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Morphological characterization of rat incisor fluorotic lesions

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ABSTRACT

The morphological characterization of fluorotic rat incisor enamel was carried out. Experimental adult animals received drinking water with 45 mg F/L of fluoride, and the control group received distilled water. Fluoride concentrations found in the control and fluorosis groups were 0.04 and 0.09 μg/mL (plasma), 0.26 and 0.66 μg/mg (whole tibia), and 0.24 and 2.3 μg/mg (tibia surface), with $P \leq 0.001$ for all comparisons between the groups. A succession of white and pigmented bands was observed in the fluorotic rat incisors. Under polarizing light microscopy, cross-sections of superficial areas corresponding to the white bands (from the surface to ~20 μm) showed high positive birefringence. These fluorotic lesions also exhibited the lowest resistance to superficial acid etching. No morphological differences in inner enamel were seen under scanning electron microscopy. In fluorotic enamel, only the surface layer related to the white areas presented lower birefringence compared with the enamel of control teeth and the surface layer of the pigmented areas (normal ones) of fluorotic teeth. In conclusion, the white bands of fluorotic rat enamel represent hypomineralized superficial areas and are not subsurface lesions. The detailed description of these lesions is important to understand dental fluorosis.

1. Introduction

Enamel fluorosis is a consequence of impaired maturation of the dental enamel by systemic fluoride, causing a poorly mineralized tissue with normal thickness.[1] Although several studies have described retention of proteins in fluorotic enamel,[2–5] the majority of these reports have employed rat enamel in the early maturation stage.

A recent review on dental fluorosis[6] includes the following hypotheses for dental fluorosis: (i) alteration in the conformation or aggregation of enamel matrix proteins, (ii) binding of fluoride ion to enamel matrix proteins and proteases, (iii) alteration in protein–crystal interaction, (iv) different processing or lifetime of proteases *in situ* and (v) alteration of proteolytic activities sensitive to calcium levels. In addition, fluoride-induced early mineralization of the enamel surface

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layer, which might impede complete removal of organic matter from the inner layers, has been suggested as a contributing factor[7] to fluorosis.

Both human and rat fluorotic enamel lesions have been histologically described as a subsurface hypomineralized area underneath a relatively well-mineralized surface layer; i.e., subsurface lesions.[8,9] Such surface layer appears radiopaque in a microradiography, and negatively birefringent in water under examination by polarizing microscopy. The description of these lesions as being radiolucent inside and covered by normal enamel[9] is consistent with the hypothesis that fluorotic enamel lesions are caused by impaired removal of organic matter from inner enamel layers resulting from the early mineralization of the surface layer of the maturing enamel.

The rat incisor is widely employed for studies on amelogenesis, but few literature reports describe the histopathology of fluorotic enamel lesions in the rat incisor. Regarding the subsurface nature of the fluorotic lesion in the rat incisor enamel, published microradiographs[9,10] do not give unequivocal evidence of the presence of a relatively well-mineralized surface layer. While the presence of this layer would suggest that a physical barrier exists in the outer enamel that might impede the removal of organic matter from the inner enamel, the lack of such mineralized layer would indicate that this physical barrier does not exist. Therefore, the in-depth characterization of the mature fluorotic enamel may aid understanding of the mechanism underlying the development of fluorotic enamel defects, particularly in the rat incisor amelogenesis model.

Therefore, we have carried out the detailed morphological characterization of fluorotic rat incisor enamel by bright field, polarized light microscopy and scanning electron microscopy (secondary and backscattered electron modes).

2. Materials and methods

This study was approved by the University's Ethical Committee for Animal Research (CEEUA – USP, protocol number 01.1364.53.4). Forty one-month-old male *Wistar* rats were used and divided into two groups: the experimental group, which received 45 mg fluoride (100 mg sodium fluoride)/L distilled water for 60 days; and the control group, which received distilled water. Water and food were administered *ad libitum*. After 60 days, the rats were killed, and their upper incisors were removed for morphological analysis. The labial surface of both control and fluorotic enamel of incisors was photographed under low magnification prior to other analyses.

