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Exposure to lead exacerbates dental fluorosis

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ABSTRACT

Aim: Our aim was to test the hypothesis that co-exposure to lead and fluoride alter the severity of enamel fluorosis.
Materials and methods: Wistar rats were allocated in four groups: control, and 3 groups that received water containing 100 ppm of fluoride (F), 30 ppm of lead (Pb), or 100 ppm of F and 30 ppm of Pb (F + Pb) from the beginning of gestation. Enamel analysis and F and Pb determinations in enamel, dentine, and bone were performed in 81-day-old animals. Fluorosis was quantified using a new fluorosis index based on the identification of incisor enamel defects (white bands and white islets, representing hypomineralization, and cavities) weighted according to their severity and quantity. Hypomineralization was validated histopathologically by polarizing microscopy and microradiography. Scores were given by two blinded calibrated examiners (intra and interexaminer kappa values were 0.8 and 0.86, respectively).
Results: The control and the Pb groups presented normal enamel. The F + Pb group presented more severe enamel defects compared with the F group ($P < 0.0001$).
Conclusions: This study shows that lead exacerbates dental fluorosis in rodents, suggesting that co-exposure to lead may affect the degree of fluorosis.

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)

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transcription,[8] other substances that inhibit these enzymes could disturb proper enamel formation. Examples of such substances are lead and cadmium.[9] Nevertheless, in vivo lead only delays amelogenesis; the final physical aspects of enamel are normal.[10] 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,[11] 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,[12] 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.[13] Since lead has been demonstrated to inhibit enamel proteinases *in vitro*[9] and has also been shown to delay amelogenesis in rodents,[10] 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,[13] 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 4 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 H_2SiF_6 (fluorosilicic acid). Animals of the group exposed to lead (Pb) received 30 ppm lead as lead acetate ($Pb(CH_3COO)_2{\cdot}3H_2O$) 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 on the basis of our group's previous studies on the exposure of rats to lead, and the concentration of lead determined in whole blood of the animals.[14] Water and food were provided *ad libitum*, and animals were maintained at 12 h/12 h light/dark cycles. Offspring were born 3–5 weeks after the beginning of the experiment. The young animals were kept under 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 *post-mortem* 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 3 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:

$$F_{rat} = \frac{B}{A} + \frac{I}{A} + \frac{C}{A} \quad (1)$$

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.

Score	Description[a]	Main feature
1	$B/A < 1$, $I/A < 1$, and $C/A < 1$	Brown and white bands
2	B $1/A = 1$, $I/A \leq 1$, and $C/A < 1$	Brown and white bands and a few islets
3	B $1/A = 1$, $I/A > 1$, and $C/A < 1$	Brown and white bands and many islets
4	B $1/A = 1$, $I/A > 1$, $C/A = 1$	Brown and white bands, many islets and occasional cavities
5	B $1/A = 1$, $C/A > 1$	Brown and white bands and many cavities

[a] B = band; I = islet; C = cavity; A = area analysed.

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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.[15] Longitudinal ground sections from the centre of the buccal surfaces were manually prepared using a lapping jip. 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.[13] One femur of each animal was totally dissolved in 6 mL of 65% HNO_3 (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 HNO_3 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 HNO_3 at 50% (v/v).

The mass of bone, dentine, and enamel of each acid extract was calculated on the basis of its phosphorus content.[16] 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,[17] dentine,[18] and bone,[19] 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% cyclohexanediaminetetraacetic acid).[19] 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.[13] 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-

