Clin Oral Invest (2012) 16:835–842
DOI 10.1007/s00784-011-0581-4

ORIGINAL ARTICLE

Effect of single-dose amoxicillin on rat incisor odontogenesis: a morphological study

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Received: 9 February 2011 / Accepted: 16 June 2011 / Published online: 30 June 2011

Abstract The effect of exposure to amoxicillin on tooth development remains to be elucidated. The purpose of this study was to investigate the effect of amoxicillin on rat incisor odontogenesis. Male Wistar rats weighing approximately 100 g were given a single intraperitoneal injection of 3.0 g/kg body weight amoxicillin. One week after injection, the rats were fixed, and the lower incisors were demineralized and prepared into paraffin sections for light microscopy (LM) and immunohistochemistry. Undemineralized samples were embedded in resin and ground for processing for contact microradiography (CMR) and scanning electron microscopy (SEM). Serum calcium, phosphate, and magnesium concentrations were measured. At 1 week after amoxicillin administration, LM, CMR, and SEM revealed a clear increase in the area of interglobular dentin, representing disruption of mineralization by odontoblasts. Immunohistochemistry demonstrated moderate levels of the small integrin-binding ligand N-linked glycoprotein family dentin matrix protein 1 in large areas of interglobular dentin. On the other hand, no morphological alteration or hypomineralization was observed in the enamel. Serum calcium values showed no significant differences between the control and experimental rats during the experimental period although both serum phosphate and magnesium levels increased at day 1 after amoxicillin injection. The results suggest that a single dose of amoxicillin specifically affects normal tooth dentin mineralization, but not enamel mineralization in rat incisor odontogenesis. The present results further our understanding of the clinical association between dentin abnormality and amoxicillin exposure during tooth development.

Keywords Amoxicillin · Rat incisor · Odontogenesis · Morphology · Immunohistochemistry

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Introduction

It has been suggested that amoxicillin, the most common antibiotic used in childhood [1–4], is associated with developmental enamel defects accompanied by diffuse opacities, a condition known as molar–incisor hypomineralization (MIH) [5]. Hong et al. [6] suggested a link between amoxicillin use during infancy and developmental enamel defect in permanent teeth and speculated that the critical time point for the effect of amoxicillin on ameloblasts was during the secretory stage. In a recent in vitro study, Laisi et al. [7] showed that the exposure of cultured mouse embryonic tooth explants to amoxicillin enhanced enamel formation, suggesting that amoxicillin interferes with ameloblast function. However, the mechanism underlying the effect of amoxicillin on amelogenesis remains to be clarified [5].

On the other hand, there are few published data on the effects of amoxicillin on dentinogenesis [7]. Therefore, the purpose of this study was to investigate whether amoxicillin adversely affected odontogenesis particularly in rat incisor dentinogenesis using light microscopy (LM), immunohistochemistry (IHC), contact microradiography (CMR), and scanning electron microscopy (SEM).

Material and methods

Animals and experimental design

Sixty male Wistar rats weighing approximately 100 g were used in this study. The animals were randomly divided into two groups as follows: group I (experimental group) received a single intraperitoneal dose of 3.0 g/kg body weight amoxicillin (Wako Chemical Ind., Tokyo, Japan) in 0.5% carboxymethylcellulose (Wako Chemical Ind.) solution (1 ml per 100 g) and group II (control group) received carboxymethylcellulose solution only (1 ml/100 g). All of the animals received a standard pellet food (Oriental Yeast Co., Tokyo, Japan) ad libitum and were allowed free access to drinking water at any time during the experimental period. All experiments were performed in accordance with the Guidelines for the Use of Experimental Animals at Tokyo Dental College (approval number 222302).

Body weight and serum analysis

All experimental and control rats were weighed at the end of the treatment. In order to analyze serum for calcium metabolism, blood from experimental (n=15) and control animals (n=15) was sampled at days 1, 3, and 7 under anesthesia. Serum calcium (Ca), phosphate (IP), and magnesium (Mg) concentrations were measured at the end of the treatment with an autoanalyzer (FUJI DRI-CHEM 3500 W, Fuji Film Co., Tokyo, Japan).

