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Histomorphometric and Microchemical Characterization of Maturing Dental Enamel in Rats Fed a Boron-Deficient Diet

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Abstract Few reports are available in the literature on enamel formation under nutritional deficiencies. Thus, we performed a study to determine the effects of boron (B) deficiency on the maturing dental enamel, employing the rat continuously erupting incisor as the experimental model. Male Wistar rats, 21 days old, were used throughout. They were divided into two groups, each containing ten animals: +B (adequate; 3-mg B/kg diet) and −B (boron deficient; 0.07-mg B/kg diet). The animals were maintained on their respective diets for 14 days and then euthanized. The mandibles were resected, fixed, and processed for embedding in paraffin and/or methyl methacrylate. Oriented histological sections of the continuously erupting incisor were obtained at the level of the mesial root of the first molar, allowing access to the maturation zone of the developing enamel. Dietary treatment did not affect food intake and body weight. Histomorphometric evaluation using undecalcified sections showed a reduction in enamel thickness (hypoplasia), whereas microchemical characterization by energy-dispersive X-ray spectrometry did not reveal alterations in enamel mineralization.

Keywords Boron · Dental enamel · Histomorphometry · Hypoplasia

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Introduction

Dental enamel is the most highly mineralized and hardest tissue covering the crowns of vertebrate teeth [1, 2]. Enamel formation (amelogenesis) is a complex process that involves the production of an extracellular organic matrix by ameloblasts. Matrix mineralization takes place almost immediately, involving: (a) formation, nucleation, and elongation of apatite crystals and (b) removal of the organic matrix and crystal maturation [3–7]. It has long been recognized that continuously erupting rodent incisors have considerable potential to serve as a model system for amelogenesis [4, 7]. This process is generally subdivided into three main functional stages universally referred to as the presecretory, secretory, and maturation stages of amelogenesis [8]. In rat incisors, it takes ameloblasts about 7.5 days to secrete the enamel layer and another 12–14 days for the enamel crystals to mature [7, 9].

Few reports are available in the literature on enamel formation under nutritional deficiencies. Vitamin A, zinc (Zn), and calcium (Ca) deficiencies have been studied, employing the rat as the experimental model [10–15]. Other minerals present in the diet such as boron (B) have received less attention [16]. Recent studies [17, 18] demonstrated the nutritional relevance of B in different physiological processes such as the formation and maintenance of mineralized structures like cartilage and bone [18–26]. However, its effects on other mineralized structures such as dental enamel are unknown. Within this context, the aim of the present study was to determine whether nutritional B deficiency affects the formation of dental enamel, employing the rat continuously erupting incisor as the experimental model. In particular, the specific aims were to evaluate maturing dental enamel histomorphometrically and by energy-dispersive X-ray spectrometry (EDS).

Materials and Methods

Male Wistar rats (International Laboratory Code Registry: Hsd:Wi-ffyb), 21 days old, were used throughout. They were housed in stainless steel cages and maintained on a 12:12 hour light–dark cycle. All animal experiments were carried out according to the guidelines of the National Institutes of Health for the care and use of laboratory animals (NIH Publication no. 85-23, Rev. 1985).

Experimental Procedure

On weaning, the animals were divided in two groups, each containing ten animals: +B (adequate; 3 mg B/kg diet) and −B (boron-deficient; 0.07 mg B/kg diet; Table 1). Feeding 3 mg B/kg to the +B group was considered nutritional because it was four times less than the 12 mg B/kg found in commercially prepared rodent diet [27]. Based on experiments with chicks [28] and rats [22, 25, 29], 3 mg B/kg diet was considered adequate to prevent boron deficiency signs. Fresh powder diet and deionized water in plastic cups were provided ad libitum. Food intake and body weight were determined.

The animals were maintained on their respective diets for 14 days and then euthanized. The mandibles were resected, fixed in 10% formalin solution, and radiographed.