2.1. Determination of fluoride ion

2.1.1. Plasma

The blood of five control and five fluorotic rats was extracted from the jugular vein and transferred to a tube containing ethylenediamine tetraacetic acid (EDTA: Sigma Chemical Co., USA), to avoid blood coagulation. The blood was centrifuged for 10 min at 2000 × *g*, and 1 mL plasma was placed on Petri dishes (Falcon 1007), to which 2 mL deionized water was added. A 0.075N sodium hydroxide (NaOH, A.R.: Sigma Chemical Co., USA) trap solution (50 μL in five drops) was placed on the Petri dish lid, and after addition of 1 mL 3N sulphuric acid (H_2SO_4: Sigma Chemical Co., USA) saturated with hexamethyldisiloxane (HMDS), each dish was immediately sealed. During overnight diffusion, fluoride ion released by acid hydrolysis was collected in the NaOH trap. The trap was then recovered and buffered to pH 5.2 with 25 μL acetic acid 0.20N (CHCOOH: Sigma Chemical Co., USA), and the recovered solution was adjusted to a final volume of 100 μL with deionized water. The fluoride concentration was determined by means of a specific electrode (Orion Research Inc., Model 96-09, Boston, USA) coupled with an ion analyzer (Orion Research Inc., Model EA 940, Boston, USA). Standard solutions (Orion #940907) in triplicates, at concentrations ranging from 0.031 to 0.5 μg F/mL, were also acid-diffused. Blanks were subjected to the same procedure.

2.1.2. Bone

The femurs of five control and five fluorotic rats were dried for 24 h at 90 °C. Then, the femurs were transversally sectioned, leading to two slices measuring approximately 5 mm from the mid-diaphysial region of each bone.[11] One slice was dissolved in acid, to obtain the whole bone fluoride concentration; the other one was used to evaluate fluoride concentration on the bone surface. For whole bone determinations, the femur slices were left at 90 °C for 24 h.[11] The slices were weighed and transferred to plastic test tubes, to which 15.55N nitric acid (HNO_3: Sigma Chemical Co., USA) was added in a 0.1 mL acid/mg bone ratio. After 24 h at room temperature, the acid was neutralized with 15.55N NaOH, and the mixture was buffered with TISAB II. The extracted fluoride was determined as described above, on the basis of a fluoride standard curve built with fluoride standard solutions (0.05–0.5 μg F/mL) prepared in the same way as the samples. The results are expressed in μg F/mg bone.

To determine fluoride concentration on the bone surface, a circular hole (2.0 mm diameter) was punched in an adhesive tape, which was applied to the surface of the bone slice.[11] A window of 3.14 mm^2 area was exposed, and 5 μL 1.6N hydrochloric acid (HCl: Sigma Chemical Co., USA) in 70% glycerol was applied for 1 min, under agitation. The extract was transferred to a tube containing 190 μL deionized water. This etching step was repeated, and the second 5 μL etching solution was directly added to the tube. In the end, 5 μL of a 70% glycerol solution was applied to the window for 30 s and added to the same tube. The extracts were neutralized with 0.105 mL 0.228N NaOH, and buffered with TISAB II. The extracted fluoride was determined as described above. The amount of dissolved bone was inferred from the amount of P present in the acid extracts, assuming that its concentration in the bone is 13.5%. The P contents of the samples were determined colorimetrically.[12] Fluoride concentrations in the bone are expressed as μg F/mg bone.

2.2. Preparation of hard tissue sections of incisors

Analyses of enamel in hand-ground longitudinal sections (100 μm thick) of five rat incisors in a pilot study showed that the thickness of the enamel varied from 100 μm to more than 250 μm in animals of the same age group. The variability in the