Statistical analyses were carried out using Stat Light 2000 software (Yukmus Co., Tokyo, Japan). The values in each group are given as the mean ± standard deviation. Differences between groups were evaluated using the Aspin–Welch t test and Student's t test.

Morphological assessment

For morphological assessment, the animals (n=30) of both groups were randomly divided into three subgroups for LM, CMR, and SEM analysis. The animals were anesthetized with intraperitoneal injection of 150 mg/kg sodium pentobarbital and transcardially perfused with fixative solution containing 2.5% glutaraldehyde and 2% paraformaldehyde (Merck, St. Louis, USA) in 0.1 M sodium cacodylate buffer (pH 7.4) for 10 min at room temperature 1 week after injection. The lower incisors were dissected out of the jaws and immersed in fixative of the same composition for 1 day at 4°C. For light microscopic observation, they were then demineralized with 10% sodium EDTA (pH 7.4) and prepared into paraffin sections. The remaining undemineralized samples were embedded in resin (Rigolac-2004, Nisshin EM Co., Ltd., Tokyo, Japan) and ground for processing for CMR or SEM.

Light microscopy For LM, the demineralized samples were dehydrated in a graded series of ethanol, infiltrated through xylene, and embedded in paraffin. Four-μm-thick longitudinal serial sections were cut and stained with hematoxylin. Images were captured with a CCD camera mounted on a Zeiss Axiophot 2 microscope (Carl Zeiss, Hallbergmoos, Germany).

Contact microradiography Undemineralized samples were dehydrated in ethanol, followed by infiltration with styrene and embedding in polyester resin (Rigolac, Nisshin EM Co., Tokyo, Japan). One hundred-μm ground sections were prepared in a direction perpendicular to the long axis of the tooth. Contact microradiography was performed with a soft X-ray generator (CMR-3, Softex Co., Tokyo, Japan) and the degree of X-ray transmittance was evaluated. The photographic conditions were 10 kV, 3 mA, film-focus distance of 44.4 mm, and photographic time of 7–25 min. A high-precision photo plate (HRP-SN-2, Konica Minolta, Tokyo, Japan) was used as the photographic plate, and the image was developed with the D-19 developer for 5 min.

Scanning electron microscopy Samples embedded in resin were ground with the Refine Polisher (Refine Tec. Ltd., Yokohama, Japan). After washing with distilled water three times and dehydrated with a series of alcohol, the specimens were coated with gold–palladium using a sputter coater (VG Microtech Ltd., Sussex, UK) and viewed with a field emission SEM (JSM-6340F, JEOL, Tokyo, Japan) at an accelerated voltage of 15 kV.

Immunohistochemical assessment

For immunohistochemical observation, additional five experimental rats and three control rats were used. Under anesthesia, all rats were perfused with 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 10 min. The lower incisors were dissected out of the jaws and immersed in the same fixative for 6 h at 4°C. After demineralization with EDTA, the tissues were rinsed in 0.1 M sodium phosphate buffer, dehydrated, and embedded in paraffin wax. Four-μm-thick longitudinal serial sections were cut. The sections were dewaxed and rehydrated. To reveal antigens, the sections were incubated in trypsin solution (Sigma-Aldrich Co., St. Louis, USA) for 20 min at 37°C.

In this experiment, the previously reported avidin–biotin–peroxidase complex method [8] was employed. The sections were immersed in blocking solution for 20 min at room temperature to reduce nonspecific immunoreaction. The sections were then incubated with the primary antibody, rabbit polyclonal antibody raised against the peptide (90-111) near the N-terminal of rat dentin matrix

protein 1 (DMP-1) conjugated with KLH (Takara Bio Inc., Shiga, Japan), overnight at 4°C in a moist chamber. This antibody specifically reacted with rat DMP-1 [9]. In the negative controls, the primary antibody was replaced with phosphate-buffered saline (PBS). After washing with PBS, the sections were incubated with a biotinylated secondary antibody for 30 min at room temperature. Labeling was visualized using the Elite ABC kit (Vector Laboratories, Inc., CA, USA) according to the manufacturer's instructions. The sections were treated with diaminobenzidine solution (Vector Laboratories, Inc.) for 5 min, followed by counterstaining with or without hematoxylin, then washed in water, dehydrated, and coverslipped for viewing by LM.

