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The effect of amoxicillin on dental enamel development *in vivo*

Juliana FELTRIN-SOUZA[a]
Fabiano JEREMIAS[b]
Satu ALALUUSUA[c]
Carin SAHLBERG[c]
Lourdes SANTOS-PINTO[b]
Jukka JERNVALL[d]
Susanna SOVA[d]
Rita de Cássia Loiola CORDEIRO[b]
Paulo Sérgio CERRI[e]

[a]Universidade Federal do Paraná – UFPR, Department of Stomatology, Universidade Federal do Paraná, Curitiba, PR, Brazil.

[b]Universidade Estadual Paulista – Unesp, School of Dentistry, Department of Pediatric Dentistry, Araraquara, SP, Brazil.

[c]University of Helsinki, School of Medicine, Department of Oral and Maxillofacial Diseases, Helsinki, Finland.

[d]University of Helsinki, Institute of Biotechnology, Developmental Biology Program, Helsinki, Finland.

[e]Universidade Estadual Paulista – Unesp, School of Dentistry, Department of Morphology, Araraquara, SP, Brazil.

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Corresponding Author:
Juliana Feltrin de Souza
E-mail: julianafeltrin@hotmail.com

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Abstract: The exposure to amoxicillin has been associated with molar incisor hypomineralization. This study aimed to determine if amoxicillin disturbs the enamel mineralization in *in vivo* experiments. Fifteen pregnant rats were randomly assigned into three groups to received daily phosphatase-buffered saline or amoxicillin as either 100 or 500 mg/kg. Mice received treatment from day 13 of pregnancy to day 40 postnatal. After birth, the offsprings from each litter continued to receive the same treatment according to their respective group. Calcium (Ca) and phosphorus (P) content in the dental hard tissues were analyzed from 60 upper first molars and 60 upper incisors by the complexometric titration method and colorimetric analysis using a spectrophotometer at 680 nm, respectively. Lower incisors were analyzed by X-ray microtomography, it was measured the electron density of lingual and buccal enamel, and the enamel and dentin thickness. Differences in Ca and P content and electron density among the groups were analyzed by one-way ANOVA. There was no significant difference on enamel electron density and thickness among the groups ($p > 0.05$). However, in incisors, the higher dose of amoxicillin decreased markedly the electron density in some rats. There were no statistically significant differences in Ca ($p = 0.180$) or P content ($p = 0.054$), although the higher dose of amoxicillin could affect the enamel in some animals. The amoxicillin did not significantly alter the enamel mineralization and thickness in rats.

Keywords: X-Ray Microtomography; Dental Enamel; Amoxicillin; Amelogenesis; Dental Enamel Hypoplasia.

Introduction

Enamel formation, also known as amelogenesis, is a complex process involving cellular proliferation and differentiation through interactions between epithelial and ectomesenchymal cells.[1,2] Amelogenesis is mainly divided into three major stages:, secretory, transition, and maturation. During the secretory stage, ameloblasts secrete large quantity of matrix proteins (predominantly amelogenin), where the enamel crystals grow. Once the full thickness of enamel has been deposited, the cells undergo a short transitional stage into maturation. During the maturation stage, there is an increase in the matrix proteins degradation and completion of the mineralization process.[1]

Whereas tooth development is a genetically regulated, it is susceptible to detrimental influences of systemic and environmental factors. A highly regulated process of the secretion and the normal development of the organic enamel matrix is a prerequisite for formation of normal mineral crystals.[3,4,5,6] Disturbances during the transition and maturation stages of amelogenesis can result in defects in the enamel structure and composition, such as hypomineralized enamel.[6] In experimental studies using animals, pathological changes of the enamel surface have been reported as a result of exposure to toxic doses of several agents, including strontium, cobalt, fluoride, environmental toxicants (such as dioxins), and tetracycline.[3,7,8]