Histologic Processing

The mandibles were processed for embedding in paraffin and/or methyl methacrylate and sectioned, at the level of the mesial root of the first molar, in a frontal plane (Figs. 1 and 2),

Table 1 Composition of the Basal Diet

Ingredient	g/kg
Ground corn, acid-washed	713.486
Casein, vitamin-free	160.000
Safflower oil	75.000
Tert-butylhydroquinone	0.014
dl-α-Tocopherol	0.200
Choline chloride	1.000
L-Cysteine	2.000
Vitamin mix[a]	4.000
Macromineral mix[b]	29.300
Trace mineral mix[c]	15.000
Total	1,000.0

Analyzed concentration of boron was about 0.07 mg (9 μmol) per kilogram. To make a 3 mg boron per kilogram diet, a mix containing 0.0172 g H_3BO_3 and 0.9828 g dextrose replaced 1.0 g of ground corn in the basal diet

[a] Composition of the vitamin mix (in milligram): vitamin A palmitate (500,000 IU/g), 16; thiamine HCL, 10; pyridoxine HCl, 15; nicotinic acid, 30; *dl*-pantothenic acid, 48; vitamin B_{12} (0.1% in mannitol), 50; folic acid, 2; biotin,1; riboflavin, 27; vitamin K (phylloquinone), 1; inositol, 50; para-aminobenzoic acid, 5; vitamin D_3 (400,000 IU/g), 2.5; and dextrose, 3,742.5

[b] Composition of the macromineral mix (in gram): $CaHPO_4$, 17.0; KCl, 7.0; and $Mg(C_2H_3O_2)_24H_2O$, 5.3

[c] Composition of the trace element mix (in milligram): NaCl, 2,000; $Mn(C_2H_3O_2)_24H_2O$, 45; $CuSO_45H_2O$, 30; $Zn(C_2H_3O_2)_22H_2O$, 84; iron powder (dissolved in HCl), 75; $NaHAs_47H_2O$, 5; KI, 0.4; $NaSeO_35H_2O$, 1.4; $Cr(C_2H_3O_2)_32H_2O$, 2; NH_4VO_3, 0.3; $(NH_4)_2MoO_4$, 1; NaF, 2; NiCl6H_2O, 3.7; $NaSiO_29H_2O$, 50; and ground corn (acid-washed), 12,700.2

allowing access to the maturation zone of the developing enamel, employing a method for locating specific stages of amelogenesis in mandibular rat incisors described by Smith and Nanci [4].

Histological Evaluation

The hemimandibles were decalcified in 10% EDTA and embedded in paraffin. Oriented histological sections (10-μm thickness) were stained with hematoxylin–eosin for histological evaluation by light microscopy.

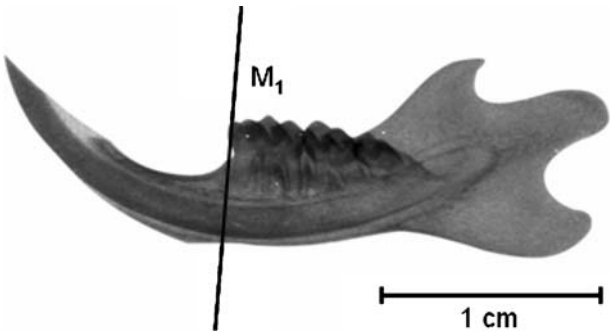

Fig. 1 Radiograph of left hemimandible. The *vertical line* shows a reference line extending from the mesial side of the first molar (M_1) perpendicular to the labial surface of the incisor

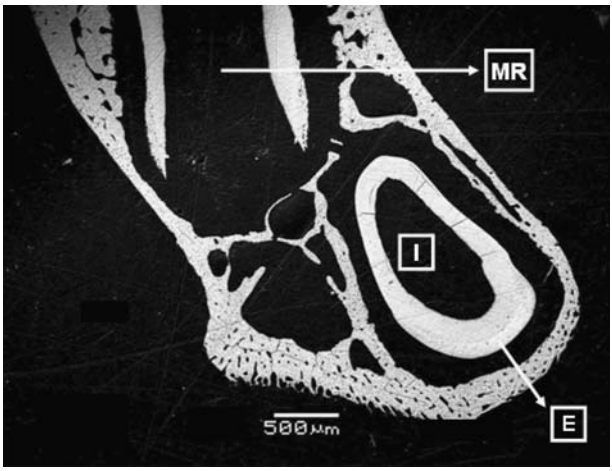

Fig. 2 SEM of an undecalcified section of rat hemimandible at the level of the mesial root of the first molar (*MR*), showing the maturation zone of the developing enamel (*E*) in the continuously erupting incisor (*I*); original magnification ×27