Results

Body weight and serum values

All experimental group rats survived the experimental period after single injection of amoxicillin. However, increase in body weight was significantly lower in the experimental group (110.6±13.3 g; n=25) (mean±SD) than in the control group (132.5±5.8 g; n=15) at day 7 after injection of amoxicillin (p<0.01, Aspin–Welch t test).

Figure 1 shows changes in serum Ca, IP, and Mg concentrations in the experimental and control animals on days 1, 3, and 7 after injection. The concentrations of serum IP and Mg significantly increased in the experimental group compared with the control group at day 1 after injection. At day 7, the level of both components in the experimental rats was almost at the same level as that in the controls. No significant difference was observed in serum Ca concentrations between the two groups during the experimental period.

LM observation

In the experimental group, the most prominent finding in the rat incisor dentin was a large number of irregularly shaped areas (interglobular dentin) which showed less staining for hematoxylin during the early stage of dentinogenesis (Fig. 2a). Concomitantly, a large number of

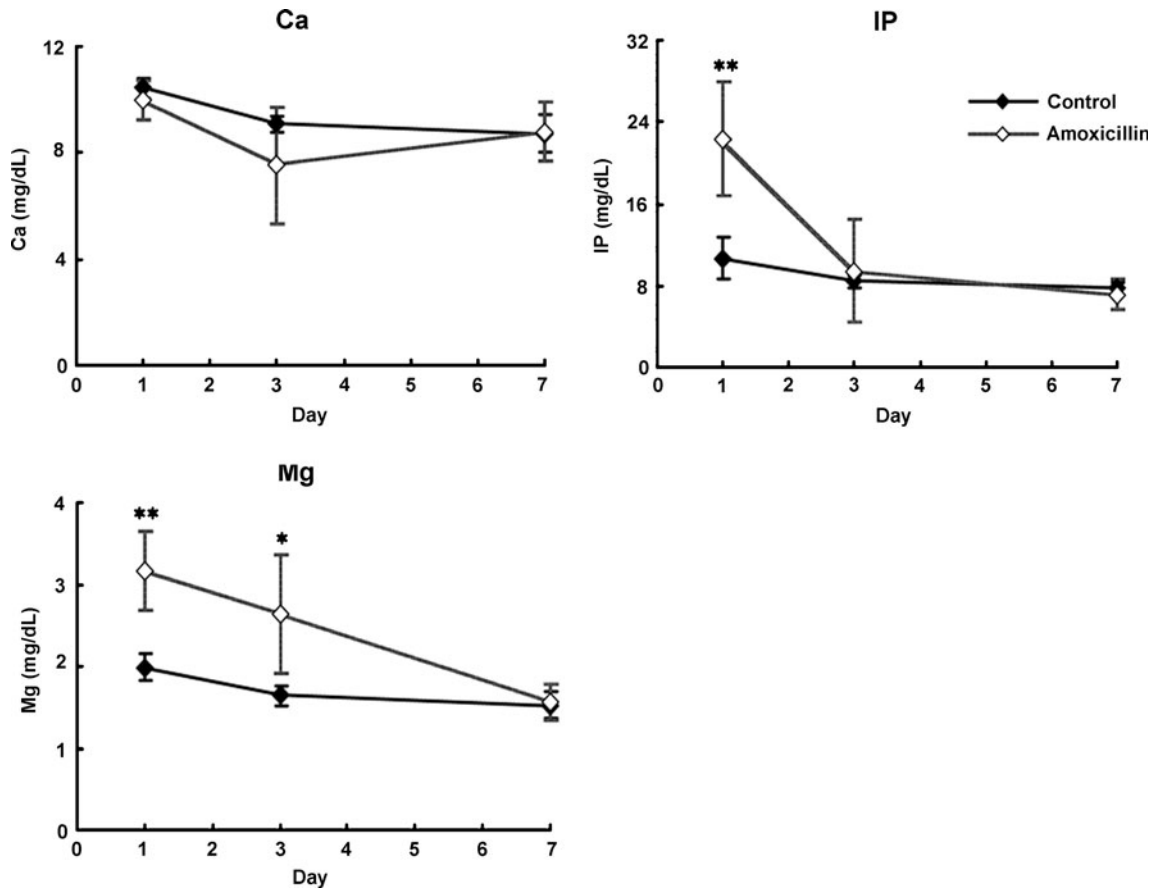

Fig. 1 Change in serum Ca, IP, and Mg levels in control and experimental rats. Values are expressed as mean±SD (n=5 in each group). *p<0.05, **p<0.01 as compared with the control group

