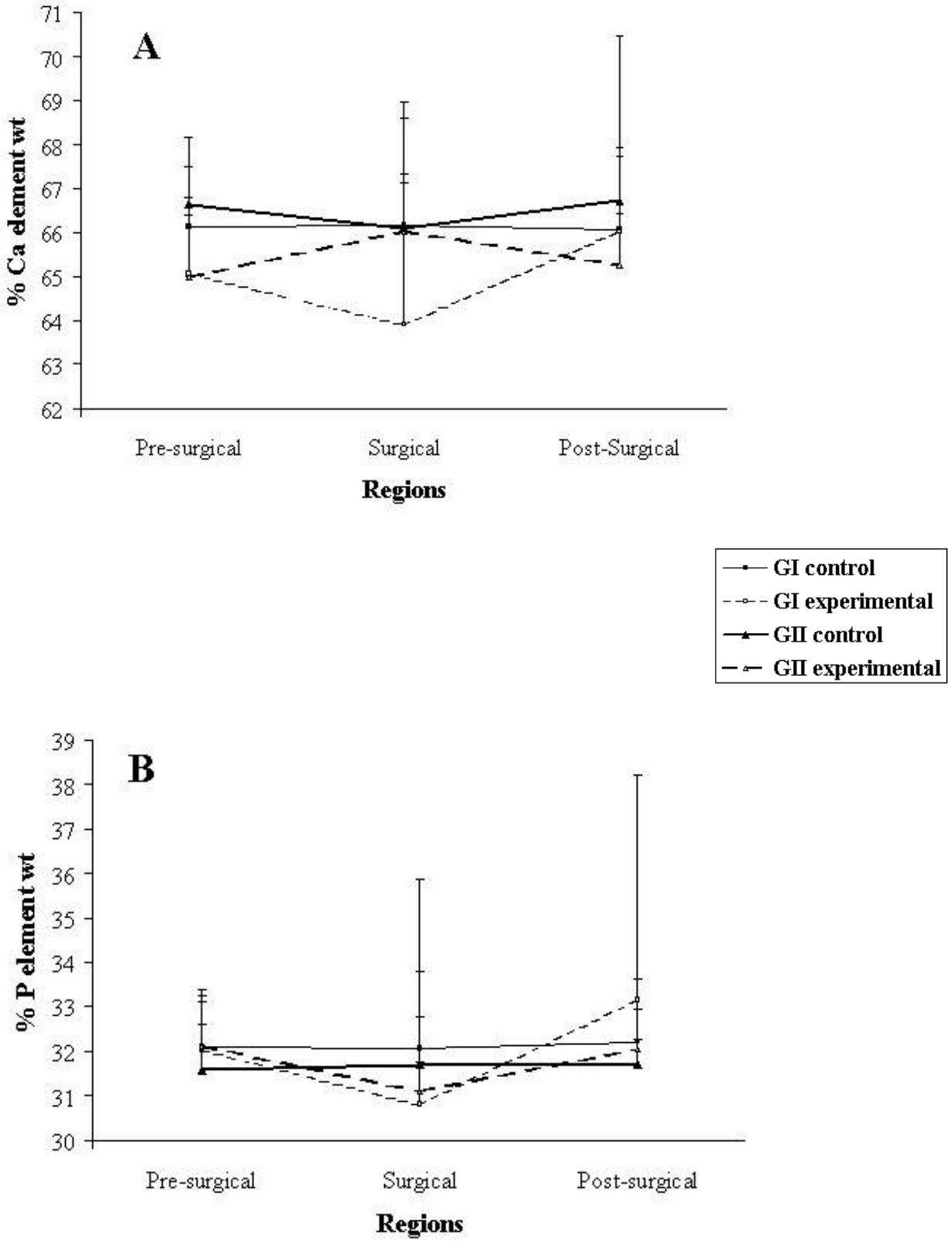

A
% Ca element wt
71
70
69
68
67
66
65
64
63
62
Pre-surgical
Surgical
Post-Surgical
Regions
GI control
GI experimental
GII control
GII experimental
B
% P element wt
39
38
37
36
35
34
33
32
31
30
Pre-surgical
Surgical
Post-surgical
Regions

