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ORIGINAL ARTICLE

How prenatal environmental factors affect rat molar enamel formation?

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Abstract

Amelogenin (AMELX) and ameloblastin (AMBN) are crucial for enamel formation, and interruptions in the production of these proteins may cause enamel defects. We investigated how prenatal environmental factors (chronic stress, bisphenol A (BPA), amoxicillin, and lipopolysaccharide (LPS)) affect AMELX and AMBN production of ameloblasts. Fifteen pregnant Sprague–Dawley rats were divided into four experimental groups and a control group. Chronic-stress group rats were exposed to a 12:12 light/light cycle (LL) from day E18 until delivery. BPA group rats were orally administered 5 µg/kg BPA daily from day E1 until delivery. Amoxicillin group rats were injected 100 mg/kg amoxicillin daily from day E18 until delivery. LPS-infection group rats were injected 125 µg/kg bacterial LPS once on day E18. Seven pups from the control group and ten pups from the experimental groups were euthanized on P10. Sections were stained with hematoxylin and eosin (H&E) and Gomori's one-step trichrome staining (GT) and incubated with rabbit polyclonal antibodies to AMELX and AMBN, to evaluate staining intensity at ameloblast stages. The surface morphology was evaluated with a stereomicroscope. AMELX ($p=0.008$, $p=0.0001$, $p=0.009$) and AMBN ($p=0.002$, $p=0.001$, $p=0.0001$) staining of all groups were significantly lower than that of the control group in the secretory, transitional, and maturation stages. Abnormal enamel matrix formation was observed in the H&E and GT staining sections of all experimental groups. Yellowish coloration of the amoxicillin group was observed in morphologic evaluation.

Keywords Enamel · Developmental biology · Enamel formation · Extracellular matrix · Histochemistry

Introduction

Dental enamel is a highly mineralized and almost acellular biological tissue. The main components of enamel consist of hydroxyapatite crystallites. Ameloblasts are epithelial cells responsible for enamel formation. Secretion and deposition of an organic extracellular matrix via ameloblasts is the first step in enamel formation [1]. Protein components of this extracellular matrix control the initiation, pattern, orientation, and organization of hydroxyapatite crystals. These protein components are encoded by tooth-specific, non-pleiotropic genes. Amelogenin, ameloblastin, and enamelin (AMELX, AMBN, and ENAM, respectively) are the three main components of enamel matrix and together are referred to as enamel matrix proteins [2].

AMELX constitutes more than 90% of total enamel protein content and is the main structural protein of enamel matrix. AMELX is crucial for the development of an enamel layer with normal thickness and composition [2].

AMBN is the second most common protein of the organic matrix and constitutes 5% of total enamel protein [2]. AMBN exhibits cell adhesion properties in addition to controlling ameloblast cell differentiation [3].

ENAM, which comprises roughly 3–5% of total enamel protein, is expressed during the three main stages of amelogenesis [2, 4]. However, its expression terminates prior to the expression of AMELX. ENAM is much less abundant than AMELX and hydrophilic in nature. It is the largest known enamel glycoprotein [2]. Previous studies have

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observed that a true enamel layer has not formed in knockout mice in which ENAM was not synthesized [5].

Developmental enamel defects are present before eruption but can only be detected after eruption of a tooth [6]. The enamel of affected teeth has a hypomineralized and porous structure. As a result, these teeth are more susceptible to breakdown and caries [7]. Studies have shown that environmental factors implicated in defects may affect individuals during prenatal, perinatal, and postnatal life [8, 9].

Emotional stress during pregnancy has been shown to affect behavioral and physiological development [10]. In the presence of accompanying adverse conditions, it has been observed that the prevalence of post-traumatic stress disorder during pregnancy can spread up to 40% [11]. Exposure to stress is one of the most common conditions that may negatively affect intrauterine life. Furthermore, different types of stress can affect various tissues [12]. The current study is thought to be the first to evaluate the effect of prenatal stress on enamel tissue.