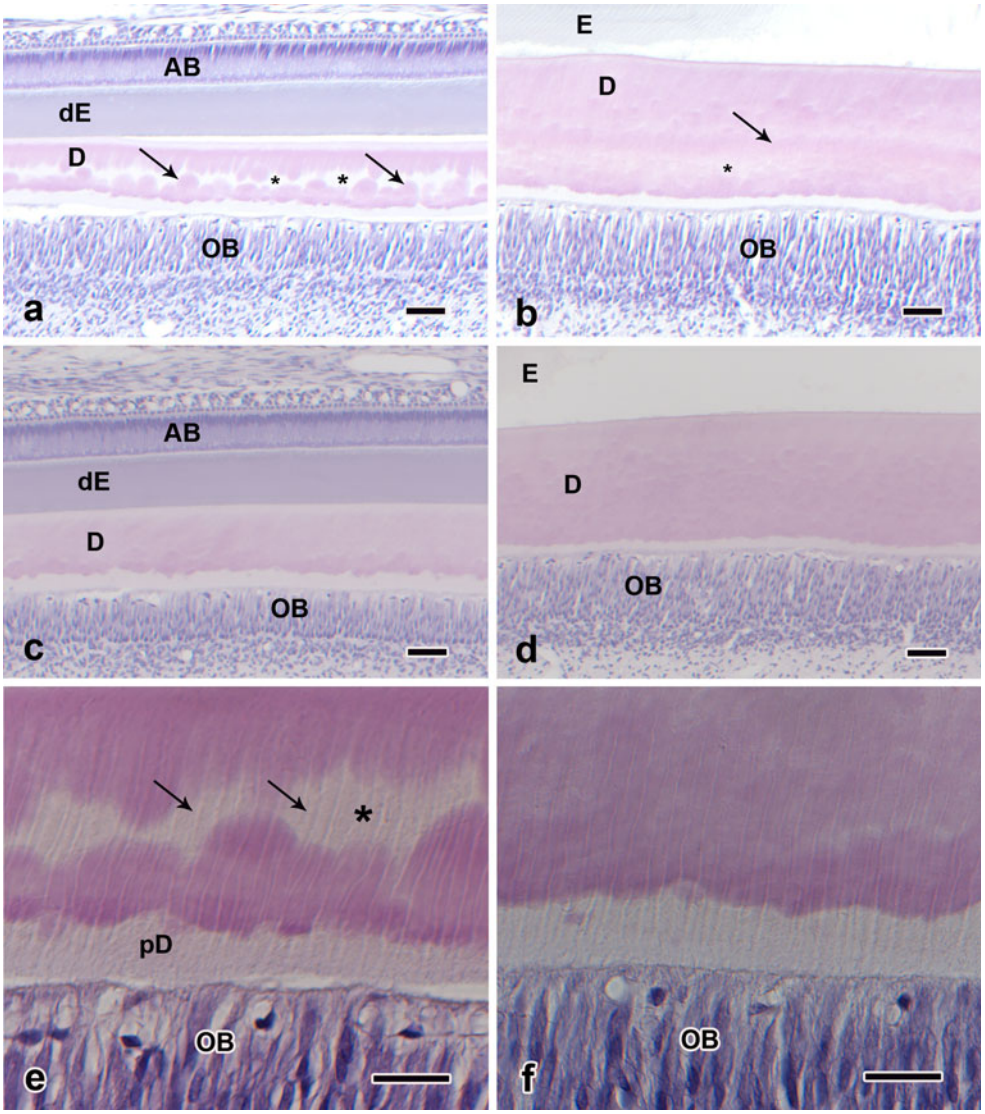

Fig. 2 Longitudinal sections from experimental (**a**, **b**, **e**) and control (**c**, **d**, **f**) rats stained with hematoxylin. **a** Note extended interglobular dentin (*asterisks*) in early stage dentinogenesis. *Arrows* indicate globular calcospherites stained with hematoxylin. **b** Band (*arrow*) stained with hematoxylin runs toward the incisal edge of dentin. Interglobular dentin (*asterisk*) formed by amoxicillin-exposed odontoblasts was less prominent at late stage dentinogenesis. **c** Odontoblasts in control rat were tall, columnar, and polarized. Secretory ameloblasts were tall, columnar with Tomes' processes extending into developing enamel. **d** Toward incisor tip, dentin gradually increased in thickness. Enamel was almost lost by tissue processing with EDTA. **e** Higher magnification views of amoxicillin-treated dentin. Note interglobular dentin (*asterisk*) and dentinal tubules (*arrows*) in experimental rats. Odontoblasts showed almost the same morphology in comparison with control rats. **f** Higher magnification view of control rat dentin and odontoblasts. *dE* developing enamel, *E* enamel, *D* dentin, *AB* secretory stage ameloblasts, *OB* odontoblasts, *pD* predentin. *Bars*=20 μm (**a**–**d**), 50 μm (**e**, **f**)

globular calcospherites (globular zones of mineralization) of various sizes were observed in the dentin, which were made by odontoblasts after amoxicillin administration (Fig. 2a, arrows). Interglobular dentin gradually decreased in area towards the incisal edge. A narrow