Figure 4

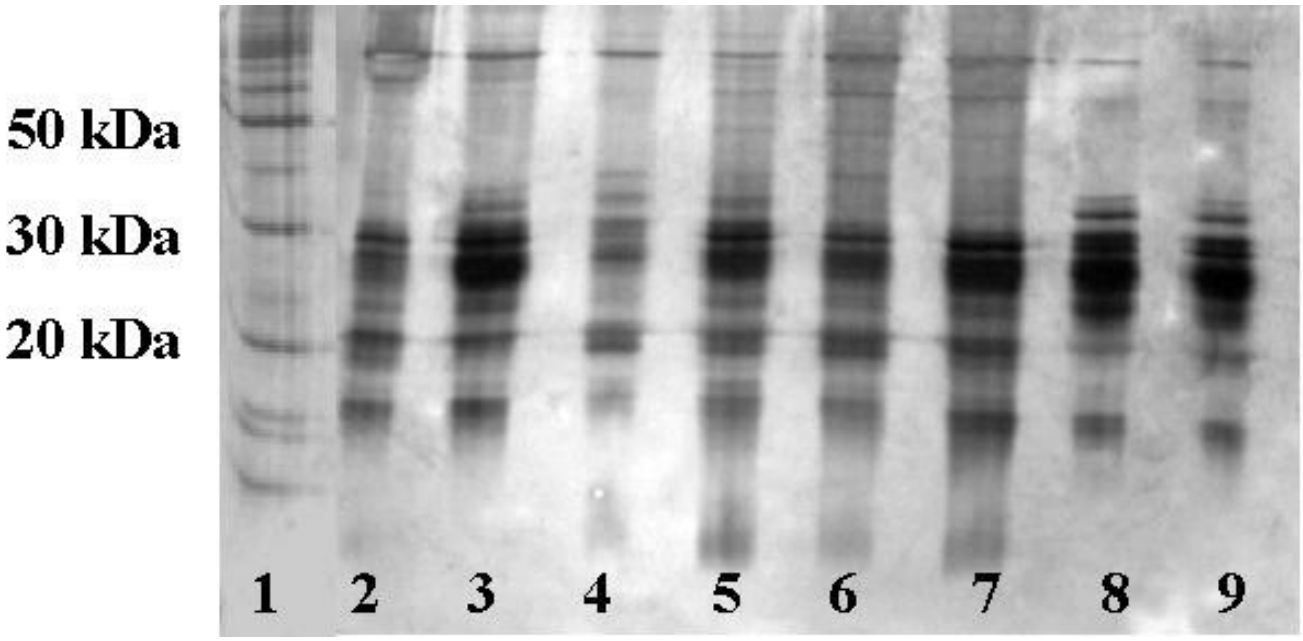
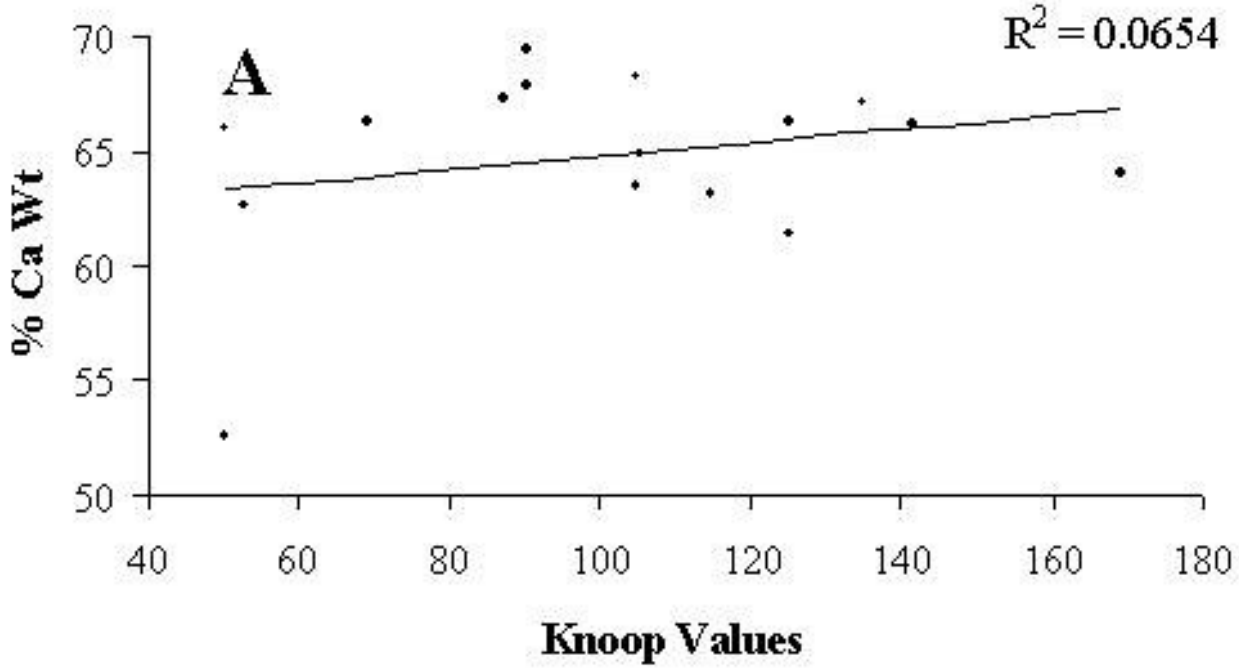
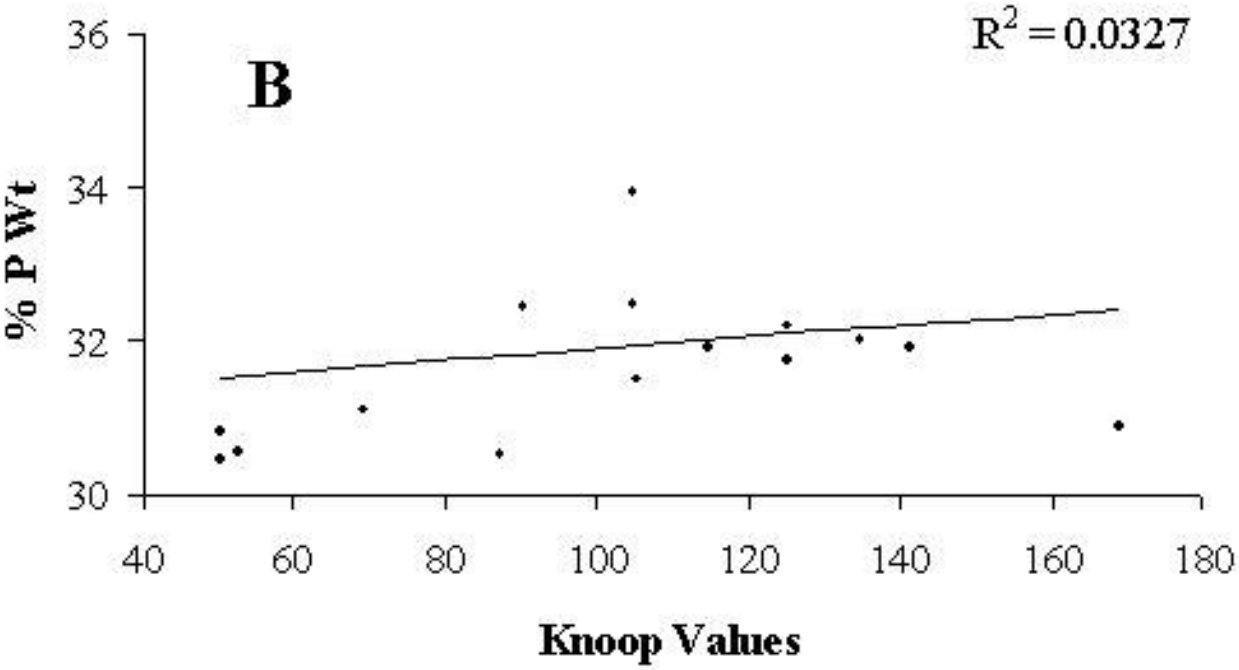