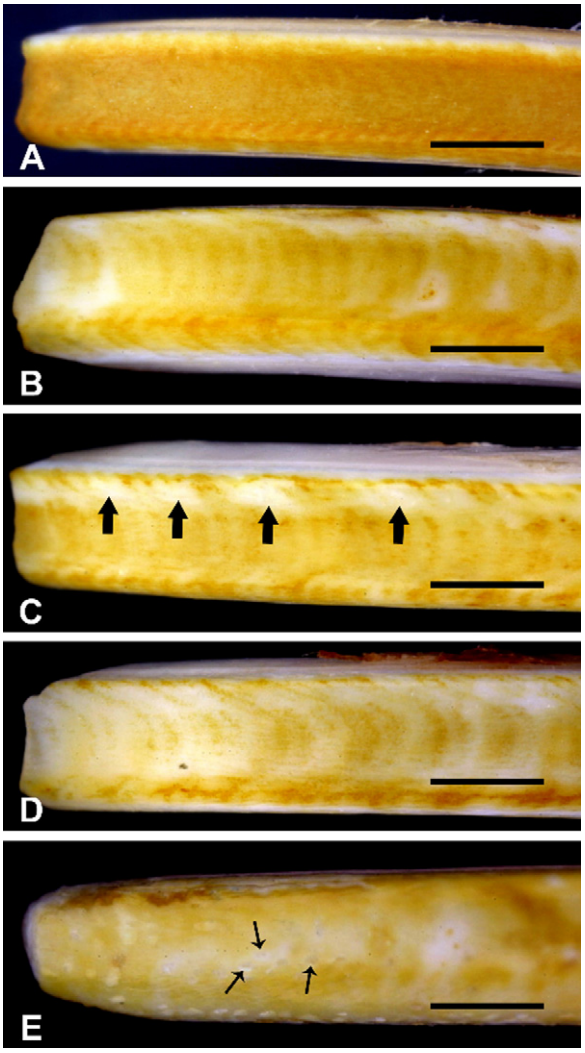

Fig. 1 – Incisor teeth illustrating the different scores used in this study. A, score 1: normal enamel without banding pattern; B, score 2: white and pigmented bands; C, score 3: white and pigmented bands with 2–5 white islets (islets are shown by the arrow heads); D, score 4: white and pigmented banding pattern still visible and more than 5 white islets; and E, score 5: diffuse large white areas with cavities (arrows) reaching dentine. Bar = 1 mm.

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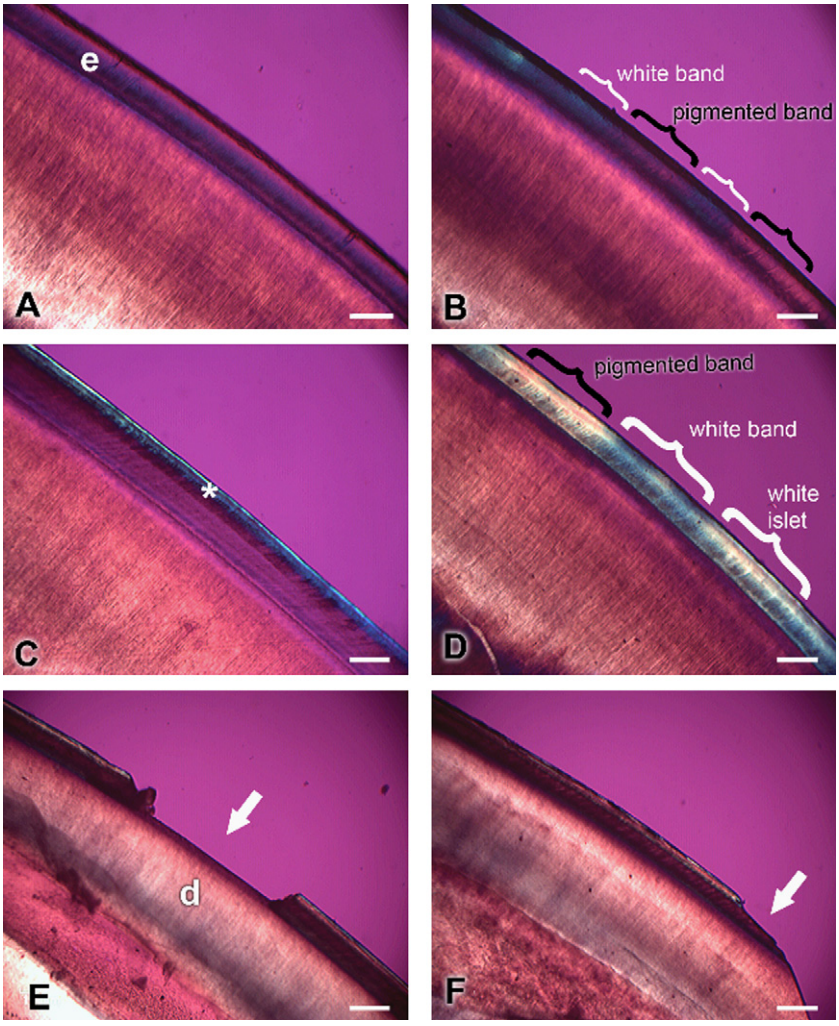

