Exposure to lead exacerbates dental fluorosis

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1. Introduction

Fluoride plays a key role in the prevention and control of dental caries. To date, no major adverse health effects have been ascribed to this substance when small fluoride doses are taken into account, so mild to moderate dental fluorosis is normally considered to be just a cosmetic problem. Dental enamel fluorosis lesions are areas of hypomineralized enamel formed pre-eruptively during the maturation stage of enamel formation.1 Excess fluoride has been shown to result in retention of amelogenin proteins during early maturation.2 However, fluoride is not the only agent leading to enamel defects. In fact, such defects can be caused by a variety of factors that adversely affect amelogenesis, probably through different mechanisms. Since amelogenesis is one of the longest formative processes taking place in our body,3 it can be influenced by a number of factors. Some of the most common causative agents of enamel defects are dioxins,4 fever, and vitamin A deficiency.5 Amoxicillin has been recently suggested to increase the prevalence of dental fluorosis, 6 indicating that larger occurrence of enamel defects may indeed be due to the synergistic action of various factors. Since enamel mineralization is reduced when enamel proteinases are not active,7 and bearing in mind that fluoride diminishes kalikrein 4 (a protease that plays a part in enamel maturation)
transcription, other substances that inhibit these enzymes could disturb proper enamel formation. Examples of such substances are lead and cadmium. Nevertheless, in vivo lead only delays amelogenesis; the final physical aspects of enamel are normal. It is conceivable that fluorotic lesions might be worsened in the presence of other substances, even when these substances alone would not give rise to enamel defects.

It has been recently described that children living in fluoridated communities are at higher risk of presenting blood lead levels (BLL) above 10 μg/dL, which was the limit defined by the Centre for Disease Control and Prevention in 1991 as the concentration that should prompt public health actions. The CDC later recognized that 10 μg/dL did not define a threshold for the harmful effects of lead, and therefore any factors that might increase the exposure of children to lead need to be investigated.

Animals co-exposed to lead and fluoride exhibited 3.4 times more lead in the whole blood, and 3.1 times more lead in the bone compared with animals exposed to lead alone, with no changes in the concentrations of fluoride in calcified tissues.

Since lead has been demonstrated to inhibit enamel proteins in vitro and has also been shown to delay amelogenesis in rodents, we hypothesized that lead might worsen dental fluorosis in rodents.

2. Material and methods

2.1. Animals

This study was approved by the Ethical Committee for Use of Animals in Research of the University of São Paulo/Ribeirão Preto (Protocol 07.1.346.53.3). The sample is the same that was utilized in our previous publication, but here the focus was on the enamel defects. Twenty-eight Wistar rats (24 females and 4 males weighing 190–210 g) were randomly divided into four groups (each one containing 6 females and one male) from the beginning of gestation (mating began when the animals started to receive the different water treatments). Control animals received water with 0.1 ppm fluoride and 0.5 μg/L lead. Animals of the fluoride group (F) received water containing 100 ppm fluoride as H₂SiF₆ (fluorosilicic acid). Animals of the group exposed to lead (Pb) received 30 ppm lead as lead acetate (Pb(CH₃COO)₂·3H₂O) in the drinking water. Animals of the F + Pb group received water containing both 100 ppm fluoride and 30 ppm lead. The Pb dose was selected because they exhibited discontinuities even in control teeth, whilst lower teeth presented the same water regimen after weaning, and they were euthanized at 81 days. All the data presented here refers to these 81-day-old animals (n = 10 for each group). Femurs as well as the lower and upper incisors from female rats were collected postmortem and stored at –20°C, for fluoride analysis.

2.2. Tooth examination and fluorosis score assessment

Upper and lower incisors from ten animals of each group were employed in this study. After analysis of all the teeth under a stereomicroscope (Nikon Instruments Inc. NK-150) using a calibrated reticule in one of the eyepieces, it was found that fluorotic enamel presented a number of morphological features on the buccal surfaces that ranged from well defined white bands, separating the pigmented area into bands, to a number of discontinuities within pigmented bands. Standardized areas on the buccal surfaces of the upper and lower teeth were chosen for reliable recording of these characteristics.