Bisphenol A (BPA) is an environmental estrogen that disrupts hormone receptors or interferes with the generation of endogenous estrogens [13]. This exogenous product leads to health problems in humans and animals as a result of the damage it causes to hormone balances and the endocrine system [14]. Recently, BPA was shown to generate enamel defects in rats [15]. However, prenatal BPA exposure in terms of AMELX and AMBN production has not been previously investigated.

The first procedure for infections that develop during pregnancy is to eliminate infection. Penicillin (penicillin V or amoxicillin) is a frequently recommended antibiotic for the treatment of fever and systemic infection during pregnancy. The penicillin group, including amoxicillin and cephalosporins, is considered safe during pregnancy [16]. Many animal studies have shown the effect of drugs, such as amoxicillin, in altering the amelogenesis process [17–20]. Additional studies have indicated that children treated with amoxicillin have an increased risk of developing enamel defects. However, the cellular and molecular cascades responsible for defects are still unknown; the data from the current study sheds light on this topic [20].

Bacterial infection is another factor that causes anomalies in the amelogenesis process. Bacteria and/or bacterial products, such as lipopolysaccharides (LPS), may cause periapical lesions and alveolar bone disorders [21]. Childhood diseases, such as pneumonia or otitis media that are accompanied by high fever, are also risk factors for the development of enamel defects [22]. High fever is a result of LPS production, so infection, rather than high fever, may be the reason for enamel defects.

The aim of this research was to investigate the effects of different prenatal environmental factors (chronic stress, bisphenol A [BPA], amoxicillin, and LPS) on AMELX and AMBN production in rat offspring during amelogenesis using immunohistochemistry. The null hypothesis was that there is no significant difference between these environmental factors in their effects on AMEL and AMBN production in rats.

Materials and methods

Animals

All experimental protocols were approved by the Marmara University Animal Care and Use Committee (Project no: 006.2017.mar). Fifteen female and five male adult Sprague–Dawley rats (250–300 g) were obtained from the Marmara University (MU) Animal Centre (DEHAMER). Quality assessment of the study was performed using the ARRIVE guidelines [23]. The sample size was determined according to similar previous studies, and the number of animals selected was the lowest necessary to obtain accurate results. Basic handling and experimental care was ensured throughout all investigation stages that used living animals [20]. The rats were randomized into five groups of four animals each (three females and one male).

The animals were allowed to acclimatize to laboratory conditions for one week before the experiments began and were housed in plastic cages with stainless steel grill tops. Female and male rats were caged together for mating at the start of the experiment. The presence of a vaginal plug was taken as an indicator of pregnancy. The female rats (three rats for each group) with plugs were placed in different cages marked with the date of breeding. Seven pups from the control group and 10 pups from the experimental groups were euthanized following birth. The total number of animals involved was 67 (20 adults and 47 pups).

Experimental procedures

Five groups of four animals were randomly separated as follows: (1) control group, (2) chronic stress group (CS), (3) bisphenol A group (BPA), (4) amoxicillin group (AX), and (5) infection group (LPS).

The control group and all experimental groups were maintained in the laboratory at 22 ± 2 °C with a relative humidity of 60–70% and were fed a standard diet throughout the experiment. Tap water was given as drinking water, and it was available ad libitum except to the BPA group. All groups were kept with a 12-h dark/light cycle except CS.

The animals in the CS group were maintained under a 12-h dark/light cycle until prenatal day 18. From prenatal day 18 until delivery, this group was then maintained with a 12-h light/light cycle to cause chronic stress.

The rats in the BPA group were placed in glass cages, and drinking water was given from glass bottles to avoid BPA contamination. During pregnancy, they were given 5 µg/kg BPA (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.0001% ethanol. The animals in the AX group were injected daily with 100 mg/kg amoxicillin (Remoxil 1 g injectable vial Ulagay IE, İstanbul, Turkey) intraperitoneally from prenatal day 18 until delivery.