Amoxicillin use during early childhood has been associated with molar-incisor hypomineralization (MIH).[3,9,10,11,12,13] MIH is defined as a hypomineralization of systemic origin of one to four first permanent molars, frequently associated with involvement of the permanent incisors.[14] In MIH, porous enamel is susceptible to break down, mainly under the influence of masticatory forces, which leaves the dentin unprotected; and thus contributes to the development of carious lesions.[14,15] The prevalence of MIH has been reported to range from 2.8 to 44% in different population.[15,16,17,18] A clear etiology of MIH is yet to be defined. A multifactorial origin, in which the systemic factors act in synergism, has been suggested.[13,19] There are evidences that changes in gene expression during dental enamel formation may be responsible for MIH.[20,21]

Several studies have described the microstructure of hypomineralized enamel as presenting less distinct prism sheaths and disorganized enamel. Moreover, the hardness and modulus of elasticity of enamel affected by hypomineralization have shown lower values than normal enamel.[6,22,23] The chemical composition of molars affected by MIH is also changed.[24,25,26]

The use of amoxicillin alone, or associated with fluoride, affects amelogenesis in cultured mouse molars, resulting in reduced enamel thickness and delayed onset of the secretory stage by ameloblasts.[3] In an *in vivo* study, dentin mineralization was found to be disturbed in rat molars after exposure to a single dose of amoxicillin.[27] De Souza et al. evaluated the effect of amoxicillin during the secretory stage of the amelogenesis in rats, and reported that exposure to amoxicillin significantly reduced enamel thickness. However, these studies did not evaluate the mineral content of enamel treated with amoxicillin during tooth development.[28]

X-ray microtomography (micro-CT) has been employed for assessment of the severity of defects in enamel and other hard tissues.[25,29] It provides high-resolution morphological information by means of an accurate and quantitative method. The micro-CT allows the imaging of structural details and quantitative analysis, such as measurement of mineral density in mineralized tissues.[29]

The enamel structure and chemical composition are complex, and different methods should be combined to evaluate them in a proper context taking into account the chemical and structural properties.[22] Therefore, the present *in vivo* investigation was carried out to assess if amoxicillin disturbs the enamel development in the incisors and first molars of rats using biochemical analyses of calcium and phosphorus content and micro-CT. Micro-CT was also used in assessing the thickness of enamel and dentin.

Methodology

Animal care

This study was performed in accordance with Brazilian animal care and national laws on animal use. The research protocol was authorized by the Ethical Committee for Animal Research (report #34/2010). The study was reported according to ARRIVE guideline.

The samples were composed of fifteen female rats (*Rattus norvegicus albinus*, Holtzman), aged 2–3 months and weighing approximately 250 g. The rats were maintained under controlled temperature (23 ± 1°C) and humidity (55 ± 5%), with a 12-h/12 h light/dark cycle and food and water available *ad libitum*.

Female rats in estrous were mated overnight with males (two females per male). In the early morning, the male rat was removed from the cage. Vaginal smears were taken from female rats and analyzed under a light microscope (Carl Zeiss, Jenamed, Germany). The presence of sperm in the vaginal smear was

deemed as pregnancy and designated as a first day of embryonic age.

The pregnant rats were single-housed in polypropylene cages and randomly distributed into three groups (n = 5 per group). In the control group, the rats received phosphatase-buffered saline (PBS). In the A100 group (A100), the rats received once-daily, oral dosing of 100 mg/kg amoxicillin. In the A500 group (A500), the rats received once-daily, oral dosing of 500 mg/kg amoxicillin. The 500 mg/5 mL amoxicillin was purchase from Eurofarma Genéricos; São Paulo, SP, Brazil. Animals were treated with PBS or amoxicillin (100 mg/kg or 500 mg/kg) at the same final volume via gavage, as described on Souza et al.[28]