Histomorphometric Evaluation

The hemimandibles were stained following Frost's bulk-staining technique [30, 31]. In brief, the specimens were immersed in 20 mL of 1% basic fuchsin in absolute ethanol. The basic fuchsin solution was changed after 8 h to eliminate the water from the specimen. Twelve hours after this, the mandibles were placed in the basic fuchsin solution in a watch glass and allowed to evaporate until dry (about 48 h). The specimens were then rehydrated during 4 days in deionized water. The undecalcified mandibles were processed for embedding in methyl methacrylate resin. The samples were then sectioned manually with a saw (Eclipse 32 TPI, Spear & Jackson, England) to obtain 500-μm slices. Ground sections, ~50 μm in thickness, were obtained by reducing the slices with equipment for polishing optical lenses (Silmar Productos Ópticos-Argentina), followed by wet sandpapering AX-51 (Abrasivos Argentinos SAIC) with glycerin for adequate superficial finishing.

The ground sections were imaged using a confocal laser scanning (Nikon D-Eclipse C1) in the fluorescence mode equipped with a HeNe laser and a Nikon Eclipse E800 microscope. Ground sections were examined using 544-nm wavelength excitation and a long-pass 570-nm emission filter. The laser was fixed to an output of 100%. A line average of four was applied when collecting images to reduce noise. Sequential optical sections at an interval of 5 μm per section were collected using a ×20 (AN0.40) objective to build a 15-μm-thick stack image. Images from the sample were collected uniformly at a resolution of 512-by-512 pixels. Images were acquired at an 8-bit resolution (0–256 gray levels) and were projected in a single image using Nikon EZ-C1 Image Analyzer software (Silver Version 3.0).

The histomorphometric assessment of enamel thickness was performed on five zones (E) shown in Fig. 3. The distance (μm) from the dentinoenamel junction to the apices of ameloblasts was determined.

Microchemical Analysis

The undecalcified sections were carbon-coated in a carbon evaporating unit (CAR 001-0045) and analyzed by scanning electron microscopy (JEOL model JSM 6480 LV), coupled to an EDS (Thermo electron, model NORAM System SIX NSS-100) to determine the elemental composition of the sample qualitatively/quantitatively. The percent atomic content of calcium

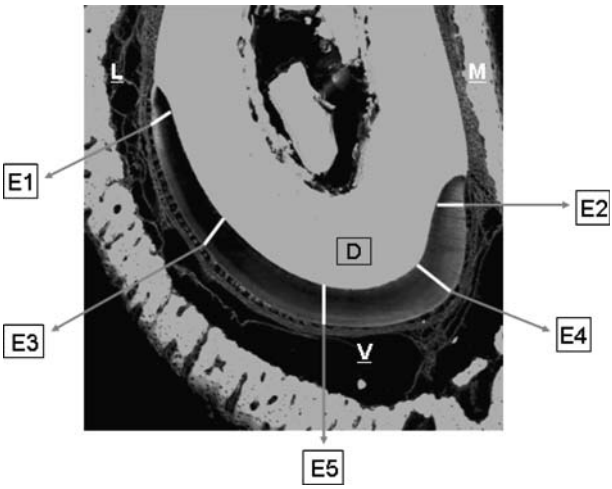

Fig. 3 Stack of images of an oriented ground section showing the five zones (*E1–E5*) selected for histomorphometric evaluation of enamel thickness. Original magnification ×10. *D* dentine, *M* mesial, *L* lateral, and *V* vestibular

(Ca) and phosphorous (P) was determined at different points of the enamel as shown in Fig. 4. The Ca/P ratio was estimated for each site.

Statistical Analysis

The data were reported as mean ± standard deviation and submitted to statistical analysis by Student's *t* test. Statistical significance was set at α=0.05 and β=0.01.