The animals in the LPS group were injected with 125 µg/kg bacterial LPS (*Escherichia coli*, 0111: B4, Sigma, St. Louis, MO, USA) intraperitoneally once on prenatal day 18. The body temperature of the animals was measured rectally and recorded before and after the injections, with intervals of one hour (as the temperature returned to normal within one hour).

After delivery, the pups of all groups were breastfed until postnatal day 10. No animals were excluded because all mothers and offspring were observed to be in good general health. Ten pups from each experimental group and seven pups from the control group were randomly selected from three mothers. The selected pups were euthanized with an overdose of ketamine (Ketalar) and xylazine (Rompun).

Mandibles were dissected at the symphysis, and left hemimandibles were used for immunohistochemical and histologic evaluations. The right hemimandibles were used for macroscopic evaluation.

Unerupted molar crowns of right hemimandibles were embedded in paraffin, inspected stereo-microscopically, and photographed.

All the above procedures were performed by one researcher who was aware of group allocation, and the samples were given codes to ensure blinding in the histological procedures.

Histological processing

The pup mandibles were resected and fixed in 10% neutral buffered formaldehyde solution for light microscopic examination. After decalcification for 5 to 7 days in 10% ethylenediaminetetraacetic acid (EDTA), the specimens were dehydrated in a graded ethanol series, embedded in paraffin, and 3 µm sections were prepared. Sections were deparaffinized and stained with hematoxylin and eosin (H&E) and Gomori one-step trichrome (GT). Some of the sections were adhered to positively charged slides for the immunohistochemical detection of AMEL and AMBN.

Immunohistochemistry

The sections were deparaffinized with xylene three times for 10 min each and rehydrated through a graded series of ethanol. Endogenous peroxidases were blocked by incubation for 20 min in a freshly prepared solution of 3% H_2O_2 in methanol. Antigen retrieval was performed in 10 mM sodium citrate buffer (pH 6.0) for 20 min in a microwave oven. Sections were then washed in 0.01 M phosphate-buffered saline (PBS, pH 7.4), and non-specific immunoreactivity was inhibited with protein blocking solution (Expose Mouse and Rabbit Specific HRP/DAB Detection IHC kit, Abcam, Cambridge, UK). Primary rabbit anti-AMBN (Bioss Antibodies Inc., Massachusetts, USA) at 1:2000 and rabbit anti-AMELX (Abcam, Cambridge, UK) at 1:2000 antibodies were applied and incubated at 4 °C overnight. After rinsing with PBS, sections were incubated with goat anti-rabbit HRP-conjugate for 15 min at room temperature. For visualization of the sections, 3–3' diaminobenzidine tetrachloride (DAB) was used, followed by counterstaining with Mayer's hematoxylin. The sections were finally rinsed with water, dehydrated, and mounted. The color reaction was terminated when the sections reached the appropriate color intensity, and the background color was faint. The negative control was performed by replacing the primary antibodies with a protein blocking solution. These control sections contained no specific immunoreactions.

The analysis of AMBN and AMELX immunoexpression were performed on rat molar teeth, including the secretory, transitional, and maturation stages of amelogenesis. (The ameloblasts assume a columnar shape with their nuclei located near the proximal ends of cell bodies and contain Tomes' processes at their distal ends at which the enamel matrix is laid down in the secretory stage. Ameloblasts are shorter in the transitional stage, and those in the maturation stage possess either ruffled or smooth profiles of their apical surfaces adjacent to the enamel; they are referred to as ruffle-ended ameloblasts and smooth-ended ameloblasts.) The intensity of AMBN and AMELX immunolabeling in each stage was evaluated as the following scores: (0) none, (1) weak, (2) moderate, and (3) strong staining. [20] Three sections from each animal were used for immunohistochemical evaluation. The sections were analyzed by one blinded researcher using an Olympus BX51 (Tokyo, Japan) microscope twice, with a 1-week interval between examinations. Each stage of amelogenesis was evaluated according to the intensity categories of immunolabeling and recorded.