line which showed moderate staining for hematoxylin demarcated the affected dentin (pulpal side) from the normal dentin (enamel side) (Fig. 2b). Odontoblasts in the amoxicillin-treated rats (Fig. 2a, b) were similar in shape, polarization, and arrangement to those in the control rats (Fig. 2c, d).

At higher magnification, no structural abnormalities in the dentin matrix, including size, distribution, or course of dentinal tubules, were observed in comparison with the controls (Fig. 2e, f). In the experimental group, no detectable structural alteration in the enamel matrix was observed (Fig. 2a). Secretory ameloblasts were observed to be mostly normal in shape, polarization, and arrangement of amelogenesis (Fig. 2a) in comparison to those of the control group (Fig. 2c).

CMR observation

Contact microradiography revealed a clear increase in the area of interglobular dentin, representing disrupted mineralization, which now occupied most of the incisor dentin during the early stage of dentinogenesis in the experimental group (Fig. 3a; compared to control rats in Fig. 3c). Disrupted mineralization was observed in the pulpal side dentin toward the incisal edge (Fig. 3b). No morphological alteration or hypomineralization was observed in the enamel throughout all the stages of amelogenesis (Fig. 3b). The thickness of the enamel was the same as that in the control rats (Fig. 3d).

SEM observation

Since no alteration in enamel was observed by LM or CMR, as described above, we focused on the ultrastructure of the dentin. Interglobular dentin was located among