Figure 5


A
$R^2 = 0.0654$
% Ca Wt
70
65
60
55
50
40
60
80
100
120
140
160
180
Knoop Values


B
$R^2 = 0.0327$
% P Wt
36
34
32
30
40
60
80
100
120
140
160
180
Knoop Values

Legend to Figures

Fig. 1. Radiographs of left incisor showing the notches made in the enamel dividing the tooth in three regions: A) one day after surgery. Bar: 5 cm; B) 30 days after surgery when the first notch appeared at the clinical crown. Bar: 7 cm. The notches limited the regions: pre-surgical (A); Surgical (B); Post-surgical (C).

Fig. 2. Cross sections of the surgical region of experimental rat left incisors stained with Haematoxylin and Eosin. AB – alveolar bone, DF – dental follicle, EM – enamel matrix, ES – enamel space, D –dentine, P – pulp. A) One day after surgery: at the incisal end of the region all enamel was removed by demineralization. B) Seven days after surgery: the dental follicle was completely regenerated and at the middle of the region, most of the enamel was removed by demineralization, but disorganized enamel matrix is seen. C) Twenty-one days after surgery: at the distal part of the region most of the enamel was removed, but a layer of enamel matrix is seen next to the dental follicle. D) Higher magnification of the enamel matrix at C. E and F) rats from group I; at the distal end of region shown in F, all enamel was removed by demineralization, while in E, a fair amount of enamel matrix is seen next to dentine. Bar: 100µm in A, B, C, E and F. Bar: 30µm in D.

Fig. 3. A) Calcium weight and B) phosphorus weight percentages in enamel of three regions of experimental and control rat incisors. There are no differences between groups and regions.

Fig. 4. Silver-stained 15% SDS-PAGE gel of enamel matrix proteins. Note that the band pattern of proteins of both groups is similar in the surgical region (lanes 2 and 3, experimental group; lanes 4 and 5, control group), showing a molecular mass between 30 and 15 kDa. In the post-surgical region of the experimental group (lanes 6 and 7) most proteins are smaller than 30 kDa, while in the control group (lanes 8 and 9) most proteins show a molecular mass around 30 kDa and a distinct band appears between 40 and 30 kDa. Lane 1:Molecular weight markers.

Fig. 5. Correlation between calcium (A) and phosphorus (B) weight percentages with microhardness values (Table 1 and 2).

Table 1. Microhardness (mean knoop values ± SD) of the enamel in three regions of experimental and control rat incisors.