Statistical analysis

The Number Cruncher Statistical System (NCSS) 2007 Statistical Software (Utah, USA) was used for statistical analysis. Descriptive statistical methods were used for frequency and percent distributions in addition to q in Fisher's exact extension of probability test, Freeman–Halton r × c table, and McNemar's test were used for the evaluation. The Freeman–Halton test was used for the evaluation of AMELX and AMBLN immunoexpression intensity in the secretory, transition, and maturation stages of ameloblasts. McNemar's

test was used to evaluate the repetitive variables. Statistical significance was accepted at a two-sided p value ≤ 0.05.

Results

Stereomicroscopic findings

The macroscopic evaluation revealed that all prenatal stress factors affected the molars in varying degrees when compared with the control group. The control group's molar enamel presented a bright, translucent, white, smooth appearance macroscopically (Fig. 1A), whereas the enamel surface of the LPS group had large, opaque, demarked areas (Fig. 1B). All enamel surfaces were also opaque and rough in the BPA group, along with vertical cleft areas on tubercles (Fig. 1C). The CS group molar teeth had enamel loss on tubercles surrounded with opaque areas (Fig. 1D), and all tubercle surfaces of the AX group's molar teeth had a hypocalcification appearance (Fig. 1E).

Histologic findings

Typical enamel, dentin matrix structure, and regular tooth development were observed in the control group sections (Fig. 2A, B). A defective enamel matrix structure with lighter enamel matrix staining was detected in the CS group (Fig. 2C and D). Similarly, an irregular and defective enamel matrix structure, in addition to ameloblast detachment, was observed in the BPA group (Fig. 2E and F). A lost attachment between the ameloblasts and enamel matrix was also seen in the AX group (Fig. 2G and H). Furthermore, vacuole-like structures were observed in the ameloblast cytoplasms. H&E staining of the LPS group presented attachment loss between the ameloblasts and enamel matrix (Fig. 2I and J).

Immunohistochemical findings

Immunohistochemical findings according to the developmental stages of the ameloblasts are shown in Fig. 3.

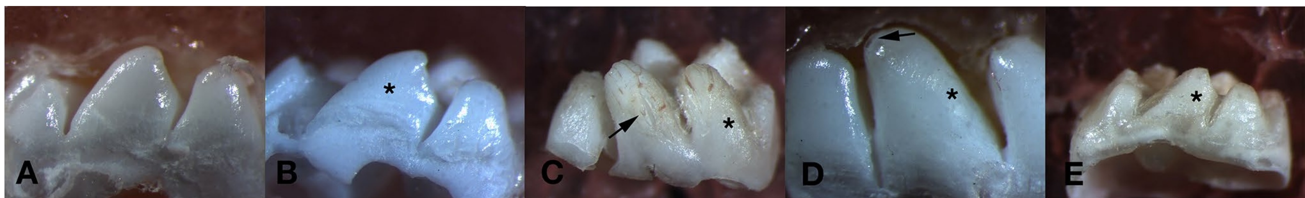

Fig. 1 Stereomicroscopic evaluation of rat molars: **A** Bright, translucent, white and smooth enamel surface of control group, **B** Demarked opacities (*) of LPS group, **C** Opaque and rough enamel surface (*) and vertical cleft areas on tubercules (arrow) of BPA group, **D** Pit like enamel loss (arrow) of CS group surrounding with hypoplasic tissue (*), **E** Hypocalsificated enamel appearance of AX group