Upper incisors presented ~12 mm of erupted enamel, whilst lower teeth presented ~9 mm. These extensions where divided into segments of 3 mm each along the long axis of the buccal surface. The more cervical segments were excluded because they exhibited discontinuities even in control teeth, making the diagnosis of fluorosis unreliable. Thus, starting from the incisal edge, 3 and 2 mm-segments were selected for analysis in the upper and lower incisors, respectively. To be able to quantify the different morphological aspects (bands, islets, and cavities), the following equation was formulated:

\[ F_{rat} = \frac{B + I + C}{A} \]  

where B is the number of 3 mm-long areas with alternating white and pigmented bands, I is the number of islets (small round white areas located within pigmented bands), and C is the number of cavities (cavities in enamel reaching dentine). A is the number of 3 mm-long areas along the long axis of the buccal surface.

Surface features (B, I, and C) of each tooth were recorded and included in Eq. (1). On the basis of the findings of the present study, a particular scoring system (Table 1) was formulated, to categorize each tooth. All the teeth were analysed under the previously calibrated stereomicroscope (magnification of 10× and calibrated reticule in one eyepiece) by two blinded examiners (intraexaminer and interexaminer kappa values were 0.8 and 0.86, respectively).

Table 1 – Fluorosis scores from the rodent fluorosis index.

<table>
<thead>
<tr>
<th>Score</th>
<th>Description*</th>
<th>Main feature</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>B/A &lt; 1, I/A &lt; 1, and C/A &lt; 1</td>
<td>Brown and white bands</td>
</tr>
<tr>
<td>2</td>
<td>B I/A = 1, I/A &lt; 1, and C/A &lt; 1</td>
<td>Brown and white bands and a few islets</td>
</tr>
<tr>
<td>3</td>
<td>B I/A = 1, I/A &lt; 1, and C/A &lt; 1</td>
<td>Brown and white bands and many islets</td>
</tr>
<tr>
<td>4</td>
<td>B I/A = 1, I/A &gt; 1, C/A = 1</td>
<td>Brown and white bands, many islets and occasional cavities</td>
</tr>
<tr>
<td>5</td>
<td>B I/A = 1, C/A &gt; 1</td>
<td>Brown and white bands and many cavities</td>
</tr>
</tbody>
</table>

* B = band; I = islet; C = cavity; A = area analysed.

2.3. **Microscopic analysis**

Hand-ground longitudinal enamel sections (100 μm thick) of three incisors from each score (scores 1–5) were prepared for microscopic analysis. Score 1 samples from both the control group and the Pb group were examined, since none of them exhibited fluorosis and both were assigned score 1. Preparation of the hand-ground incisor sections is critical for microscopic analysis, as shown by us before, and details how these sections were prepared can be found elsewhere. Longitudinal ground sections from the centre of the buccal surfaces were manually prepared using a lapping jig. The thickness of the samples (~80 μm) was measured to the nearest 2 μm with the sample positioned edge-on in a compound transmission light microscope equipped with an eyepiece containing a calibrated reticle. Qualitative analyses of the ground sections were performed by means of a polarizing light microscope equipped with a Red I filter under water immersion (after immersion in distilled water for 24 h), followed by analysis under immersion in Thoulet’s solution (solution of potassium iodide and mercurial iodide in water) with a refractive index of 1.62 (after immersion in Thoulet’s solution 1.62 for 48 h). The refractive indexes of the immersion solutions were determined in an Abbe refractometer. Representative pictures of the qualitative analyses were taken.

2.4. **Microradiography**

The same ground sections analysed under light microscopy were mounted on high definition photoplates (2000 lines/mm) and exposed to X-rays in a Faxitron MX20 machine operating at 30 kV and 0.3 mA for 90 min. Digital images of developed photoplates were obtained by a light microscopy in bright field for qualitative analyses.

