Abstract

Some evidence in vitro suggested that amoxicillin and fluoride could disturb the enamel mineralization. Objective: To assess the effect of amoxicillin and of the combination of amoxicillin and fluoride on enamel mineralization in rats. Methodology: In total, 40 rats were randomly assigned to four groups: control group (CG); amoxicillin group (AG - amoxicillin (500 mg/kg/day), fluoride group (FG - fluoridated water (100 ppm -221 mg F/L), and amoxicillin + fluoride group (AFG). After 60 days, the samples were collected from plasma and tibiae and analyzed for fluoride (F) concentration. The incisors were also collected to determine the severity of fluorosis using the Dental Fluorosis by Image Analysis (DFIA) software, concentration of F, measurements of enamel thickness, and hardness. The data were analyzed by ANOVA, Tukey’s post-hoc test, or Games-Howell post-hoc test (α=0.05). Results: Enamel thickness of the incisors did not differ statistically among the groups (p=0.228). Groups exposed to fluoride (AFG and FG) have higher F concentrations in plasma, bone and teeth than those not exposed to fluoride (CG and AG). The groups showed a similar behavior in the DFIA and hardness test, with the FG and AFG groups showing more severe fluorosis defects and significant lower hardness when compared with the AG and CG groups, with no difference from each other. Conclusion: The rats exposed to fluoride or fluoride + amoxicillin developed dental fluorosis, while exposure to amoxicillin alone did not lead to enamel defects.

Keywords: Dental fluorosis. Rats. Amoxicillin. Enamel defect.
Introduction

The dental enamel development, known as amelogenesis, is a dynamic process resulting from the interplay between the epithelial and mesenchymal tissues.1-2 Even though genetic factors3,4 may play an important role in this process, extracellular disorders may cause changes in dental enamel.5-6 Developmental defects of enamel have been associated with recurrent health problems and medications in the first years of life, especially with conditions that cause high-grade fever.6-8 Amoxicillin is the main antibiotic used to treat bacterial infections in children. Amoxicillin has been previously demonstrated to affect the enamel formation in animals.9-11 Thus, whether the damage is caused by the disorder itself or by the medication use is still unclear, as both occur simultaneously and in close association.

The excessive chronic intake of fluoride may also affect amelogenesis, causing dental fluorosis with variable intensity.12 Besides the time of exposure to fluoride, its concentration, and bioavailability in blood plasma,12,13 the intensity of fluorosis has been associated in humans14 and rodents15 with genetic polymorphism as well as with hormonal,16,17 and environmental18 factors in humans. Animal studies suggest that enamel defects in rats exposed to a combination of fluorine and bisphenol-A were more severe than exposure to either agent alone.19 Exposure to lead also appears to exacerbate fluorotic lesions in rats.20 On the other hand, vitamin D and calcium associated with fluoride seem to protect against more severe forms of fluorosis in humans.21

Children chronically exposed to high dose of fluoride at the age between one and three years seem to be more prone to develop fluorosis.5,12 The mechanisms that clearly underlie this susceptibility remain unknown, but the increased risk of developing fluorosis was observed when fluoride intake was associated with treatments that used amoxicillin, especially in patients aged two years or less.22,23

Most experimental in vivo studies show dental effects in rodents exposed to amoxicillin.24-27 Some of them suggest amoxicillin can affect amelogenesis when administered in the prenatal period.11,26,29 An in vitro experiment showed that amoxicillin in combination with sodium fluoride can cause changes in amelogenesis.28 However, the effect of combining fluoride and amoxicillin on amelogenesis has not been assessed in controlled studies in vivo. Therefore, this in vivo study aimed to investigate the effect of amoxicillin and its combination with fluoride on amelogenesis in rats.

Methodology

Study groups and experimental design

The study was approved by the Animal Research Ethics Committee of Araraquara Dental School (CEUA-FOAr process no. 22/2012).