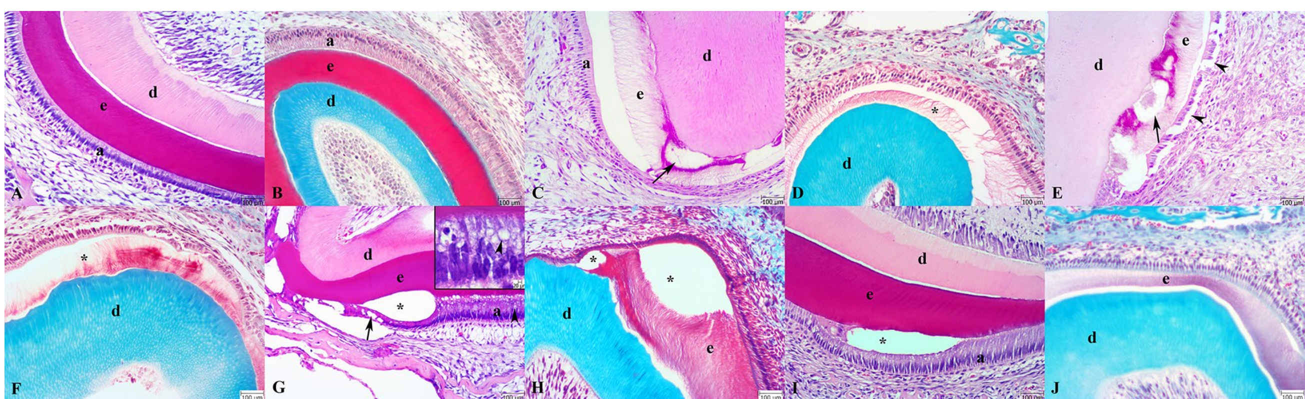

Fig. 2 **A** and **B** Regular structure of ameloblast (a), enamel (e) and dentin (d) in control group, **C** Defective enamel matrix structure of CS group in H&E (arrow) stainings, **D** Defective enamel matrix structure of CS group in GT (*) stainings, **E** Defective enamel matrix structure with ameloblast deattachment of BPA group in H&E (arrow) stainings, **F** Defective enamel matrix structure of BPA group in GT (*) stainings, **G** Defective separation of enamel matrix and ameloblasts (*), ameloblast deattachment (arrow) and vacuole-like structures in ameloblast cytoplasms (arrowhead) of AX group in H&E stainings, **H** Defective separation of enamel matrix and ameloblasts (*) of AX group in GT stainings, **I** Defective separation of enamel matrix and ameloblasts (*) of LPS group in H&E stainings, **J** Defective separation of enamel matrix and ameloblasts (*) of LPS group in GT stainings. a: ameloblast, e: enamel, d: dentin matrix