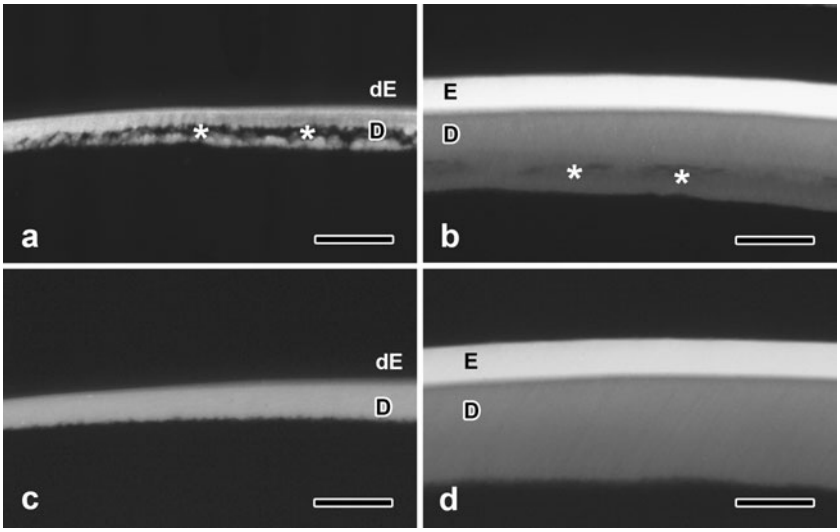

Fig. 3 Microradiograph of rat mandibular incisor from experimental (**a**, **b**) and control (**c**, **d**) groups. **a** Radiolucent irregular band (*asterisks*) corresponding to interglobular dentin as indicated in Fig. 2**a** was observed during the early stage of dentinogenesis. **b** Dentin (*D*) formed after amoxicillin injection showed poorly calcified area (*asterisks*) at late the stage of dentinogenesis. No hypomineralization or defect was observed in enamel (*E*). **c** Normal dentinogenesis was observed at early stage. **d** At late stage in dentinogenesis, highly mineralized enamel (*E*) was observed on matured dentin (*D*). *dE* developing enamel. *Bars*=200 μm

calcospherites, becoming abnormally prominent in amoxicillin-exposed rat incisor dentin (Fig. 4a). However, the size and distribution of the dentinal tubules in the experimental rats (Fig. 4b) were similar to those in the control rats.

IHC observation

Immunohistochemistry was used to determine the specific localization of DMP-1 in the rat incisor dentin. The specific immunoperoxidase labeling of DMP-1 was observed in the interglobular dentin, as well as the widened area of predentin formed after amoxicillin administration (Fig. 5a). The intensity of immunostaining in the interglobular dentin was lower than that in the predentin. The dentinal matrix surrounding the interglobular dentin (calcospherites) showed no reaction products. Osteocytes in alveolar bone matrix used as a positive control sample for DMP-1 showed intense

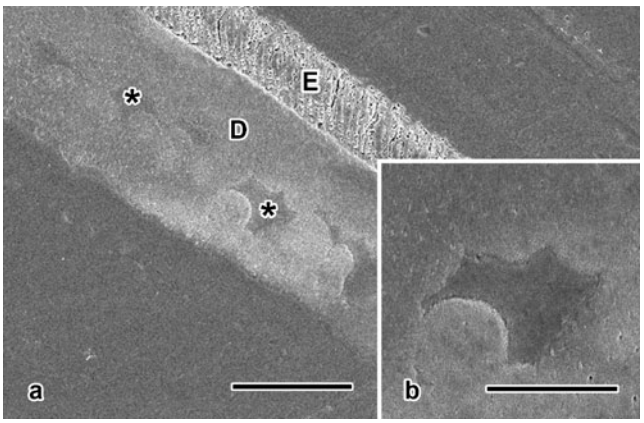

Fig. 4 Scanning electron microscopy of altered dentin. **a** Large amount of interglobular dentin (*asterisks*) was observed under enamel (*E*). **b** Higher magnification view of interglobular dentin in **a**. *Bars*=100 μm (**a**), 50 μm (**b**)