For estimation of the ideal sample size, a 95% confidence interval, 80% power, and previous parameters described in the literature were considered. The measures of central tendency and of dispersion used were the Dental Fluorosis by Image Analysis (DFIA)30 index and the hardness index.31,32 The ideal sample size ranged from four to 10 animals per group. In total, 40 male rats (Rattus norvegicus, albinus, Holtzman) aged 2-3 months and weighing around 200 g were selected. The rats were housed in polyethylene cages bedded on wood shavings, with at most two animals per cage, kept at the animal facility of Araraquara Dental School at controlled temperature (23±10ºC) and relative humidity (55±5%), artificial lighting with fluorescent lamp under a 12/12 h light/dark cycle, and fed Biotec pellets without fluoride (Biobase, Santa Catarina, Brazil) and water ad libitum.

After a 5-day adaptation period, the rats were randomly assigned to four groups of 10 rats each, with administration of the following substances every 24 h: CG (control group) – intragastric administration of deionized water in similar volumes to those of AG; AG - amoxicillin (Eurofarma Genéricos, São Paulo, Brazil), given intragastrically at the dose of 500 mg/kg of body weight, as oral suspension; FG - deionized water containing 100 ppm of fluoride ad libitum, and intragastric administration of deionized water with 100 ppm of fluoride ad libitum; AFG – intragastric administration of 500 mg/kg of amoxicillin per body weight, and daily administration of deionized water containing 100 ppm of fluoride ad libitum.

The substances were administered for 60 days, period during which incisors could fully develop again.33 Amoxicillin was prepared by diluting the content of the flask in deionized water at the concentration of 500 mg in 5 mL and that administration was done as described by Mihalaş, et al.34 (2016) and Souza, et
Fluoride was prepared by diluting sodium fluoride in deionized water (221 mg NaF/L), obtaining 100 ppm of fluoride (100 mgF/L). After 60 days, the rats were euthanized with an overdose of 10% ketamine hydrochloride (Cetamin®-Syntec do Brasil Ltda, São Paulo, Brazil) in combination with 2% xylazine hydrochloride (Xilazin®- Syntec do Brasil Ltda, São Paulo, Brazil). Blood was collected by cardiac puncture using vacutainer blood collection tubes containing heparin as anticoagulant (BD Vacutainer Blood Collection Tubes, SST II Plus; BD Biosciences, New Jersey, USA). Upper and lower incisors were extracted and one tibia was removed from each animal, cleaned, coded for blind analysis, and stored at 60 °C for 24 h.

Photographic analysis

In total, 10 lower incisors from each group were photographed and analyzed by the quantitative method known as Dental Fluorosis by Image Analysis (DFIA). Before being photographed, the incisors were fixed on paraffin-embedded slides, positioning the buccal surface at the incisal edge of the crown parallel to the macro lens (Medical 100 Macro, Canon, New York, USA), which was positioned and vertically attached 7 cm above the tooth. The standardized images were made with a digital camera (EOS Rebel XTI, Canon, New York, USA). An area of 690 × 170 pixels was selected from the image, converted to a grayscale, with dark and light areas, and analyzed by a software program specifically developed for that. The DFIA index was estimated by the difference between $I_h$ (mean of pixels with the highest intensity, corresponding to the dark areas in the original image) and $I_l$ (mean of pixels with the lowest intensity, corresponding to light-colored areas in the original image). The DFIA index was proposed by Catani, et al. (2010). The higher the index, the more severe the fluorosis.

Analysis of enamel thickness

In total, 10 upper incisors from each group were transversely sectioned in three regions (incisal, medial, and apical) using a diamond bur at high speed. Each region was fixed on an acrylic resin block, with the cross-sectional tooth surface parallel to the horizontal plane. Three enamel thickness measurements were made in each region using 10× magnification on a microhardness tester (Future Tech, FM-7 model, Kawasaki, Japan) coupled to FM-ARS software.