The treatments were performed from day 13 of pregnancy, which corresponds to the beginning of development of the first upper molars in rats and when amoxicillin crosses the placenta and umbilical cord.[28,30] After birth, the offsprings from each litter continued to receive the same treatment according to their respective group, phosphatase-buffered saline (PBS), 100 mg/kg amoxicillin (A100) or 500 mg/kg amoxicillin (A500), from postnatal day 1 until postnatal day 40, as described on Souza et al.[28] On day 40, a total of ten offspring per group were euthanized by an overdose of 10% ketamine (Cetamin®, Syntec do Brasil Ltda, São Paulo, Brazil), and 2% xylazine hydrochloride (Xilazin®, Syntec do Brasil Ltda, São Paulo, Brazil). The upper and lower jaws were extracted, cleaned, and coded for analyses.

Micro-CT analyses

Lower jaws of 15 animals, five from each group (A100, A500 and control) were randomly selected and examined by X-ray microtomography. The jaws were cleaned, dehydrated, and scanned using a SkyScan 1272 desktop micro-CT system (Bruker microCT N.V., Kontich, Belgium). The samples were imaged with 90 kV acceleration voltages and a source current of 111 µA. The filter applied was Al 0.5 + Cu 0.038, and the scaled image pixel size was 20 µm (0.05 pixel/µm). Projections were acquired over a full circle of rotation steps at 0.4 degrees angle intervals, and each projection was composed of the average of 4 transmission images. The data from the tomography projection scans were reconstructed using SkyScan NRecon and then analyzed using ImageJ (Wayne Rasband, National Institutes of Health, USA) software. Two minerals, fluorapatite ($Ca_5(PO_4)_3F$) and quartz (SiO_2) were used for standardization and calibration of measurements. The mean gray-value of the mineral grains were set to as the electron densities of 3.17 g/cm^3 (fluorapatite) and 2.65 g/cm^3 (quartz). From the first molars, lingual and buccal enamel densities from the thickest areas were measured (Table 1). From the incisors, the measurements were made along a straight 200-µm wide rectangle area along the length of the enamel that reached from the mesial surface of the first molar to the tip of the incisor to a length of 10000 µm. The outermost tips of the incisors were disregarded because the wear of the rat teeth and abrasion of the enamel influences the values (Figure 1). The analysis was performed once in each tooth due to the high accuracy measurements of the micro CT.[22,25,29,31] The thickness of enamel and dentin and the electron density of dentin were measured from the CT-scans' central section of the incisors at the point just in front of the margin of the lower jaw bone (Figure 2).

Biochemical analysis for determination of calcium and phosphorus content

For determination of calcium (Ca) and phosphorus (P) content in the dental hard tissues, a total of 60 upper first molars and 60 upper incisors from the randomly selected 30 animals (10 animals per group) were dried, separately, for 24 h at 60°C, then pulverized. The resulting powder was sieved to obtain particles in the range of 140 to 1.000 µm and dried at 60°C for an additional 24 h. Aliquots (30 ± 0.01 mg) were then

Table 1. Mean (SD) electron density on the lingual and buccal surfaces of the molars in each group. A100 = amoxicillin 100 mg/kg/day and A500 = amoxicillin 500 mg/kg/day.

Groups	Electron density	
	Lingual	Buccal
	Mean (SD)	Mean (SD)
Control	2.95 (0.04)	2.95 (0.04)
A100	2.99 (0.04)	2.96 (0.02)
A500	2.95 (0.01)	2.95 (0.02)

No significant difference between the three groups ($p > 0.05$, ANOVA). SD: standard deviation. Electron density in cm^3.