Fig. 2 – Representative 100 μm midsagital sections photographed in polarizing light microscopy under water immersion, at the position of maximum birefringence with Red I plate. E, enamel; D, dentine; bar = 100 μm. A, enamel with score 1 (control group) showing a low positive birefringence. B, enamel with score 2 (fluoride group) showing, from right to left, alternating white and pigmented bands: white band with a high positive birefringence (blue area, white bracket), and pigmented band with low birefringence (redish area, black bracket). C, enamel with score 4 (lead + fluoride group) showing an area covered by extensive white bands with high positive birefringence (vivid blue) in the outer layer. D, enamel with score 3 (lead + fluoride group) showing, from right to left: a pigmented band with low positive birefringence (red area), a white band with a high positive birefringence (vivid blue area) followed by an islet with a bit lower birefringence (light blue area) in the outer layer, and another white band with high birefringence (vivid blue area). E and F, enamel with score 5 (lead + fluoride group) showing a cavity reaching the dentine and adjacent to an enamel area with high positive birefringence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

cant 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 Thoulet́s 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 Thoulet́s 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

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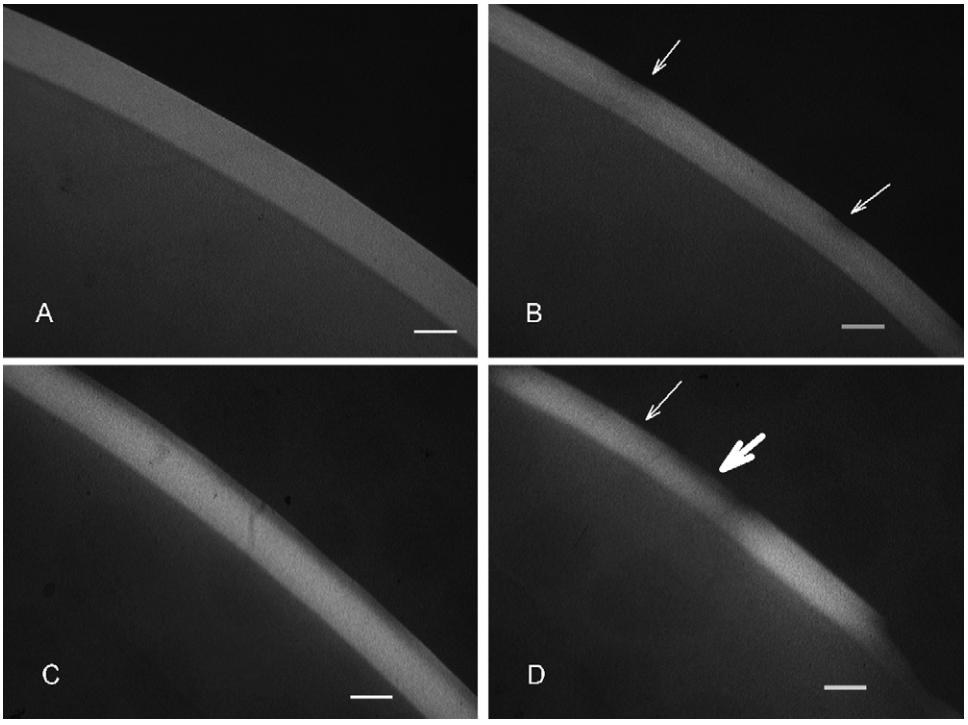

Fig. 3 – Microradiographs of representative 100 μm midsagital sections. A, enamel with score 1 (control group, Fig. 2A) showing uniform mineralization. B, enamel with score 2 (fluoride group, Fig. 2B) showing hypomineralization in the surface layer (arrows, white bands) intercalated with areas of higher 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.

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

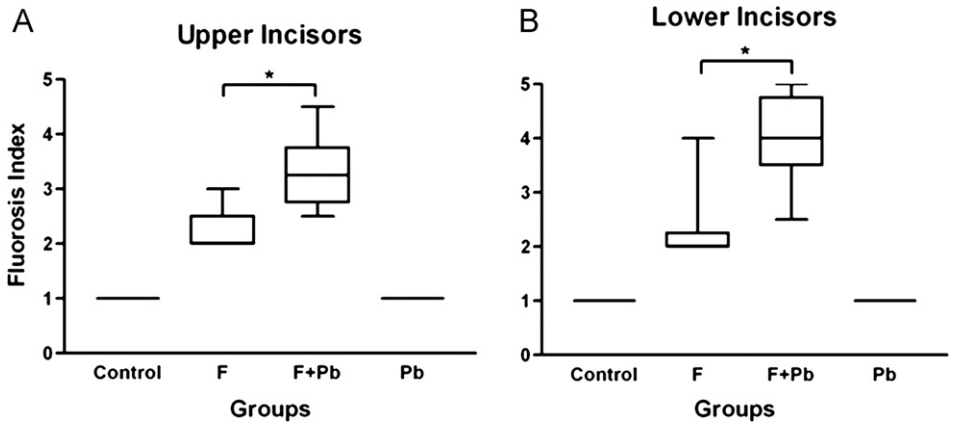

Fig. 4 – Median box and whiskers plot showing median, 25th, 75th, maximum and minimum values for fluorosis index for upper (A) and lower (B) incisors. *P < 0.0001.