Hardness analysis

In total, ten upper incisors from each group were embedded on acrylic resin discs with their long axes parallel to the disc surface (Arotec EMB-30, Arotec Ind. Com. LTDA, São Paulo, Brazil). Subsequently, they were abraded up to the center of the tooth using 400-grit wet-dry sandpapers (Arotec APL-4, Arotec Ind. Com. LTDA, São Paulo, Brazil) and polished with higher-number sandpaper grits (600 to 1200) and then with a felt polishing wheel soaked in a 1-μm diamond suspension. Enamel hardness was measured by a microhardness tester (Future Tech, FM-7 model, Kawasaki, Japan) and Knoop indenter, coupled to FM-ARS software, in three tooth regions (incisal, medial, and apical). Each region was assessed at five depths from the outer surface of the enamel (10 μm, 20 μm, 30 μm, 50 μm, and 70 μm). The Knoop indenter was applied using the axial axis of the diamond parallel to the outer surface of the enamel with a load of 25 g for 5 s. Three indentations were performed at each depth and the mean of each depth was estimated. The data were expressed in Knoop hardness number (KHN), that were result from measurements performed by the microhardness tester using the length of the longest diagonal of the diamond ($d$), the constant related to the frame formed by the base of the indenter (C) and the load applied to the indentation (c), using the formula: $KHN=C.c/d^2$.

Fluoride concentration in blood plasma

The plasma samples were obtained after centrifugation at 2,000 g for 10 min. One milliliter of each sample was buffered with TISAB III (Orion Research, Massachusetts, USA) and subjected to analysis of fluoride concentration using an ion-selective electrode (Orion Research 96-09; Orion Research Incorporated, Massachusetts, USA) coupled to a potentiometer (Orion Research EA 940; Orion Research Incorporated, Massachusetts, USA). Prior to the analyses, the electrode was calibrated by a standard curve for fluoride concentration. The results were expressed as μg F/mL of plasma.

Fluoride concentration in bone specimens

Ten tibiae from each group were dried at 60 °C for 24 h, transversely sectioned into 5-mm slices in the diaphysis area. A section of each specimen was...
powdered, sifted to obtain particles between 1 mm and 140 μm, and placed in an oven for another 24 h. Ten milligrams (±0.01 mg) of powder was transferred to test tubes containing 0.5 M HCl and shaken for 1 h. At the end of this period, the tubes were centrifuged, the supernatant was collected, with addition of 1.0 mL of TISAB II (1.0 M acetate buffer, pH 5.0 with 1.0 M NaCl and 0.4% CDTA), containing 0.5 M NaOH, followed by agitation. Fluoride concentration was determined using an ion-selective electrode (Orion Research 96-09; Orion Research Incorporated, Massachusetts, USA) coupled to a potentiometer (Orion Research EA 940; Orion Research Incorporated, Massachusetts, USA). The results were expressed as μg of F/g of bone.

Fluoride concentration in teeth

Ten lower incisors from each group were transversely sectioned into three segments (apical, medial, and incisal), placed in an oven at 60 ºC and weighed separately thereafter, powdered, and sifted to obtain particles between 140 μm and 1,000 μm, and placed in an oven for another 24 h. Subsequently, 5 mg (±0.01 mg) of powder was transferred to test tubes containing 0.5 M HCl and shaken for 1 h. At the end of this period, the tubes were centrifuged and the supernatant was collected for quantification of fluoride, as described for the concentration of fluoride in rat tibiae.

Statistical methods

Analyses of variance (ANOVA) were performed using Statistica version 8.0 (StatSoft Inc, Oklahoma, USA). When differences were observed between the groups, multiple comparisons of the means were made by Tukey's test or by the Games-Howell test, deemed appropriate in the presence of heterogeneity of variance. The significance level was set as α=0.05. The relationship between surface depth and hardness was determined by linear regression procedures, computing the angular coefficient, which represents the variation in hardness units (KHN) by unit of depth, and by the linear regression coefficient (r).