2.5. **Fluoride analysis of calcified tissue samples**

Calcified tissue samples for fluoride analyses were obtained as previously described. One femur of each animal was totally dissolved in 6 mL of 65% HNO3 (ultrapure grade). This acid extract was utilized for fluoride and phosphate determination. Enamel samples were acquired using surface enamel etching with acid, a procedure performed in a 0.5 mL Eppendorf tube containing 300 μL HNO3 at 1.8% (v/v). The labial face of the incisal third of the lower incisor was maintained in contact with the acid for 20 s (the tube was inclined at 35'). A dentine fragment obtained from the lingual aspect of the incisor root was completely digested in 500 μL HNO3 at 50% (v/v). The mass of bone, dentine, and enamel of each acid extract was calculated on the basis of its phosphorus content. All the samples were assayed in triplicate. The mass (g) of enamel, dentine, and bone was determined assuming phosphorus contents of 17.0%, 15.97%, and 13.5% in enamel, dentine, and bone, respectively.

For fluoride analysis, 100 μL of the acid extract were mixed with 900 μL deionized water buffered with 100 μL TISAB II (1.0 M of acetate buffer, pH 5.0 with 1.0 M NaCl and 0.4% cyclohexanediiminetetraacetic acid). Fluoride was determined in an ion-specific electrode, calibrated with standard fluoride solutions (0.5–5.0 μg/mL).

2.6. **Lead analysis of blood and calcified tissues**

Whole blood and calcified tissues were collected for determination of Pb levels. Blood samples were withdrawn using metal-free syringes with lyophilized heparin. A detailed description of the applied technique can be found in our previous report. Pb levels were obtained as μg of Pb/dL of whole blood or as μg of Pb/g of calcified tissue.

2.7. **Statistical analysis**

Enamel, dentine, and bone lead and fluoride concentrations were compared by ANOVA followed by Bonferroni’s Multiple Comparison Test. Fluorosis scores were compared by Kruskal–Wallis test. Differences were considered statistically signifi-
cantly at $P < 0.0083$ (5% significance level divided by 6 comparisons).

3. Results

This study aimed to compare the enamel characteristics in the different groups. In order to do that, a fluorosis, or better, an enamel defect index comprising 5 categories of defects was proposed. Representative pictures of the 5 scores suggested for this index are shown in Fig. 1, and a detailed description of each score is displayed in Table 1.

From a histopathological viewpoint, all the normal and fluorotic teeth presented positive birefringence in water and negative birefringence in Thoulets 1.62. Sharp changes in enamel birefringence were detected with increasing fluorosis scores, and these alterations consisted of enhanced positive birefringence in water and decreased (less negative) negative birefringence in Thoulets 1.62. The most remarkable contrast between white and pigmented bands was found upon water immersion and with the target area at the position of maximum birefringence, using the Red I plate.

Normal enamel displayed low positive birefringence in water (Fig. 2a) and a homogeneous mineralization in the...
microradiograph (Fig. 3a). White bands exhibited higher positive birefringence, seen as blue bands (Fig. 2b), and lower radiopacity (Fig. 3b) compared with pigmented bands. Islets appeared as a band with slightly higher positive birefringence compared with normal enamel, presenting a pale blue colour, adjacent to bands with even higher positive birefringence (white bands) presenting vivid-blue colours (Fig. 2d), and as a band with low radiopacity adjacent to bands with an even lower radiopacity (thin arrow in Fig. 3d). Some teeth had somewhat long extensions along the main axis of the buccal surface without pigmented bands, where the superficial enamel layer uninterruptedly displayed higher positive birefringence with a vivid blue colour (Fig. 2c) and lower radiopacity (Fig. 3c) compared with normal enamel. Cavities

![Microradiographs of representative 100 μm midsagittal sections. A, enamel with score 1 (control group, Fig. 2A) showing uniform mineralization. B, enamel with score 2 (fluoride group, Fig. 2B) showing hypomineralization in the surface layer (arrows, white bands) intercalated with areas of higher mineralization (pigmented bands). C, enamel with score 4 (lead + fluoride, Fig. 2C) showing a lengthy hypomineralized surface layer. D, enamel with score 3 (lead + fluoride, Fig. 2D) showing an area with severe hypomineralization (heavy white arrow, white band), spreading from the surface layer to the enamel–dentine junction, adjacent to an area of higher mineralization (pigmented band) to the right and to another area with less severe hypomineralization (light white arrow) to the left. Bar = 100 mm.](image)

![Median box and whiskers plot showing median, 25th, 75th, maximum and minimum values for fluorosis index for upper (A) and lower (B) incisors. *P < 0.0001.](image)
with the bottom in dentine (enamel–dentine junction) were seen in some teeth, outlined by enamel with higher positive birefringence compared with normal enamel (Fig. 2e and f).