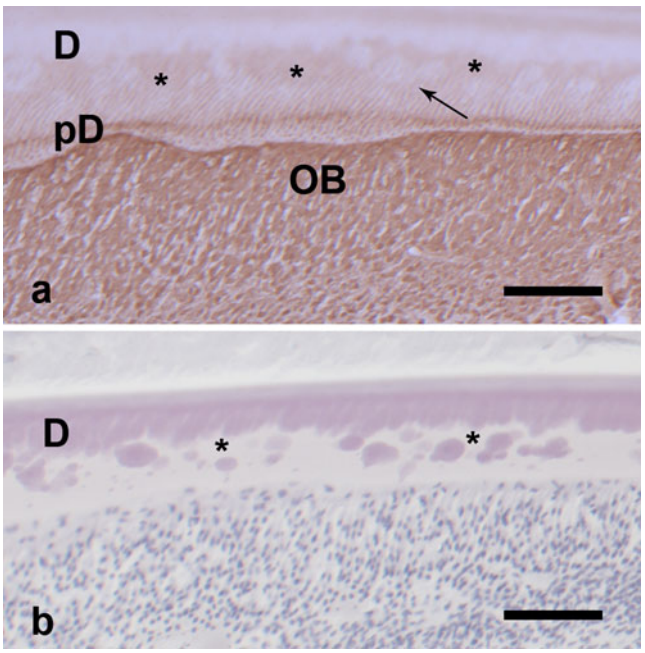

Fig. 5 Immunohistochemical localization of DMP-1 in rat incisor tooth exposed to amoxicillin. **a** Interglobular dentin (*asterisks*) as well as predentin (*pD*) were positively stained with anti-rat DMP-1 antibody. Odontoblasts (*OB*) were also positively stained. Dentin (*D*) and calcospherites (*arrow*) were not stained with antibody. **b** No immunoreaction products were observed in negative control sections. *Asterisks* indicate large areas of interglobular dentin. *D* dentin. Control section was counterstained with hematoxylin. *Bars*=100 μm

immunoreaction products (data not shown). The negative staining of samples for DMP-1 showed no immunoreaction (Fig. 5b).

Discussion

A previous morphological study in permanent first molars from children with MIH showed a high frequency of interglobular dentin with no structure alteration in odontoblasts [10]. In the present study, we found an abnormality in dentinogenesis in rat incisor after single-dose amoxicillin administration. The most conspicuous finding was an increase in the area of interglobular dentin, indicating depression of odontoblastic activity in dentin mineralization during the experimental period. To our knowledge, this is the first study to report that amoxicillin can affect dentin mineralization in vivo. Intensity of dentin alteration was observed to vary between rats in the amoxicillin-treated group depending on individual differences, particularly body weight. Rats with lower body weight showed increasing areas of interglobular dentin.

In another recent culture experiment, however, exposure to amoxicillin showed no effect on dentin in explant [7]. This earlier study evaluated morphological change in dental hard tissue using demineralized paraffin sections, though, so information on mineralization in the explant would have been lost during tissue preparation.

Although interglobular dentin is considered a normal histological feature, the dentin became abnormally prominent with disruption of mineralization such as that seen in vitamin D deficiency, vitamin D-resistant rickets, or severe fluorosis [11]. Similar findings were also reported in animals, including parathyroidectomized rats [12], in rats with chronic calcitonin deficiency [13], and in deer exposed to excess fluoride [14]. An increase in predentin, as well as alterations in dentin mineralization evidenced by an irregular mineralization front and the presence of numerous isolated calcospherites in thyroparathyroidectomy rats, has also been described by others [15]. To explain these findings, Chardin et al. [15] postulated two alternative hypotheses: (1) a delay in mineral deposition together with an alteration in the mineralization pattern due to hypocalcemia and (2) change in the relative quantities of the collagenous and non-collagenous components of predentin due to an alteration in the rate of secretion.

Serum levels of IP and Mg, both known to be important elements in dentin mineralization, in the experimental group were transiently higher than those in the control group. In respect to serum levels of Ca, no significant difference was observed between the experimental and control rats during the experimental period. Interpretation of the present results is very difficult. However, alteration in the kidneys such as vacuolation of tubular epithelia or pyknosis of the glomeruli resulting from a toxic dose of amoxicillin [16] may have been the leading cause of the highly elevated levels of serum IP and Mg seen here. In contrast to the conclusions of Chardin et al. above, we believe that sufficient amounts of serum Ca and/or IP were circulating in the blood vessels during the experimental period for dentin biomineralization to take place. Therefore, we speculated that odontoblastic transport of ions to the mineralized front of dentin was transiently and selectively disturbed after amoxicillin administration, resulting in the formation of large areas of interglobular dentin.