Results

A total of 40 rats (10 per group) were assessed. No statistical difference (ANOVA: p>0.05) was observed between the group means for water intake (42.1±5.6 mL) and for body mass (309.8±64.8 mg) among the rats included in the study.

Table 1 shows the means and standard deviations of enamel thickness (in μm) and of the DFIA index, which determine the severity of fluorosis, according to the experimental groups assessed. No significant differences were found between the means obtained for enamel thickness between the groups. On the other hand, the means of DFIA for the groups exposed to fluoride (FG and AFG) presented significantly higher values than control (CG) or amoxicillin (AG) groups. Exposure to amoxicillin (AG) alone or with fluoride (AFG) did not influence the DFIA values. Figure 1 shows the diagram of DFIA index for quantification of fluorosis through the differences between the dark and light areas.

Table 2 shows the means and standard deviations of Knoop hardness, angular coefficients, and correlation coefficients of two linear regressions for depths of 10 to 30 μm. The groups exposed to fluoride (AFG and FG) had significantly lower mean hardness than that of non-exposed groups (AG and CG) at all depths. We can observe that the fluorosis was more severe on the outer enamel surface (10 μm), since the results are the same and greater for depths from 20 to 70 μm. Regarding exposure to amoxicillin, the enamel hardness values from rats only exposed to amoxicillin (AG) did not differ from the control group (CG), and are similar to the groups exposed to fluoride and amoxicillin (AFG) and only fluoride (FG).

The regressions show significant linear increase in hardness at approximately 10 μm to 30 μm in groups not exposed to fluoride (CG and AG), with an increase by 7.9 and 7.1 KHN (Knoop hardness number) per μm, compared to an increase by 4.0 and 5.4 KHN per μm in the FG and AFG, respectively. Figure 2 shows the area of the three indentations that were performed, namely: incisal, medial, and apical regions.

Table 3 shows the analysis of variance, indicating significant difference between the mean fluoride
Inc – Lower incisor, indicating the area demarcated for analysis. Black arrow – indicating the highest pixel intensity. White arrow – indicating the lowest pixel intensity. In the image of the rat tooth with dental fluorosis showing striated white bands distributed in the orange enamel. In the gray scale image, the black area corresponds to the original orange bands and the white area corresponds to the original white bands. For each segmented area, the method computes the average of the pixel intensities in the input image.

**Figure 1** - Schematic diagram of DFIA index for quantification of fluorosis.

**Table 2** - Mean (SD) of Knoop hardness (KHN). Estimates of angular coefficient (AC) and of standard error (SE): AC(SE) and correlation coefficient (r).

| Group | Depth (μm)* | AC (SE)** | r*** |
|-------|-------------|------------|------|
|       | 10  | 20  | 30  | 50  | 70  |       |
| CG    | 148.1 (47.6)c | 233.6 (43.6)c | 306.3 (46.8)c | 354.3 (29.6)c | 352.2 (31.3)c | 7.9 (1.0) | 0.83 |
| AG    | 130.5 (35.0)bc | 202.5 (31.2)bc | 271.8 (29.1)bc | 336.5 (22.0)bc | 336.6 (31.9)bc | 7.1 (0.7) | 0.89 |
| FG    | 79.5 (18.4)a  | 114.0 (23.9)a  | 158.7 (54.3)a  | 191.4 (46.0)a  | 179.1 (47.0)a  | 4.0 (0.8) | 0.69 |
| AFG   | 102.5 (22.5)ab | 145.6 (27.9)ab | 211.3 (54.8)ab | 226.0 (43.4)ab | 212.3 (44.7)ab | 5.4 (0.9) | 0.78 |

* Group means in the columns followed by identical letters are not significantly different according to Tukey’s test (p>0.05). The result is the same for the depths of 20 to 70 μm.
** Linear regression at 10 to 30 μm.
*** Linear correlation coefficient (r: p<0.05).