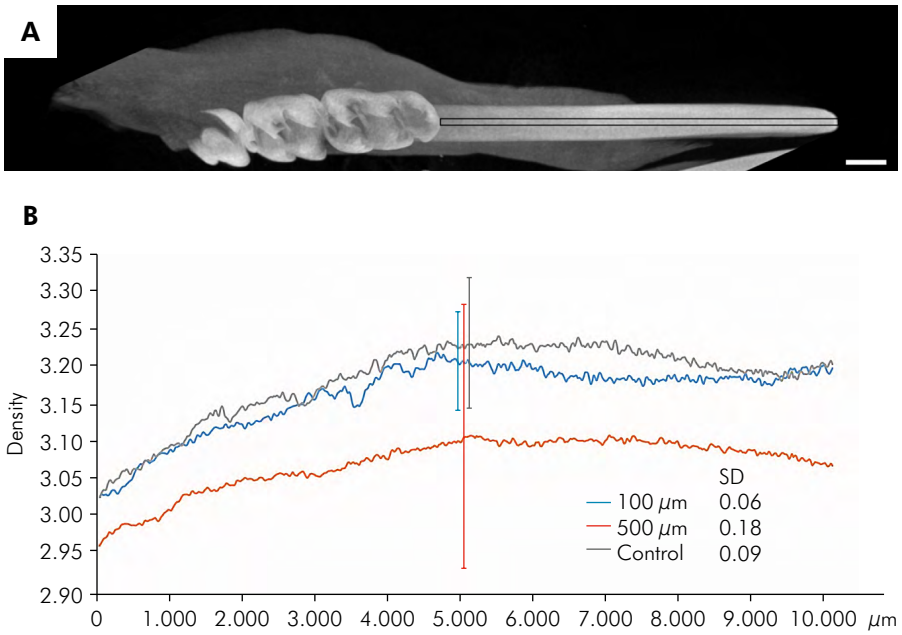

Figure 1. A. ImageJ projection of a half mandible and right side incisor from a rat given 100 mg/kg amoxicillin. Inserted the area from which the density was measured. bar: 10 000 μm, white dashed line represents the area used to calculate the SD reported in Figure 1B. B. Scatter plot of mean enamel density values from five incisors/experiment. The measurement was made from the mesial surface of the first molar towards the tip of the incisor. The SD was measured at 5000 μm from the molar mesial surface, approximately at the point where the incisor enamel has reached full mineralization (dashed line in A).

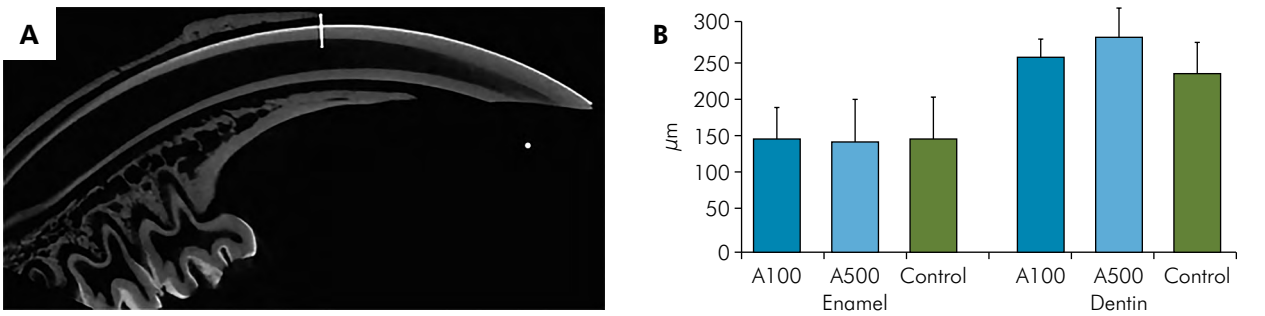

Figure 2. A. Longitudinal section of an incisor exposed to 100 mg/kg amoxicillin. The white bar indicates the point of measurement. B. A graphical representation of mean thickness of enamel and dentin with confidence intervals (95% CI). There are no significant differences between the groups. A100 = amoxicillin 100 mg/kg/day and A500 = amoxicillin 500 mg/kg day.