Region	Group	Experimental	Control
Pre-Surgical	I (n=14)	293.56 ± 49.85Aa*	309.25± 45.30Aa
	II (n=8)	342.80 ± 37.37Ab*	338.29 ± 24.61Aa
Surgical	I (n=12)	94.81 ± 29.98Ba**	299.22 ± 30.98Aa*
	II (n=10)	114.68 ± 39.22Ba**	340.17 ± 21.62Ab*
Post-Surgical	I (n=8)	280.74 ± 50.31Aa	262.38 ± 44.37Aa*
	II (n=7)	309.32 ± 47.66Aa	322.73 ± 33.28Ab*

For each group (see text) comparison between experimental and control incisors is indicated by capital letters and between groups of each region by small letters. Different letters indicate statistical significance: *$p<0.05$ and **$p<0.0001$.

Table 2. Mean ± SD of calcium and phosphorus weight percentages in enamel of three regions of experimental and control rat incisors.

		% Ca wt		% P wt	
Region	Group	Experimental	Control	Experimental	Control
Pre-Surgical	I (n=7)	65.08 ± 1.71	66.13± 1.38	32.04 ± 1.08	32.09 ± 1.30
	II (n=7)	64.99 ± 1.39	66.64± 1.50	32.12 ± 1.12	31.59 ± 1.02
Surgical	I (n=9)	63.90 ± 5.08	66.15 ± 2.45	30.80 ± 7.07	32.07 ± 1.71
	II (n=7)	66.01 ± 1.31	66.11 ± 1.70	31.08 ± 0.67	31.70 ± 1.07
Post-Surgical	I (n=8)	66.02 ± 1.93	66.08 ± 4.39	33.15± 5.03	32.21 ± 1.39
	II (n=7)	65.25 ± 1.17	66.72 ± 1.18	32.03± 0.93	31.72 ± 0.54

Comparison between regions and groups did not show statistical differences.

Table 3. Protein content (mean µg protein/mg enamel matrix ± SD) of the enamel in two regions of experimental and control rat incisors, one week later of the surgery.

Region	Experimental	Control
Surgical (n=8)	94.82 ± 84.30Aa*	112.10 ± 30.04Aa**
Post-Surgical (n=10)	135.46 ± 87.67Ba*	333.67 ± 90.22Ab**

Comparison between experimental and control incisors are indicated by capital letters and between regions by small letters. Different letters indicate statistical significance: *p<0.05 and ** p < 0,001.

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We would like to acknowledge the help we received from the staff and the permission to use equipments from the Biochemistry Laboratory - FOP/UNICAMP. We are grateful for the technical assistance of Mrs Maria Aparecida Santiago Varella and Mrs Eliene Orsini Navraes Romani from the Histology Laboratory – FOP/UNICAMP. This study was supported by FAPESP, grants 03/05452-5 and 03/13340-2.

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3. CONCLUSÃO

Os resultados deste estudo permitem concluir que:

1. Os ameloblastos do estágio de maturação não interferem no influxo de minerais.

2. A ausência dos ameloblastos parece interferir na organização e crescimento dos cristais minerais, o que é evidenciado pelo baixo valor da microdureza mostrado pelo esmalte presente na região cirúrgica do grupo experimental.

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5. APÊNDICES

Editorial Manager(tm) for Archives of Oral Biology
Manuscript Draft

Manuscript Number:

Title: Enamel mineralization after partial removal of post-secretory ameloblasts in the rat lower incisors

Article Type: Original Paper

Section/Category:

Keywords:

Corresponding Author: Dr Isabel Maria Porto, DD

Corresponding Author's Institution: FOP/UNICAMP

First Author: Isabel Maria Porto, DD

Order of Authors: Isabel Maria Porto, DD; Jose Merzel, DD, MS, PhD; Raquel Fernanda Gerlach, DD, MS, PhD

Manuscript Region of Origin:

Abstract:

COMPARISON OF THREE METHODS FOR ENAMEL PROTEIN EXTRACTION IN DIFFERENT DEVELOPMENTAL PHASES OF RAT LOWER INCISORS.

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Running Title: Protein extraction from rat incisor enamel

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PORTO IM, LINE SRP, LAURE HJ, GERLACH RF.

Comparison of three methods for enamel protein extraction in different developmental phases of rat lower incisors.

Eur J Oral Sci

Abstract: Protein extraction methods (urea, trichloroacetic acid (TCA), and acetic acid) were compared for protein recovery from rat incisor developing enamel in S phase (intermediate/final secretion), M1 phase (initial maturation), M2 phase (intermediate maturation) and M3 phase (final maturation). We compared the protein recoveries with the percentage of enamel matrix dry weight burnt off by incineration. Our results indicate that TCA and urea were equally efficient for extraction of S (85% and 90% recovery, respectively), while Urea was the best for M1 stage proteins (92% recovery), and TCA was the best for M2 (99% recovery) and M3 proteins (60% recovery). The other methods yielded less than 30% recovery in comparison to incineration for M2 and M3 stages. The fact that urea extraction works well in the S and M1 stages and not thereafter is probably related to the changes in the proteins during enamel development, and the amount of mineral that needs to be dissolved. TCA is the single method that effectively recovered proteins from all developmental stages of the rat incisor enamel. Supported by FAPESP, grants 03/05452-5 and 03/13340-2.