A – Upper incisors embedded in acrylic resin discs for hardness analysis. B – Schematic diagram of hardness analysis in the incisal, medial, and apical regions at different depths of the enamel surface. For each depth, the values of Hardness were analyzed between the groups.

**Figure 2** - Rat teeth organized for hardness analysis.
Table 3- Mean (SD) concentration of F, expressed as μg/g of bone or tooth and as μg/mL of plasma

| Group* | Blood plasma | Bone | Tooth |
|--------|--------------|------|-------|
| CG     | 0.02 (0.01)  | 97.3 (20.5) | 35.6 (10.0) |
| AG     | 0.05 (0.04)  | 81.6 (27.7) | 45.6 (20.0)  |
| FG     | 0.38 (0.15)  | 2250.3 (270.7) | 1907.0 (732.8) |
| AFG    | 0.25 (0.13)  | 2243.4 (470.3) | 2560.2 (894.7) |

* Group means in the column followed by identical letters did not show any differences in the Games-Howell test (p>0.05).

Concentrations in plasma, bone, and teeth. Groups exposed to fluoride (AFG and FG) have higher concentrations than those not exposed to fluoride (CG and FG) with no effect of amoxicillin on F concentrations.

Discussion

This *in vivo* study showed the effect of chronic exposure to fluoride and to amoxicillin on dental enamel. To the best of our knowledge, this is the first animal study that compared the effect of fluoride alone and of fluoride + amoxicillin on amelogenesis, considering previous hypotheses described in the literature. The methods presented herein allowed detecting dental fluorosis caused by chronic exposure to excessive fluoride. The methods applied in this study by DFIA index and hardness presented compatible results according to the experimental groups, validating the study model to the fluoride exposure.

The DFIA method was validated to detect the increase in the severity of fluorosis, determining the difference between light-colored and orange striated patterns observed in the exposure to fluoride. Our findings showed that high levels of fluoride caused dental fluorosis in rats, as observed by Catani, et al. (2010). Although the literature showed that amoxicillin can exert some influence on fluorosis, this study showed no differences between the control group and the group exposed to amoxicillin, despite the high doses of amoxicillin. One possible explanation could be that the rat strain used in this study is not sensitive to 500 mg amoxicillin/kg.

The rats exposed to fluoride presented severe fluorosis in the image analysis (DFIA index) and in surface hardness test, as expected, since the model for the induction of dental fluorosis in rodents used in this study is already known. The severity of fluorosis can be quantified and correlated with the level of injury caused by fluoride based on its plasma concentration. In this study, the analysis of fluoride on plasma and bone was performed to demonstrate the experiment’s validity. Bone tissue presents a highly dynamic behavior, with a constant rate of remodeling, and, therefore, fluoride exposure can affect the bone composition, its three-dimensional structure, and bone cells. Thus, the concentration of fluoride in bone tissue was higher than in the enamel. This can be explained by the higher bone metabolism of growing animals, since a higher proportion of fluoride is retained in calcified tissues in young people due to their high rates of remodeling bone. The effect of amoxicillin on F concentrations regardless of the tissue considered was not found.

Hardness values obtained for those groups treated with fluoride, either with or without association with amoxicillin (FG and AFG), showed severe fluorosis are related to high fluoride concentrations in the plasma, bone, and teeth, corroborating the findings of other authors. Moreover, after fluoride is absorbed, it remains in the bone tissue for a while and, consequently, fluoride is released into the bloodstream and locally to the tissues contiguous with the enamel in the mineralization stage, possibly increasing the severity of fluorosis.

Considering the hardness results, our findings show a significant linear increase in hardness at the depths of 10 to 30 μm in all groups, suggesting that exposure to fluoride caused changes in the enamel, which were more prominent at the first 10 μm of the enamel surface. These findings corroborate those observed *in vivo* by Shinoda (1975), who described a reduction in enamel surface hardness at 10 μm caused by exposure to fluoride, demonstrating that this could be related to changes in the size, shape, and quality of enamel rods.