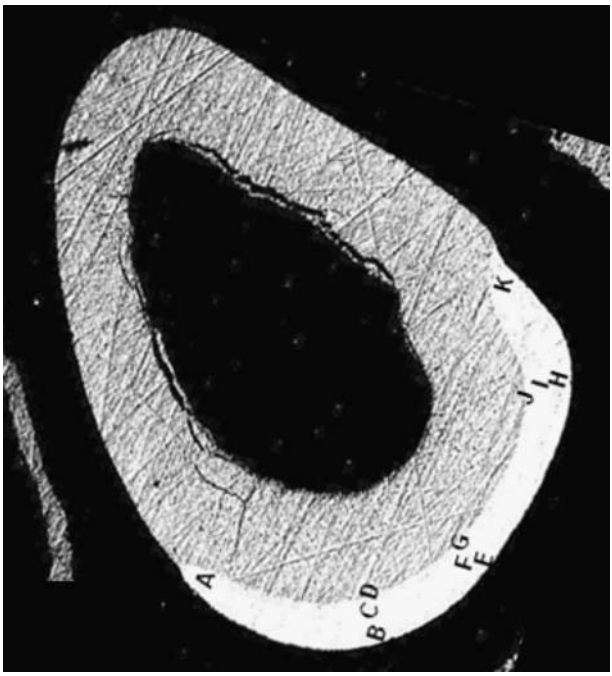

Fig. 4 SEM of transverse section of rat mandibular incisor, showing the different points of the enamel (*A–K*) considered for the microchemical analysis by EDS; original magnification ×30

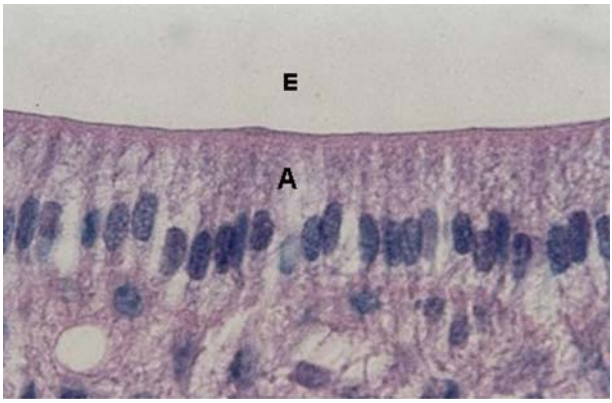

Fig. 5 Microphotograph of the frontal section of the enamel of rat mandibular incisor. Note the enamel space (*E*) lined by mature ameloblasts (*A*; hematoxylin–eosin stain; original magnification ×1,000)

Results

Body Weight and Food Intake

No statistically significant differences were observed in food intake between groups (results not shown). The final body weight was similar in both +B and −B groups (131±5 vs. 131±9 g, respectively).

Histological Evaluation

Both groups exhibited a palisade of ameloblasts in direct relation with the negative image of the enamel (Fig. 5). Their characteristics were those described for the maturation stage, i.e., tall, columnar cells, with a vacuolized cytoplasm above the nucleus. The prominent, oval, basophilic nucleus was located in the distal pole [3, 6, 32, 33].

Histomorphometric Evaluation

The bulk-staining specimens in alcohol-soluble basic fuchsin in combination with confocal laser scanning microscopy allowed rapid nondestructive optical serial sectioning of thick ground undecalcified sections providing optimally thin optical sections useful to perform histomorphometric analysis of enamel thickness. Histomorphometry found a 25±3% reduction in enamel thickness in zones E1 and E2 in group −B compared to group +B. Although not statistically significant, enamel thickness was lower at zones E3, E4, and E5 in the animals that were fed a B-deficient diet (−B group) compared with controls (+B group). Histomorphometric data on enamel thickness are presented in Table 2.

Table 2 Histomorphometric Evaluation of Enamel Thickness (μm)

	E1	E2	E3	E4	E5
+B	64±10*	77±14*	108±4**	129±11**	117±9**
−B	49±2	56±6	107±9	121±6	110±12

*$P<0.05$; **$P>0.05$ (values are means ± SD)

Microchemical Analysis

EDS analysis of the maturing dental enamel revealed that the contents of Ca and P for the −B group did not differ significantly from the values seen for the +B group (data not shown). The spectra obtained by EDS are shown in Fig. 6. The values corresponding to the Ca/P ratio for the different sites of analysis are presented in Fig. 7. No statistically significant differences were observed in homologous localizations between groups ($P > 0.05$).