Key Words: enamel, protein extraction, maturation, rat, incisor

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Dental enamel is the hardest tissue found in mammals. By weight, the amount of enamel mineral content increases from about 36% in late secretory stage to 95% when the majority of proteins have been removed (1). Crystal growth during enamel maturation occurs at the expense of the organic matrix proteins and water (2). Proteolysis is responsible for the changes in the enamel matrix proteins (3), transforming enamel matrix proteins into small polypeptide fragments which are believed to leave the matrix as maturation of enamel proceeds (4). Proteolysis also changes the chemical character of the bulk organic matrix, which is essentially insoluble at the early stages of enamel development (5).

The rat incisor is by far the most studied model of enamel development, but its protein content has not been fully characterized yet, particularly regarding protein extraction procedures. The major obstacle in studying the proteins of mature enamel is their scarcity (6). Even though protein extraction procedures have been used in many studies on developing enamel proteins (6-11), a study specifically designed to compare extraction procedures for rat incisor enamel is lacking.

Therefore, the aim of the present study was to evaluate three different methods for protein extraction from different phases of rat lower incisor maturing enamel.

Material and methods:

The rat lower incisor of male Wistar rats (~250 g) was used, because it has a continuous growth, and, in the same tooth, several phases of developing enamel are present. The animals were treated in accordance with the international principles for the use of laboratory animals, and the protocol for this study was approved by University's Ethical Committee for Animal Research (CEEA-IB-UNICAMP, Protocol number: 556-1).

The mandibles were removed and the incisors carefully dissected out. A contiguous series of 2-mm enamel samples were then dissected from the late secretion stage to the maturation stage until the enamel became too hard to cut. The samples were designated S (late secretory stage), M1 (early maturation), M2 (intermediate maturation stage) and M3 (late maturation stage). To better characterize these stages, we determined

changes in water, organic content of dry weight and increase in inorganic content of dry weight of enamel samples according to the stages used in this study.

Developing enamel samples from each of these stages were placed in 1.5 ml microcentrifuge tubes as a pool of samples from two teeth per tube. The samples were weighed, lyophilized for 15 h and weighed again. Subtracting the pre-value from the post-value, the weight corresponding to water was obtained, as well as the dry weight of each sample. Some samples from each group were incinerated, so that the dry weight mass corresponding to organic matter (most of which proteins, burnt off by incineration) could be determined for each stage. These values were used later as a gold standard (mass burnt off = 100%) for comparison to the different protein extraction methods tested, so that an estimate of the recovery could be made.

A total of 38 samples from S phase were used, and 28 samples for each of phases M1, M2 and M3, randomly assigned to one of the following groups: incineration, 0.5 M acetic acid, 12% TCA, 6M urea.

Acetic acid

In this group, 1ml of 0.5 M acetic acid was added to each tube of lyophilized sample, containing 1 mM of the following protease inhibitors: PMSF (Phenylmethyl-sulphonylfluoride), NEM (N-ethylmaleimide) and phenanthroline (1,10-Phenanthroline). After matrix dissolution, samples were concentrated by centrifugal ultrafiltration (Amicon Microcon YM 3000, Millipore, Bedford, MA, USA), washed three times with water, using centrifugation at 4°C. The samples were then concentrated 5-fold using a vacuum concentrator (Concentrator 5301, Eppendorf, Hamburg, Germany).

Trichloroacetic acid

In this group, 198 µl 12% trichloroacetic acid (TCA) containing PMSF (1 mM), NEM (1 mM) and phenantroline (1 mM). were added to the lyophilized samples. One hour later, 2 µl of sodium deoxycolate (20 mg/ml) were added (12). The samples were stored overnight at 0°C, and then centrifuged at 4ºC at 2500 g for 45 min. The pellet was redissolved in 200 µl of 6M urea containing PMSF, NEM and phenanthroline (all at 1 mM).

Urea

In this group, 200 µl 6M urea containing PMSF, NEM and phenanthroline (all at 1 mM) were added to the lyophilized samples. The samples were stored overnight at 4°C, and then centrifuged at 1200 rpm for 5 min at room temperature. The supernatant was transferred to another tube and the pellet discarded.

After extractions, protein recovery was determined by the Coomassie Blue protein assay (13) (BioRad, Hercules, CA, USA) using microplates. The total amount of protein obtained from each sample was calculated by multiplying the amount of protein obtained by the total volume of extraction solution. This amount of protein was then used to calculate the percentage of mass representing proteins obtained by each method of extraction. To be able to compare the extraction methods, results of the protein recoveries obtained with the different extraction protocols were compared with the total amount of organic matter burnt off by incineration (675°C for 1 h).

To observe whether the different methods showed qualitative differences in extraction of proteins, 25 µg of protein from one sample of each group were used for separation by electrophoresis. Samples precipitated with TCA were neutralized with 1.0 M NaOH prior to electrophoresis. LAEMMLI's discontinuous buffer system SDS-PAGE was carried out in 15% slab gels (14). Samples were heated at 100ºC for 10 min prior to application. Electrophoresis was performed at 25 mA. After runs were completed, gels were fixed and stained with silver (15). Determination of sample protein molecular mass was possible by comparison with a molecular weight marker run together with the samples (Invitrogen, Carlsbad, CA, USA).