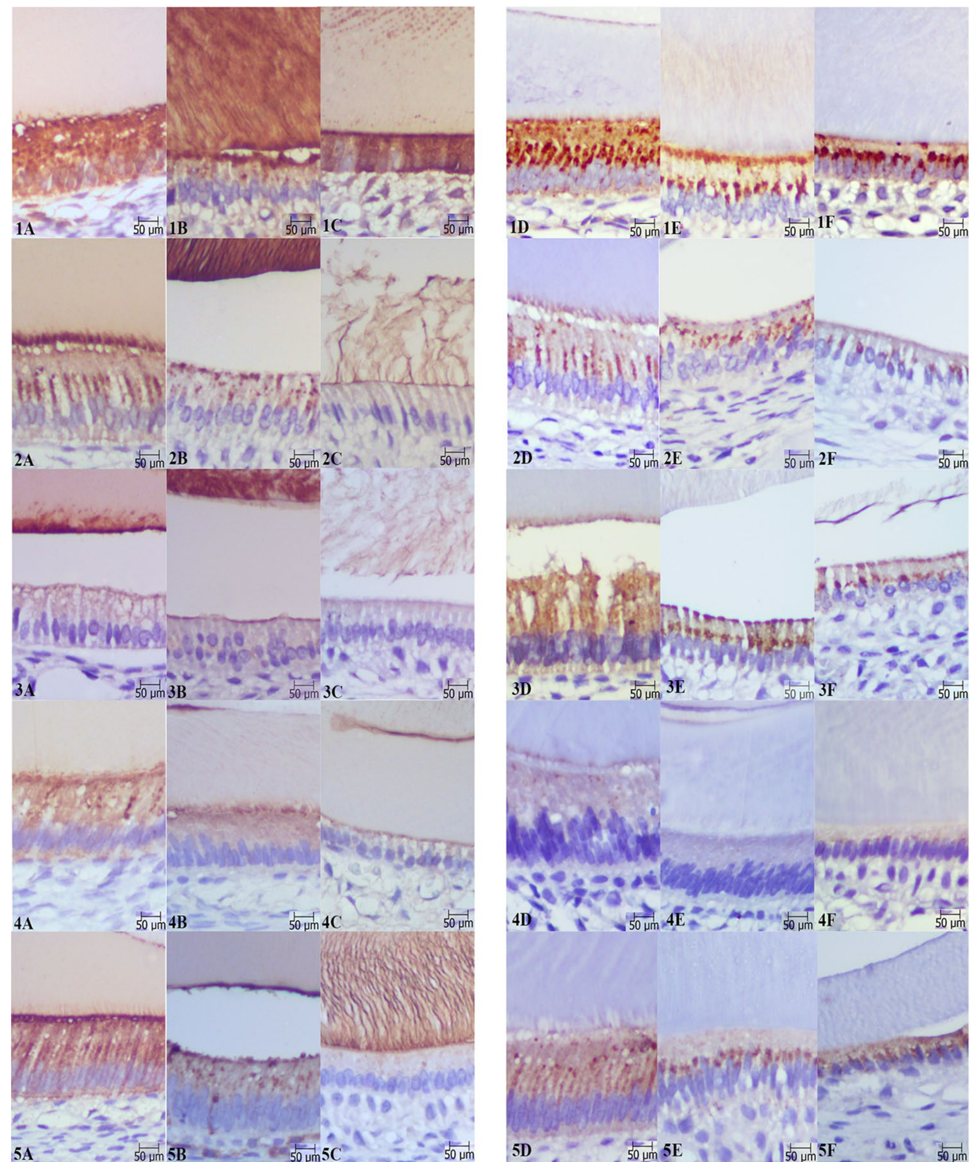

Fig. 3 AMELX immunexpression of **1A.** Control group secretory stage **1B.** Control group transition stage **1C**. Control group maturation stage **2A.** CS group secretory stage **2B.** CS group transition stage **2C**. CS group maturation stage **3A.** BPA group secretory stage **3B.** BPA group transition stage **3C**. BPA group maturation stage 4**A.** AX group secretory stage **4B.** AX group transition stage **4C**. AX group maturation stage **5A.** LPS group secretory stage **5B.** LPS group transition stage **5C**. LPS group maturation stage. AMBN immunexpression of **1E.** Control group secretory stage **1F.** Control group transition stage **1G**. Control group maturation stage **2E.** CS group secretory stage **2F.** CS group transition stage **2G**. CS group maturation stage **3E.** BPA group secretory stage **3F.** BPA group transition stage **3G**. BPA group maturation stage 4**E.** AX group secretory stage **4F.** AX group transition stage **4G**. AX group maturation stage **5E.** LPS group secretory stage **5F.** LPS group transition stage **5G**. LPS group maturation stage

AMELX staining intensities between the secretory, transitional, and maturation stages of ameloblasts were not significantly different in the control group, whereas differences were detected in the CS, BPA, AX, and LPS groups ($p = 0.013$, 0.04, 0.004, and 0.0001, respectively). AMELX intensity in the maturation stage was weakest in the CS and LPS groups, in contrast to the BPA group, in which the intensity was strongest. The secretion stage had the strongest intensity between the developmental stages in the AX and LPS groups.

Differences among groups were detected once each development step was evaluated separately. The AMELX staining intensity of the BPA group in the secretory stage was weaker than the LPS, control, AX, and CS groups ($p = 0.009$, 0.0092, 0.021, and 0.036, respectively). The greatest differences were observed in the transition stage. All groups except CS were weaker than the control group. The staining intensity of the BPA group was weaker than that of the LPS, CS, and AX groups ($p = 0.0001$, 0.003, and 0.013, respectively). The intensity of the maturation stage was significantly weaker in the control group than in the CS and AX groups ($p = 0.018$ and 0.006, respectively).