Discussion

The present study evaluated the effects of nutritional boron (B) deficiency on the maturing dental enamel, employing the continuously erupting incisor of the rat as an experimental

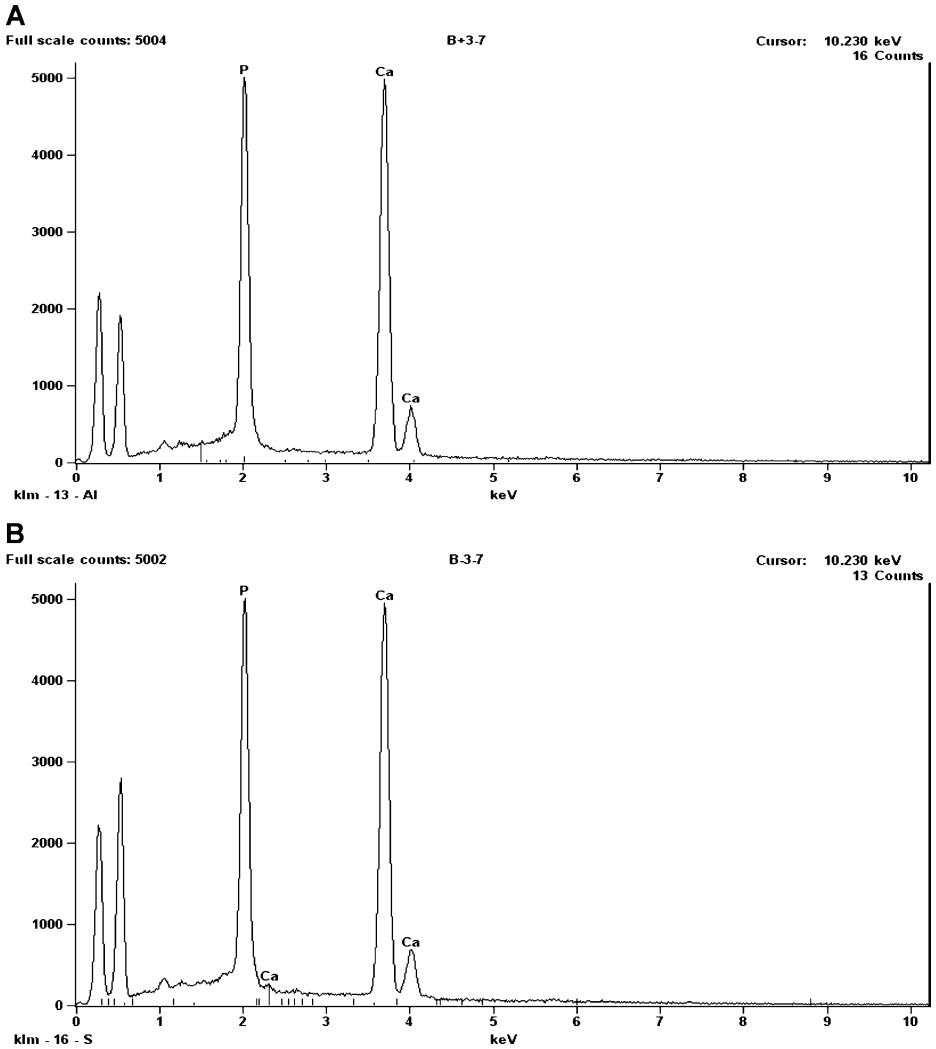

Fig. 6 EDS spectra of maturing dental enamel in +B (**a**) and −B animals (**b**)