Amoxicillin is an antibiotic widely used in the treatment of bacteremia, and its most frequent side effects are related to dysregulation of the gastrointestinal tract. Studies in humans also point to the possible interference of amoxicillin on tooth enamel. Laisi, et al. (2009) reported that 16.3% of preschool children with molar incisor hypomineralization (MIH) used antibiotics during the first year of life, particularly amoxicillin. This finding was corroborated by Wuollet, et al. (2016), who found that children exposed to amoxicillin in the first 3 years had 2.58 times the risk of developing MIH. However, these studies are not...
retrospective and influenced by confounders between the exposure and the outcome.

The 500 mg/kg daily dose of amoxicillin ministered in this study was estimated by simulating the exposure of infants/children to amoxicillin in their first years of life, according to the American Academy of Pediatrics.\textsuperscript{37} The total daily dose was ministered once a day, conveniently, since the effects on tooth development seem similar when the total daily dose was divided into 3 times a day\textsuperscript{10} or in a single dose.\textsuperscript{38}

The amoxicillin did not influence the enamel hardness and thickness in this study. No significant difference was observed between the groups exposed to fluoride + amoxicillin (AFG) and the fluoride group (FG) on the DFIA and hardness, as well as no difference between the AG and CG groups. Therefore, we suggest that exposure to amoxicillin did not exhibit interaction on the effect of fluoride on \textit{in vivo} dental enamel. These data are not in accordance with other previous published data.

In animals’ study, Gottberg, et al.\textsuperscript{11} (2014) observed that the side effect caused by amoxicillin on dental enamel was dose-dependent in rodents. Souza, et al.\textsuperscript{26} (2016) have evidenced in a histological analysis that, during the secretory stage of the enamel matrix (seven days), exposure to amoxicillin reduced enamel thickness, but in the subsequent stage, at the end of secretion and at the outset of mineralization (12 days), this reduction in thickness was not observed between the exposed and non-exposed groups, thus suggesting a one-off effect of amoxicillin or a possible change in the secretion rate of the enamel matrix, which was later restored. Our results do not show statistically significant effects of amoxicillin on dental fluorosis in Holtzman rats, in accordance with de Souza, et al.\textsuperscript{25} (2020) who used the same rat strain and observed none effect of amoxicillin on the enamel mineralization.

Regarding enamel thickness, no difference among the groups was identified. The exposure to fluoride leads to changes in enamel mineralization, causing enamel fluorosis without morphological changes in the secretory stage, which is in line with the assumptions about the pathogenesis of dental fluorosis.\textsuperscript{30,41,42} However, \textit{in vitro}\textsuperscript{28} and \textit{in vivo}\textsuperscript{24,25} studies have shown that exposure to amoxicillin, to fluoride, or to both can affect enamel thickness during the initial secretory stage of enamel matrix formation. These controversial results should be analyzed in future studies that should also analyze the bioavailability of amoxicillin in the organism of the animal and the possible intestinal environment of absorption of the drug, as previously suggested by Chesa-Jíménez, et al.\textsuperscript{43} (1994).

Previous studies also demonstrate that some endogenous factors\textsuperscript{15} and exogenous molecules\textsuperscript{31} may modulate fluoride effects on dental enamel underlying the necessity for further studies with dose-response effects on various strains with careful considerations to external conditions. In rodents, the association between fluoride and bisphenol-A seems to increase severity of fluorosis in rats.\textsuperscript{19}

Conclusion

We conclude that chronic exposure to fluoride caused significant changes in dental enamel, compatible with the plasma concentrations of fluoride and with the reduction of enamel hardness, especially at the first 10 μm. Amoxicillin associated to fluoride did not influence the severity of fluorosis. Moreover, the exposure to amoxicillin alone has not affected amelogenesis in our rat model.

Declarations of interest

The authors declare no competing interests.

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Authors’ contributions

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