Statistical Analysis

All results are given as means ±SD. Analysis of differences in extraction efficiencies were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. A probability value of $p<0.05$ was considered the minimum level of statistical significance acceptable.

Results

Fig. 1 shows the changes in organic content of dry weight and increase in inorganic content of dry weight of maturing enamel samples according to the stages labelled S (late secretion), M1 (early maturation), M2 (maturation) and M3 (late maturation) used in this study. Fig. 2 indicates the steady decrease in organic content of enamel dry weight during enamel development.

Table 1 shows the results as percentage of recovery obtained with each extraction procedure in comparison to the amount of organic matter burnt off by incineration (most of which is protein), i.e. the mean of the incineration values obtained for each stage was considered 100% protein recovery. TCA and urea were equally efficient for extraction of S stage proteins (85% and 90% recovery, respectively), and were superior methods in comparison with acetic acid ($p<0.001$). Urea and TCA yielded M1 stage protein recoveries of 92% and 70%, respectively, which were statistically different from the M1 stage protein recovery obtained by the acetic acid protocol ($p<0.001$). Urea was most efficient than TCA for extraction of M1 stage proteins ($p<0.05$). For recovery of M2 stage proteins, TCA was found to be the most efficient method, yielding 99% recovery ($p<0.001$, TCA versus acetic acid and urea). TCA was also the best method to recover M3 stage proteins, with a yield of 60% (statistically not different from values obtained by incineration ($p>0.05$). Acetic acid and urea yielded less than 20% recoveries of M3 stage proteins in comparison to incineration (<0.001). Acetic acid dissolution of enamel matrix followed by filtration using Microcon devices was a poor method to recover enamel proteins from all developing stages.

Electrophoretic profiles of proteins obtained with the different methods revealed the bands of proteins expected and confirmed that the amount of protein revealed by protein determination was in accordance with the amount of protein observed in the gel (Fig. 3).

Discussion

Lyophilization followed by incineration of the samples made it possible to ascertain the stages of rat incisor developing enamel we were dealing with. In late secretory stage, the organic content found was 63.19%, reaching 7.23% in late maturation stage, in accordance with SMITH *et al*. (4). Mineral content increases gradually during amelogenesis, starting at 30% mineral (by weight) in early secretory stage, until enamel is fully mature, containing about 95% mineral (by weight).

Based on this study, TCA is the best method to recover proteins from all developmental stages found in rat incisors. Urea enables better protein recovery than TCA for M1 stage proteins, and was equal to TCA in recovery of S stage proteins. The high recoveries of early amelogenesis stage proteins obtained by urea indicate different behaviors of proteins found in the different stages. It is known that the protein composition changes as enamel matures. In earlier stages of enamel mineralization the more insoluble protein scaffold is composed of more intact proteins, most of which amelogenins, which are cleaved soon after their secretion into forming enamel (4). Amelogenin proteins are the most abundant proteins in early stages of amelogenesis (8). The full-length molecule of amelogenin was shown to bind hydroxyapatite through its anionic carboxy-terminal motif (17), which is cleaved off in the tissue, decreasing the binding affinity of the molecule for hydroxyapatite and the solubility of the protein. Thus, proteins with a highly hydrophobic character might predominate in samples from M1 used in this work, which were better recovered by a strong denaturing agent as urea. In latter stages of enamel development, though, as mineralization proceeds, smaller pieces of the major proteins appear, many of them more soluble, but possibly trapped as a bulk in the highly calcified enamel found in these latter stages. The only method that worked well to recover proteins found in the latter stages of amelogenesis was TCA, possibly because it simultaneously decalcified the matrix freeing any trapped proteins, and it also precipitated the extracted proteins, avoiding extra steps like filtration. Reducing the time during recovery of proteins is paramount, since proteinolysis can greatly decrease recoveries. Furthermore, avoiding transfer to other tubes or use of filtration devices decreases the chances that the proteins adhere to the tubes or

membranes. In this regard, TCA precipitation avoids the need to use dyalisis membranes or centrifugation devices to separate proteins from decalcification solutions in tubes. Therefore, it shall greatly facilitate recovery of proteins from maturation stage enamel. Finally, TCA is a less expensive, less laborious and more rapid method in comparison to descalcification/filtration for latter stages of amelogenesis.

The low protein recoveries obtained with all methods in M3 stage are probably related to the small amount of protein found in this stage and the fact that most enamel proteins are already processed into smaller polypeptide at this stage, which do not precipitate well and are also not retained by the filtration membrane (nominal cut-off of 3.000 kDa).

The low recoveries by acetic acid followed by filtration may be related to adherence of diluted protein solutions to plastics and membranes. Work with rat enamel almost inevitably results in diluted protein solutions, which are prone to significant protein losses due to adherence to charged surfaces. In spite of our results, a previous study (17) showed that when membranes with nominal cut-off of 4 kDa were used, the loss of neutral soluble proteins and peptides is less than 5% in all preparations.