weighed out and transferred to tubes containing 1.0 mL of 0.5 M nitric acid. After 1 h, this solution was divided into two parts for Ca and P determination. The Ca content was quantified by complexometric titration. EDTA (disodium ethylenediaminetetraacetate) and the indicator Calcon® (Sigma-Aldrich, St. Louis, USA), were added to the sample. The Ca content was expressed as mg of Ca per g of tooth mass (mg Ca/g tooth). The P content was determined by the colorimetric method using a spectrophotometer at 680 nm (DR 2500, Hack, Loveland, USA). Sodium molybdate (10 mL) and 5 M ascorbic acid (1.0 mL) were added to the sample. The spectrophotometer had been previously calibrated with standards of known P concentration. The P content was expressed as mg P/g tooth. The analyses were performed three times in each specimen and the mean of the three measures was given as results.

The Micro-CT analyzes, and the biochemical analysis for determination of Calcium and Phosphorus

content were performed after due training and their interpretations made by an expert researcher.

Statistical analysis

The data was analyzed using the Statistica version 8.0 software (StatSoft Inc, Tulsa, USA). The dependent variables were represented by the values of Ca and P content and electron density, which were analyzed according the values distribution by Kolmogorov-Smirnov test. The values of Ca, P and electron density presented a normal distribution ($p > 0.05$). The dependent variables were compared among the groups (independent variables) by one-way ANOVA. The significance level of 5% was adopted. Additionally, to obtain exact *p* values, we tested electron density differences in the incisor micro-CT data after treatment assignments were randomized 10,000 times (means of density values from 0 to 10 mm towards the incisor tip).

Results

The mean (SD) of enamel electron densities on the buccal and lingual surfaces of the lower first molars for the control, A100 and A500 groups are given in Table 1. The statistical analysis showed that there was no significant difference among the groups ($p > 0.05$).

Scatter plot presents the mean of density values (SD) from each incisor group (Figure 1B). At the point where the incisor enamel has reached full mineralization, (approximately 5000 μm from the molar mesial surface) the density values of the controls ranged from 3.14 to 3.31 g/cm^3 and those of the A100 group from 3.12 to 3.20 g/cm^3. Enamel's electron densities in the A500 rats ranged from 2.93 to 3.30 g/cm^3. The values of A500 group were marginally different from the other groups ($p = 0.081$).

The thickness of enamel and dentin was measured from the micro-CT scans (Figure 2). There was no significant difference in enamel thickness among the three groups and only a slightly greater thickness of dentin was observed in rats exposed to 500 mg/kg/d amoxicillin. Electron density values of dentin were very similar to the mean, 2.84, 2.84 and 2.83 g/cm^3 in the control, A100 and A500 groups, respectively.

Statistically significant differences in the Ca and P content were not observed among the groups ($p > 0.05$) (Table 2).

Discussion

Amoxicillin use in early childhood has been associated with molar-incisor hypomineralization.[3,9,10,11,12,13] *In vitro* studies have demonstrated changes in cultured mouse embryonic tooth explants after amoxicillin exposure.[3,12] In our previous study, same doses of amoxicillin reduced the enamel matrix thickness during secretory stage.[28] However, the effect of amoxicillin exposure *in vivo* on the mineral content of rat teeth was unclear.

According to Schour and Massler,[30] the first rat molars begins its formation by day 13 of intrauterine life, and its mineralization is completed on day 12. In the present study, the animals were exposed to amoxicillin during every stage of amelogenesis until day 40. At this age, the enamel of the incisors is 80 μm thick, allowing manipulation and mineral content analysis.[30] The doses of 100 and 500 mg/kg/d amoxicillin were selected to simulate amoxicillin exposure in children during the first years of life pursuant to recommendations of the American Academy of Pediatrics Subcommittee on Management of Acute Otitis.[32] Since rat and human metabolism are different, it is necessary a higher dose of amoxicillin to determine its effect on the development of dental

Table 2. Mean (SD) Calcium (Ca) and Phosphorus (P) content in mg/g of tooth. A100 = amoxicillin 100 mg/kg/d and A500 = amoxicillin 500 mg/kg/d.