Dentin contains a large amount of collagen and a lesser amount of non-collagenous proteins, including members of the small integrin-binding ligand N-linked glycoprotein (SIBLING) family. Milan et al. [17] reported that fluoride altered the biochemical structure of dentin phosphoprotein, which is believed to be involved in the nucleation and control of mineralization in dentin [18], resulting in the disruption of dentin mineralization. Large areas of unmineralized dentin were also observed in dentin sialophosphoprotein knockout mice [19]. In the deciduous teeth from hypophosphatemic children, low molecular weight non-collagenous proteins such as dentin sialoprotein, osteopontin, and osteocalcin accumulated in non-mineralized interglobular spaces, leading to abnormalities in the mineralization process [20]. These low molecular weight molecules in the dentin were considered to have resulted from alteration in the post-translational processing of molecules or partial degradation of matrix molecules.

To test this hypothesis, proposed by Boukpessi et al. [20], we performed immunohistochemical examination using specific antibody for rat DMP-1 (N-terminal), a member of the SIBLING family. The results showed moderate immunoreaction products in interglobular dentin occurring after amoxicillin administration. It has been reported that the NH_2-terminal fragments of DMP-1 localized to predentin and not mineralized dentin matrix in rat molar [21], indicating that it as an inhibitor of dentin mineralization. Zhang et al. [22] demonstrated that DMP-1 N-terminal fragments were located not only in normal rat predentin, but also in widened areas of predentin in Hyp mouse. Therefore, another possible explanation for the formation of a large number of unmineralized areas in the dentin observed here is that amoxicillin injection may directly and/or indirectly affect the SIBLING family, including DMP-1, leading to disruption of dentin mineralization, as suggested by Boukpessi et al. [20].

An additional goal of this study was to investigate whether a single dose of amoxicillin induced change in ameloblasts during enamel development in vivo. In this study, no morphological alteration was observed in ameloblasts during the secretory stage at 7 days after a single

dose of amoxicillin. In addition, no developmental enamel defect accompanied by hypomineralization was observed, even at a toxic level dose. Therefore, these findings do not support an earlier study suggesting that antibiotic amoxicillin treatment during early childhood causes enamel defects in maxillary central incisors as a result of dysfunction in secretory stage ameloblasts [6]. In a recent in vitro study, Laisi et al. [7] showed that amoxicillin at a concentration of 4 mg/ml increased enamel thickness in exposed explants, indicating enhancement of ameloblastic function. Our results are not in accord with those of this earlier study. This discrepancy, however, may be accounted for by the experimental design employed (in vivo animal model study vs in vitro explant study), type of animal used (rat vs mouse), and drug dosage (a single intraperitoneal injection of 3.0 g/kg body weight amoxicillin in rat vs exposure to 4 mg/ml amoxicillin in explant).

On the other hand, Jälevik and Norén [23] suggested that damage to early maturation stage ameloblasts caused severe hypomineralization of the enamel in permanent first molars. Enamel hypomineralization has also been considered to represent a qualitative change in the matrix produced by disorders in maturation stage ameloblasts [24]. In this context, kallikrein 4, a protease secreted from maturation ameloblasts, has shown to degrade the residual enamel matrix, facilitating its removal from the enamel [25, 26]. This leads to the supposition that exposure of maturation stage ameloblasts to amoxicillin leads to their dysfunction, resulting in hypomineralization of the teeth. However, this issue cannot be resolved based on the data obtained from our rat incisor animal experiments (a single intraperitoneal injection) alone. Therefore, further study on maturation stage ameloblasts from rat molar (daily oral administration) is required to fully elucidate the mechanism involved in enamel hypomineralization in human MIH.

Conclusion

In conclusion, within the limits of this animal study, the present results demonstrate that single injection of a toxic dose of amoxicillin induces dentin abnormality in rat, with disruption of dentin mineralization, in particular. Unexpectedly, we found histologically that exposure to amoxicillin caused no morphological alteration during the secretory stage of ameloblast development within the experimental period. However, further research is needed to assess the association between dental enamel defect/hypoplasia and amoxicillin during tooth development.

Acknowledgments We would like to thank Associate Professor Jeremy Williams, Tokyo Dental College, for his editing and language assistance for the improvement of the manuscript.

Conflict of interest The authors declare that they have no conflict of interest.

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