LPS was the only group in which AMBN intensity between developmental stages was found to be significantly different ($p = 0.003$). The transitional stage staining of this group was shown to have the weakest intensity.

The AX group was shown to have the weakest AMBN intensity in both the secretory and maturation stages. Staining intensity in the transition stage for all groups was significantly weaker than the control group, whereas the intensity difference between the groups was not significant.

Discussion

Developmental enamel defects are predisposing factors for tooth decay and early tooth loss due to the high prevalence of these defects. Numerous etiological factors related to defect formation have been previously studied; however, the high incidence of molar and incisor teeth defects that develop during the prenatal process highlights the importance of further study of this period [24]. The aim of our study was to determine the effects of prenatal stress, BPA toxicity, amoxicillin, and LPS-induced infection on amelogenesis. The effects of prenatal BPA toxicity and amoxicillin on enamel have been shown by previous studies [25, 26]; however, the current research is the first study to investigate the effects of stress and infection on amelogenesis.

AMELX and AMBN were selected for immunohistochemistry due to their effects on enamel formation, as reported in numerous studies. The important role of AMELX in enamel formation has been demonstrated by many human genetic analyzes and AMELX-null mice studies. AMELX regulates enamel crystal organization and elongation [27]. In vivo-targeted gene knock-out strategies have provided researchers with the opportunity to understand the direct role of AMELX in controlling mineralization.

In the absence of AMELX, the teeth of mice were seen to have discolored, disorganized, and hypoplastic enamel. The authors suggest that AMELX is essential for elongation of enamel crystals and achievement of proper enamel thickness, even if it is not required for the initiation of mineralization, since the observed thickness of AMELX-null mice enamel is only 10%–20% the thickness of normal enamel [28]. AMBN, which is the second most common protein in the organic matrix, cannot be isolated from in vivo sources because it is rapidly resorbed after secretion [29]. The direct function of AMBN in apatite mineralization could not be detected using knock-out mouse studies due to the lack of true enamel layer formation [30].

The disturbances during amelogenesis result in developmental enamel defects. Previous investigations have documented that the systemic influence of chemical substances has teratogenic effects on the fetus. Exposure to environmental factors during enamel morphogenesis results in enamel defects. Fluoride, dioxin, tetracycline, or antiepileptic drug intake during pregnancy leads to low birth weight, respiratory distress, rickets of prematurity, neonatal infections, and maternal conditions such as preeclampsia and diabetes. These issues have been reported as etiological factors that cause enamel defects [31].

Amelogenesis can be disturbed, and enamel quality can be altered by exposing rats to different environmental factors; thus, rats constitute a good model for studies on environmental factors. When developmental enamel defects are to be characterized, such an approach could be used as a predictive model of potential pathological impacts of novel pollutants.

Mental disorders are among the most common morbidities of pregnancy. Previous studies have investigated various maternal mental disorders that cause stress, including depression, anxiety disorders, post-traumatic stress disorder, eating disorders, and personality disorders. [32]. The effects of different stress types on bone development and function have been reported in various studies [33, 34]. Teeth have also been suggested as a means of measuring early-life adversity and subsequent mental health risk [35]. Therefore, chronic stress was included in our experimental groups as a potential etiological factor.

A study that investigated the effects of circadian rhythm on amelogenesis used a 12/12 light rat model similar to that used in the current study. AMELX immunoexpression reduction and significant delay in the histological development of enamel were reported, similar to the findings of this study [36]. In light of the existing literature, two possible mechanisms could have caused amelogenesis defects in this study. A drop in melatonin receptor mRNAs due to disrupted circadian rhythm may have resulted in low amelogenin levels, affecting enamel development [36]. On the other hand, increased cortisol levels as a result of stress could have led to a decrease in insulin-like growth factors (IGFs); as IGFs contribute to the amelogenesis process, increased cortisol levels could likewise decrease amelogenesis [37]. The positive relationship between maternal stress during pregnancy and childhood dental caries, as shown by another study, may be an indicator of affected enamel structure, although the study associated it with neglect [38].