Group	Calcium		Phosphorus	
	Incisors	Molars	Incisors	Molars
Control	271.0 (18.1)	258.9 (36.7)	135.1 (7.0)	136.1 (5.7)
A100	289.2 (17.3)	258.3 (27.2)	133.6 (6.7)	133.1 (5.3)
A500	260.1 (16.2)	250.2 (58.8)	136.0 (6.9)	143.1 (7.5)

No significant difference among the three groups ($p > 0.05$, ANOVA). SD: standard deviation.

enamel in rats; this approach was also used in previous studies to evaluate the effect of other substances such as fluoride, macrolides and other factors in amelogenesis.[7,33,34]

Dental enamel has a number of properties that makes it a unique structure. It is the hardest structure in the mammalian body and has a very high proportion of inorganic matter, mainly hydroxyapatite. The ameloblasts have limited reparative capacity; therefore, disturbances during the mineralization of enamel can result in permanent defects in enamel structure. Defects in mature enamel have important implications in understanding their relation with etiological factors.[22]

During amelogenesis, elements and compounds are transported to mineralizing areas.[35] The elemental composition pattern of dental enamel varies during the differentiation stages of the ameloblasts.[1,7] Calcium and phosphorus, in the form of phosphate, are the major components of hydroxyapatite in all hard tissues. Studies have been using the Ca/P ratio to determine the calcium content of hard tissues, which was found to be constant along the developing tooth organ.[22,24,26] If this ratio is altered, it is likely that the mineral phase is altered or that significant ion substitution may have occurred.[24]

Several methods can be used to quantify the mineral content of hypomineralized enamel.[22,25,29] The present study utilized quantitative chemical analysis and micro-CT to determine the mineralization state of enamel after amoxicillin exposure. Micro-CT provides a high-resolution, non-destructive, and non-invasive method for the analysis of dental hard tissues. This method promotes the three-R, the replacement, refinement e reduction of animal samples. It has been constantly cited as a reliable method for measurement of the mineral density of normal and hypomineralized enamel.[22,25,29,31]

In the present study, the amoxicillin-treated groups did not differ significantly from the control group in the calcium and phosphorus content, suggesting that amoxicillin exposure did not influence the mineralization and maturation stages. Studies *in vitro* with mouse molar germs have demonstrated that amoxicillin affects ameloblasts during the secretory stage, causing alterations in ameloblast morphology and promoting a reduction in the enamel matrix.[3,12,36] Similarly, mice exposed to amoxicillin/clavulanic acid (50, 100 or 150 mg/kg/day) showed increase in the amount of disorganization and clear vacuoles in maturation stage ameloblasts.[34] In another *in vivo* study, the animals exposed to amoxicillin (100 or 500 mg/kg/d) during the secretory stage of amelogenesis (7-day-old-rats), presented a significant reduction in the enamel thickness. Otherwise, the enamel thickness in the amoxicillin-treated rats did not differ from the control group during mineralization stage (12-day-old-rats),[28] supporting our results. In conclusion, these suggest that ameloblasts are especially sensitive to amoxicillin exposure during the secretory stage, but not during the mineralization stage. Fluoride exposition has also been observed to influence the ameloblasts and enamel formation at different stages of the life cycle, resulting in distinct enamel defects types.[7]

Another point is the possible recovery of ameloblasts during mineralization. There is evidence that rat pups exposed to a calcium-deficient diet developed enamel hypomineralization in incisors, with thinner crystallites and presence of organic matrix.[37] However, when normal diet was returned, enamel mineralization was restored.[37]

Recently, an *in vivo* study has reported structural changes in the enamel development of mice incisors after chronic administration via subcutaneous injections of amoxicillin and clavulanic acid.[34] Significant reduction in the mineral content of calcium, fluoride and phosphate was also detected by X-ray spectroscopy analysis. These results are not in line with our findings probably due to the clavulanic acid that could act in synergism with amoxicillin interfering with the enamel formation. We also used a different route of administration of amoxicillin.