In this study we showed that 12% TCA was the single method that effectively recovered proteins from all enamel developmental stages of the rat incisor, probably due to its ability to both precipitate proteins and dissolve mineral. This method is easy to perform and cheap in comparison to decalcification with acetic acid followed by filtration with microcons or dyalisis using membranes. TCA recovery of enamel proteins may be of great value in the study of rat enamel proteins, since this model offers small amounts of starting material.

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Figure 1

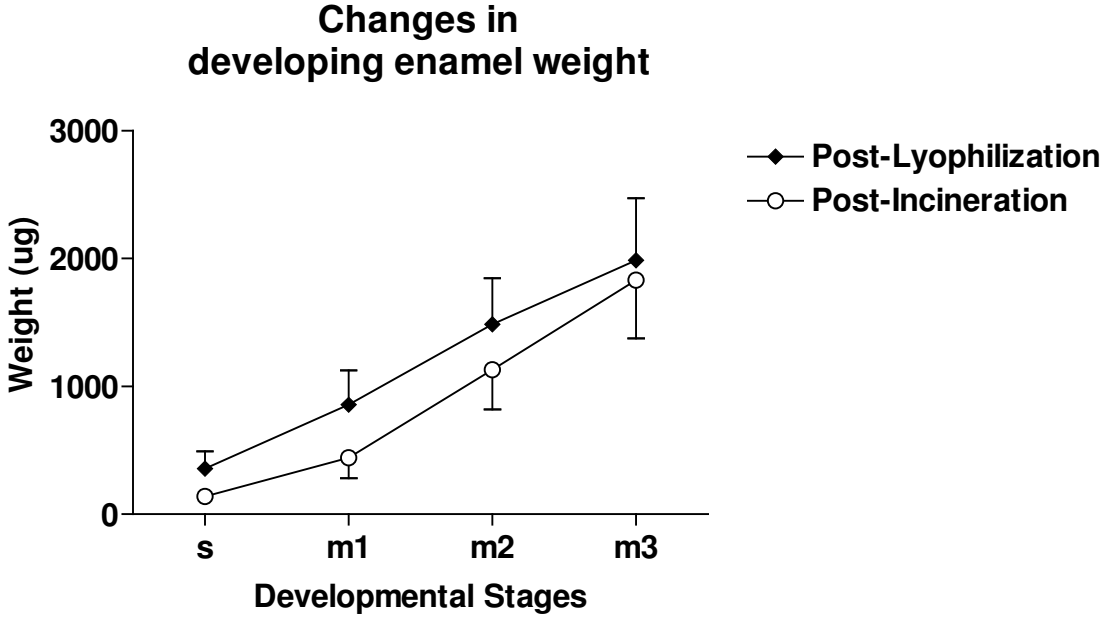


Figure 2

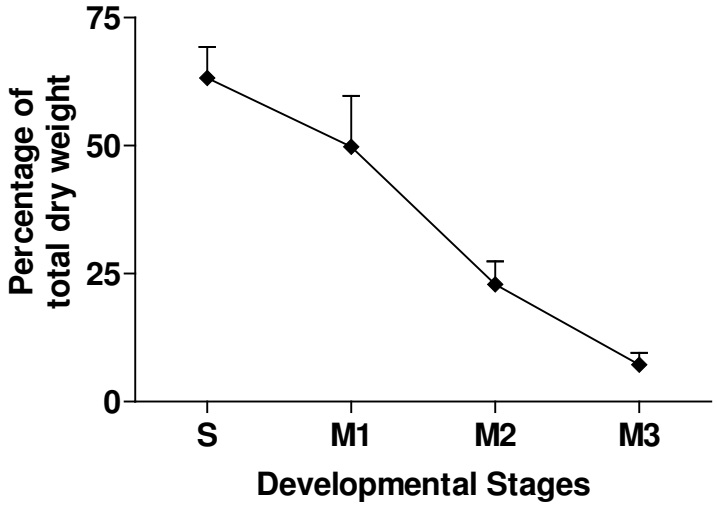
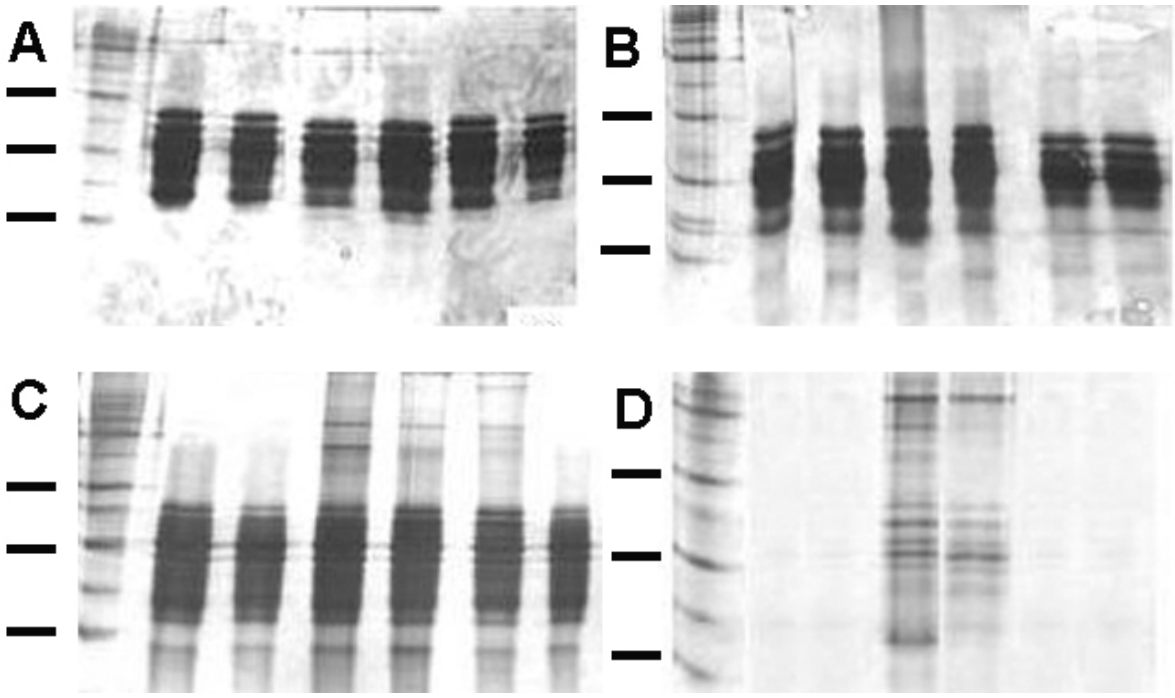