BPA is a chemical that binds to estrogen and is widely used in the plastics industry; its annual production increased from 5 million metric tons in 2010 to 8 million metric tons in 2016 [39, 40]. It is a slightly soluble compound in water and can leak from plastics [41]. In recent years, researchers have investigated the prenatal effects of BPA on the highly sensitive amelogenesis process due to its damage to many tissues and organs [15]. Animal studies have shown that prenatal BPA intake affects enamel protein content and causes defective enamel structure. These defects have similar patterns to the molar incisor hypomineralization of human teeth [15, 42].

According to the results of the current paper, AMELX levels were higher in the secretory phase ameloblasts than in the transition and maturation phases. No difference was observed in AMBN levels between the development phases. The data support the results of previous studies [15]. Conversely, according to another study, no change was observed in amelogenin, ameloblastin, tuftelin, and matrix

metalloprotein (MMP)-20 levels, while enamelin levels showed an increase, and kallikrein-related peptidase (KLK4) levels decreased [26].

Amoxicillin is a broad spectrum semi-synthetic antibiotic with bactericidal effects against Gram-positive and Gram-negative bacteria [25]. The pregnancy category is defined as "B" by the Food and Drug Administration (FDA) [43]. This antibiotic is the first choice for respiratory and gastrointestinal tract, skin, and neurological infections [25]. It is also used for dentoalveolar abscesses, sinus track infections, and bacterial endocarditis [44]. The most common side effects of this antibiotic are hypersensitivity and gastrointestinal disorders [25]. Clinical case reports and epidemiological studies have suggested that amoxicillin causes dental pathologies, such as dental fluorosis [45, 46]. On the other hand, another study reported that the risk of developing defects as a result of amoxicillin use is similar to that of other antibiotics; it claimed that the reported defect could be related to the disease state necessitating antibiotic use [47]. The severity of the defect is thought to be dose-dependent. High doses resulted in defects similar to tetracycline-induced defects, while defective enamel rates decreased with low doses [25]. Current data, including this study, indicate a relationship between prenatal amoxicillin use and enamel defect development. Thus, the use of amoxicillin in pregnancy should be carefully evaluated with a risk–benefit analysis.

Infection is one of many hereditary and environmental etiological factors thought to be responsible for enamel defects [48]. Hypertension, cardiomegaly, obesity, and Alzheimer's disease as a result of prenatal LPS-induced infection have been reported in previous studies [49, 50]. Additionally, *E. coli* LPS was shown to cause alveolar bone resorption through increasing osteoclast number and activity [51]. LPS activity affects osteoclasts and may also affect ameloblast cells, which are known to be very sensitive to environmental factors. LPS may also disturb enamel matrix secretion or cause ameloblast death [21]. The reduction in AMEL secretion during the transition and maturation phases in ameloblasts in the current research supports these findings.

Conclusion

Early diagnosis and noninvasive treatment of enamel defects—one of the main subjects of pediatric dentistry—is possible by detecting the etiological factors involved in causing these defects. The current study, using histological and immunohistochemical evaluation, clearly demonstrated the effects of prenatal environmental factors on enamel structure. Defective enamel formation due to environmental factors may also be a type of biomarker that indicates toxic effects in children. Detection of the affected tooth due to prenatal BPA or amoxicillin intake also provides an opportunity to investigate possible damage and diseases affecting other tissues. Human studies are needed in addition to animal experiments to support these data.

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Author contributions CD contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; NO contributed to conception and data acquisition, performed all histologic experiments, and critically revised the manuscript; AM contributed to conception, design, performed all statistical analyses and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

Conflict of interest The authors have no conflict of interest.

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