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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 fluorotic 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,[13] 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.[15] White lines and white islets were defined as hypomineralization, as evidenced by the altered birefringence detected by means of polarizing microscopy, in agreement with a recent report,[15] and by the lower X-ray absorbance seen on microradiographs. These features, along with elevated fluoride content in mineralized tissues,[13] characterize enamel fluorosis. The higher lead levels in blood and calcified tissues observed in the F + Pb group compared with the other groups[13] indicate higher availability of lead and higher incorporation of this metal into tissues when it is associated with F.

Hypomineralization was shown starting from the very surface of enamel (i.e., no subsurface lesions), reflecting the condition of rat enamel during the final wave of mineralization at the maturation stage.[20]

The cavities have also been described in the case of hypomineralized mouse enamel formed in the absence of the gene for kallikrein 4.[21] The presence of cavities can be explained by the interaction between mechanical loading and the hypomineralized enamel. An improvement in motor activity in rats exposed to Pb[22] and the reduced enamel hardness resultant from hypomineralization[23] are consistent with a higher probability of brittle fracture and cavity formation in enamel. In this context, it is important to note that cavities were demonstrated to be surrounded by hypomineralized enamel (Fig. 2e–f).

In the literature, rodent enamel fluorosis has been scored by means of a macroscopically applied shade guide, so as to measure increasing whiteness of the incisor buccal surface.[24] In relation to ours, such scoring system, which was validated by quantitative light-induced fluorescence on the non-sectioned buccal surface, poses three major limitations: (i) it cannot be used to localize a single fluorotic lesion; (ii) the surface features are not related to inner histological ones, and (iii) the number of cavities is not taken into account. Spatially-resolved correlations between surface and internal enamel defects might be helpful for a deeper understanding of the mechanism of enamel fluorosis.

Rises in fluoride concentrations do not seem to be responsible for the appearance of the more severe defects in the F + Pb group, since no increased amounts of fluoride could be detected in the calcified tissues of the animals co-exposed to lead. Furthermore, fluorosis severity has been shown to be influenced by a variety of factors, such as the genetic background in rats.[24] The more severe defects observed in the F + Pb group would more likely be caused by an additive or synergistic effect of the co-exposure to fluoride and lead. Lead alone did not produce any alterations. Although it is known that lead concentration in calcified tissues is 2–3.4 times higher in the F + Pb group compared with the Pb group,[13] these concentrations still would not elicit enamel defects in the absence of fluoride. Lead given to rats at 34 and 170 ppm in the drinking water for 70 days did not modify the superficial physical properties of mature enamel,[10] even though enamel mineralization was delayed, and more protein was found in the secretory early maturation stage compared with controls.

Lead is recognized as being an inhibitor of enzymes, the most widely known example being the inhibition of delta-aminolevulinic acid dehydratase.[25] It has already been shown that lead inhibits enamel proteinases (including metalloproteinases) *in vitro*.[9] Impaired enamel maturation has been reported in MMP-20 (the metalloproteinase of enamel) null mice.[7] Fluoride, on the other hand, has been shown to decrease levels of kallikrein 4 in enamel organ cells,[8] to induce disturbance in the protein synthesis in ameloblastos,[26] to increase apoptosis in ameloblast-like cells,[27] and to reduce the number of lysosomes in ameloblasts.[28] Therefore, the more severe defects found in the group exposed to F + Pb may stem from the fact that impaired protein removal (a prerequisite for proper mineralization) during amelogenesis is caused by fluoride and lead.

The dose of 100 ppm fluoride has been used here because it is known that this fluoride dose results in fluorotic defects in rats. However, in rats this dose results in serum fluoride concentrations achieved in the case of humans consuming water containing 5–10 ppm fluoride.[29] Therefore, results cannot be directly transposed to humans.

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.

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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.[32] 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.[16,33] 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.[34]

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,[35] 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.[36]

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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