Figure 3



Legend to Figure 1

Fig. 1. Changes in developing enamel weight. Each sample corresponds to the pool of 4 lower incisors. Samples were weighed after lyophilization (♦) and incineration (o). Each point represents the mean and standard deviation of n=6, n=8, n=8 and n=5 in stages S, M1, M2, and M3, respectively.

Legend to Figure 2

Fig. 2. Percentage of developing enamel dry weight corresponding to organic components (mostly proteins) that were burnt off by incineration in different stages of enamel development.

Legend to Figure 3

Fig. 3. Silver-stained enamel matrix proteins run on 15% SDS-PAGE gels. **A:** intermediate/final secretion. **B:** initial maturation; **C:** mid of maturation; **D:** final maturation. Lane 1: molecular mass standard; lanes 2 and 3: extraction with 6M Urea; lanes 4 and 5: precipitation with 12% TCA; lanes 6 and 7: Acetic acid dissolution followed by filtration in Amicon Microcons. Molecular mass markers are indicated on the left, from top to bottom: 50 kDa, 30 kDa, and 20 kDa.

Table 1. Protein recovery percent from different enamel matrix developmental stages according to the extraction method tested.

	S	M1	M2	M3
Acetic Acid	8.18 ± 3.51 **a**	13.28 ± 6.65 **b**	25.20 ± 17.85 **e**	13.35 ± 35.39
	(n=7)	(n=10)	(n=13)	(n=8)
TCA	84.85 ± 17.71	70.11 ± 21.46 **c,d**	99.30 ± 19.82	60.43 ± 26.13
	(n=9)	(n=8)	(n=5)	(n=6)
Urea	89.97 ± 18.32	92.25 ± 12.38	26.92 ± 17.42 **f**	19.59 ± 35.08
	(n=7)	(n=8)	(n=6)	(n=7)
Incineration	100.00 ± 9.63	100.00 ± 19.98	100.00 ± 19.73	100.00 ± 32.07 **g**
	(n=8)	(n=10)	(n=13)	(n=8)

a *P<0.001* all *versus* acetic acid in S phase

b *P<0.001* all *versus* acetic acid in M1 phase

c *P<0.05* TCA *versus* urea in M1 phase

d *P<0.01* TCA *versus* incineration in M1 phase

e *P<0.001* Acetic Acid versus TCA and incineration in M2 phase

f *P<0.001* Urea versus TCA and incineration in M2 phase

g *P<0.001* Incineration versus acetic acid and urea in M3 phase

"ENAMEL VII" PROCEEDINGS IN EUR. J. ORAL SCI.

Manuscript No. EN7-038

"Comparison of Three Different Methods for Enamel Proteins Extraction in

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==========Additional comments from the editor-in-chief (see also Comments on the scanned MS) that have to be incorporated into the revised version:

1) My comments are obvious in the pdf file. In general, the English is a bit weak in this MS.

2) One major point concerns what is written on p. 8, namely that "urea gives higher protein recovery" but it is not statistically significant

3) Another major point is that they give the same data in Table 1 as in Figs. 3-6, so the Figs. must go.

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A receipt notification of this message will be greatly appreciated.

Hoping to hear from you soon,

Dr. D.M. Lyaruu,
Guest Editor,
Enamel 7 Proceedings

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