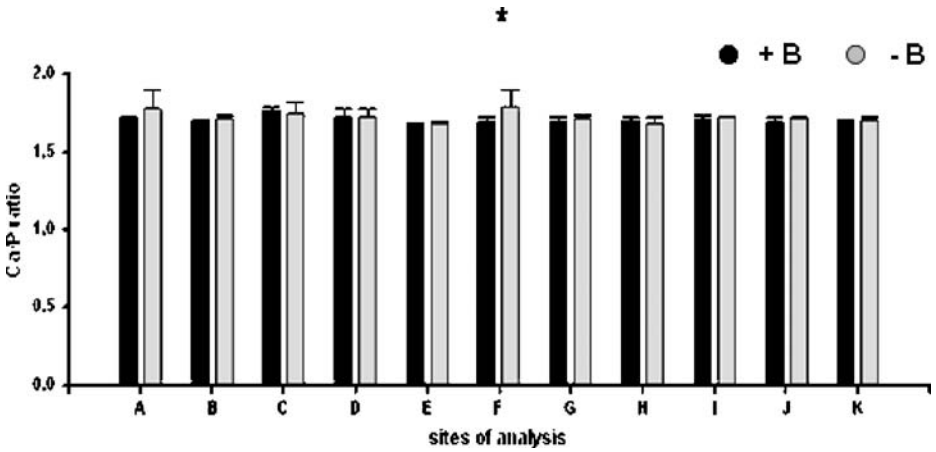

Fig. 7 Ca/P ratio for the different sites of analysis. Note that no statistically significant differences were observed in homologous localizations between groups (*P>0.05)

model. The histomorphometric evaluation showed a reduction in enamel thickness (hypoplasia), whereas microchemical characterization by EDS did not reveal alterations in enamel mineralization.

Hypoplasia is a disorder characterized by the malformation of enamel matrix. The main alteration involves a reduction in matrix thickness, leading to changes in dental contour. Thus, teeth acquire a different shape and become more sensitive to caries and dentine hypersensitivity [34–36].

The effect of B on the development of dental enamel of the rat ever-growing incisor has been studied by Wessinger and Weinmann [16], who reported that a single subcutaneous dose of B (>200 mg/kg) does not elicit enamel hypoplasia.

In the present study, the animals that were fed a diet with sufficient B (3 mg B/kg) for 14 days did not exhibit enamel hypoplasia, whereas the animals that were fed a B-deficient diet exhibited a reduction in enamel thickness. It is noteworthy that the B-deficient regimen was not long enough to detect a statistically significant difference at all enamel zones evaluated. Probably, rats exposed to B deficiency during lactation and/or a longer period of B deprivation postweaning may have resulted in a significant finding.

Boron nutritional deficiency did not elicit alterations in developing dental enamel mineralization in the present study. Not finding a difference in the calcium (Ca) and phosphorous (P) content of the enamel is not surprising because B deficiency has only small effects on the Ca and P content of other mineralized structures such as bone, even after long-term boron deprivation [22]. Our results from the analysis of enamel Ca/P ratio by EDS were consistent with those of Sasaki et al. [37] in continuously erupting rat incisor enamel (1.52±0.01) and with the data of Arnold and Gaengler [38] in mature enamel (2±0.15) of developing human teeth obtained from fetuses at 16 weeks of gestation.

The presence of B in dental enamel of primary and permanent human teeth was reported by Torrisi et al. [39] and Shashikiran et al. [40]. Data were extremely variable, conceivably because the samples came from different geographical locations (Italy, India), and/or different analytical techniques were employed to determine B content. Torrisi et al. [39] reported values of 32–36 and 25–28 μg/g of the isotope ^{11}B in human dental enamel of primary and permanent teeth, respectively, whereas Shashikiran et al. [40] reported values

of 4±0.50 and 5.50±0.30 μg/g of B in primary and permanent teeth, respectively, by atomic absorption spectrometry, a less sensitive method of B analysis.

Reports discussing the role of B in dental enamel are controversial. Losee and Ludwig [41], Curzon et al. [42], and Curzon [43] suggested a cariostatic role for B based on less incidence of caries in areas with abundant B in drinking water and food. However, Liu [44] found that B did not reduce enamel caries activity in an experimental rat model. The lack of an effect may be related to Liu determining the effect of B supplementation on postdevelopmental molar caries activity in B-adequate rats. Liu [44] also found that B administered in combination with fluoride (F) in the drinking water had a partially antagonistic effect on the cariostatic action of F. The antagonism may be the result of the formation of the anionic complex BF_4, which was suggested to be the mechanism through which B counters F toxicity [45].

With the method employed in the present study, the results suggest that nutritional B deficiency would affect ameloblasts during the secretory stage when final enamel thickness is determined [6]. Within this context, our findings contribute to the knowledge of the effects of B in the pre-eruptive stage of teeth. Future studies are warranted to study the response of ameloblasts to nutritional B deficiency in rats at a cellular and molecular level.

In conclusion, the results of the present study provide evidence, for the first time, that boron nutritional deficiency affects amelogenesis in rats, leading to a reduction in enamel thickness (hypoplasia) without altering enamel mineralization.

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