Another concern is the mineral content analysis of enamel defects and its implications. In human, the molars affected by MIH have shown lower hardness than normal enamel due to the disorganized structure of the hypomineralized enamel; however, mineral content findings were controversial.[22,25,38] Some studies have reported that molars affected by MIH exhibited lower calcium and phosphorus

content than in enamel of the control group.[25,26] On the other hand, other studies have shown that Ca/P ratio in enamel affected by MIH was similar to control molars.[22,24] Similar results were found by Sucheela and Bhatnagar[8] evaluating fluoride exposure in rabbits and rats. It is possible that, in hypomineralized enamel, calcium and phosphorus are presented not only in an organized crystalline structure but also in amorphous matter.[8,24] Thus, mineral content should be interpreted with caution, and additional methods should be combined for its analysis. Also it is generally accepted that the serum calcium concentration is controlled within a very narrow range by hormones, such as calcitonin and parathyroid hormone, and it plays important role in the maintenance of a normal heart beat as well as mineralized tissue metabolism.[31]

A recent study evaluated if treatment with antibiotics and non-steroidal anti-inflammatory drugs could disturb the enamel mineralization in mice.[39] The authors evaluated amoxicillin (5 mg/d of amoxicillin), amoxicillin/clavulanate (2.5/0.31 mg/d), erythromycin (5 mg/d), acetaminophen (5 mg/d), ibuprofen (2.5 mg/d) and celecoxib (0.12 mg/d) on the Ca, P, Al, K, Na, Mg and Cl content by EDX-analysis, as well as on the COX2 expression. The amoxicillin groups did have any effect on the calcium and phosphate content, in agreement with our results. The authors suggested that COX2 is involved on the enamel formation. Amoxicillin, amoxicillin/clavulanate and erythromycin administration reduced the quantity of COX2 present in the enamel organ of mouse incisor during the maturation period, but not its activity.

In the present study, electron density values in the molars ranged from 2.83 to 2.99 g/cm^3. These values were similar to the electron density of normal human enamel, which is approximately 2.8 to 3.0 g/cm.[3,29] Molar surfaces, which have been severely affected by MIH, have shown reductions of up to 20% in mineral density when compared to normal enamel.[25] Our findings corroborate with a recent study, which evaluated the effect of amoxicillin (doses of 50 mg/kg/d and 90 mg/kg/d) in enamel mineral content in piglets.[40] The authors did not observe significant differences between the groups. In this study, the electron density values of enamel in the molars of controls and treated piglets were lower than ours and ranged from 2.41 to 2.66 g/cm^3.

We found that the enamel of incisors presented more variation of electron density values than the molars. It could be explained by the differences on the enamel developmental of the incisors, which presents a continuous metabolism and growth. Although no significant difference was detected, the A500 group presented lower values of densities in some animals ($p = 0.08$) in the incisors. However, the variability between the animal response to the exposure could be due the biological variability and sensitivity among the experimental animals in developing defects of enamel, which appeared first in rats exposed to a higher dose of amoxicillin. Kumazava et al.[27] reported that the intensity of dentin alteration was observed to vary between rats in an amoxicillin-treated group depending on individual differences, particularly body weight. This may explain the greater variability observed in the A500 group during our study.

In this study, the sex information of the offspring was not collected. Although it can be a limitation, to the best of our knowledge, there is no evidence that sex is associated to dental enamel defects. The data of Ca and P have small variation, the difference in mean values of Ca and P among the groups were also small, thus a larger sample size may provide a statistically significant result. However, our sample size was similar to previous studies in animals.[8,27,34]

Conclusion

Electron density in molars was similar among the groups. In incisors, the higher dose of amoxicillin decreased markedly the electron density in some rats, but the difference among the groups was not statistically significant. Further studies are needed to understand the amoxicillin effects on the maturation stage of enamel.

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