As illustrated in Fig. 3, control and Pb group animals did not display signs of fluorosis in their teeth (score 1). All the animals from the F or F + Pb groups, on the other hand, presented enamel with various degrees of defects (Fig. 4). Whilst the F group animals had the typical rodent florotic enamel appearance (scores 2–4), the animals exposed to F + Pb exhibited significantly higher degree of fluorosis as evidenced by the Enamel Defect Index proposed in this study ($P < 0.001$). The median of the F group animals was 2.0 (2.0; 3.0) (minimum; maximum) in upper incisors, and the F + Pb group animals furnished a median score of 3.25 (2.5; 4.5) ($P < 0.0001$). For the lower incisors, higher fluorosis scores were also obtained in the F + Pb group animals: the F-exposed animals presented a median of 2.0 (2.0; 4.0), whereas the F + Pb group animals had a median of 4.0 (2.5; 5.0) ($P < 0.0001$, Fig. 4).

### 4. Discussion

This study shows for the first time that the fluoride effects on enamel formation can be altered by the co-exposure of rats to lead, resulting in worse enamel defects in both lower and upper incisors. Data on F and Pb tissue levels have been reported previously, and it was demonstrated that: (i) animals from F and F + Pb groups exhibited increased concentrations of fluoride in calcified tissues compared with the control and Pb groups, in all analysed tissues ($P < 0.0001$) (Fig. 3 of Sawan et al., 2010$^{13}$); (ii) there were no differences between the F and F + Pb groups ($P > 0.1$) in terms of the concentrations of fluoride in whole bone, dentine, or enamel; and (iii) Pb levels in blood and calcified tissues were higher in the F + Pb group (blood Pb level of 76.7 ± 11 μg/dL) compared with the other groups (blood Pb level of 22.6 ± 8.5 μg/dL in the Pb group and below 5 μg/dL in the control and F groups) ($P < 0.001$) (Figs. 1 and 2 of Sawan et al., 2010$^{13}$).

The modified Fluorosis/Enamel Defects Index for rodent teeth employed here allowed for discrimination of a wider range of defects than that previously observed in rat fluorosis. White lines and white islets were defined as hypomineralization, as evidenced by the altered birefringence compared with normal enamel (Fig. 2e and f). This study suggests that the development of fluorosis may be susceptible not only to the influence of drugs$^{4,6,30}$ or genetic factors$^{24,31}$ but also to other inorganic compounds present in the environment, particularly lead.
Exacerbation of dental fluorosis by lead (in teeth with increased concentrations of lead but not fluoride) may be a useful morphological aspect for detection of populations at risk of higher exposure to lead. In recent years, there has been a rise in the prevalence of enamel fluorosis in the U.S.A. Therefore, investigations to observe whether increased prevalence of fluorosis is associated with elevated exposure to lead in the early childhood must be conducted. Perhaps, some contribution to this might be achieved by obtaining information on lead from superficial acid etch biopsies, which would be useful to identify children and areas with increased lead exposure. Fluoride and lead can be both determined in such superficial samples, and this 20 s etching procedure is not detrimental to the primary tooth enamel.

Our results may also be important to describe fluorosis in wildlife, since some species are exposed to large amounts of environmental lead. Fluorosis has been demonstrated in free-ranging deers in Europe, and the highly polluted regions from which some of the deer teeth were obtained (North Bohemia, Czech Republic) are areas in which some lead mining occurred.

In conclusion, our results suggest that lead may exacerbate dental fluorosis in rodents co-exposed to high concentrations of fluoride.

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Conflict of interest: There are no conflicts of interest in this study.

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