Submitted: 09 March, 2023 Accepted: 13 April, 2023 Published: 03 July, 2023 DOI:10.22514/jocpd.2023.039

ORIGINAL RESEARCH

Journal of Clinical Pediatric Dentistry

Iron level participates in the pathological damages of dental caries in infant rats by affecting enamel mineralization

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Abstract

Iron deficiency anemia (IDA) is a common nutritional disease associated with early childhood caries. This study aimed to explore the role of iron levels in pathological changes of dental caries in childhood. Rats were divided into four groups based on their iron content: IDA, positive control (PC), high iron (HI), and negative control (NC). Except for the rats in the NC group, rats in the other groups were inoculated with *Streptococcus mutans* and fed cariogenic high-sugar fodder to induce caries. Three months later, the caries status of the molars was evaluated at both the smooth and sulcal surfaces according to Keyes scores. Scanning electron microscopy (SEM) was performed to reveal microstructural changes in caries. Energy-dispersive spectroscopy (EDS) was used to determine the elemental composition of the enamel and dentin. In addition, the histopathology of the salivary gland was detected using hematoxylin and eosin (HE) staining.The results showed that rats in the PC group exhibited obvious carious lesions. The carious score was significantly higher in the IDA group than in the PC group but was lower in the HI group. SEM revealed complete destruction of the enamel and damage to the middle dentin in the IDA group. In contrast, the molars in the HI group exhibited some degree of enamel demineralization, but the underlying dentin was almost intact. In addition, the elemental compositions of the enamel and dentin were similar among the four groups, and iron was detected only in the HI group. No differences were observed in the morphological structures of the salivary glands of rats from the different groups. In conclusion, ID enhanced the pathological damage of caries, whereas HI weakened it. Iron may participate in the pathological damage caused by childhood caries by affecting enamel mineralization.

Keywords

Caries; Children; Iron; Enamel demineralization; Salivary gland

1. Introduction

Dental caries is caused by the interaction of specific bacteria with saliva, carbohydrates, and other factors in biofilms, which can damage mineralized tooth tissues [1]. As a highly prevalent oral disease, dental caries pose a considerable burden on the economy and on quality of life. The Global Burden of Diseases, Injuries, and Risk Factors Study in 2015 estimated that permanent tooth caries affects more than 10% of the global population in 2015 [2]. This study also showed an increased prevalence of people affected by permanent and deciduous dental caries in 2005 and 2015 of 14.6% and 4.5%, respectively [2]. Consequently, the public health challenges associated with dental caries cannot be overlooked.

Anemia, a common nutritional disease, affects approximately 2.36 billion population worldwide in 2015. Iron deficiency (ID) is the most common cause of anemia, accounting for more than half of all cases [2]. Insufficient iron intake and absorption may lead to iron deficiency anemia (IDA). Estimates suggest that 56% of pregnant women and 46% of children aged 5–14 years have anemia, which is becoming a major global public health problem [3]. Some researchers have reported that caries are related to nutritional levels and that children with low weight and nutritional deficiencies have a high prevalence of caries [4–6]. Notably, many epidemiological and clinical studies have shown a positive association between early childhood caries (ECC) and increased odds of developing IDA [7, 8]. Based on 12 case-control studies, a meta-analysis performed by our research group revealed that ID was more prevalent in children with ECC [9]. However, scientific evidence of the causal relationship between ID and ECC remains limited [8]. The pathological damage caused by dental caries in response to iron levels is also not well understood.

Streptococcus mutans (*S. mutans*) is one of the main bacterial pathogens causing caries [10]. This pathogen produces

extracellular glucans from sucrose using glucosyltransferases, which enhance local accumulation of microorganisms and promote the formation of cariogenic biofilms on the tooth surface [11, 12]. Iron, one of the most important elements in the human body, is essential for the survival and proliferation of almost all microorganisms. However, studies have shown that iron has a significant inhibitory effect on the growth of *S. mutans* [13, 14]. Berlutti *et al*. [13] reported that iron-loaded saliva (Fe^{3+} > 1 mM) inhibits *S. mutans* aggregation and biofilm formation as compared with normal saliva (Fe^{3+} from 0.1 to 1 mM), while ID saliva (Fe^{3+} < 0.1 mM) increases both phenomena. Pecharki *et al*. [14] showed that iron can reduce the growth of *S. mutans* in plaque biofilms and the demineralization of enamel. Based on these studies, we speculated that iron levels may affect the development of dental caries in children.

In this study, the effects of different iron levels on the pathological characteristics of carious lesions were explored using a rat model. Our findings provide new guidelines for preventing IDA-related dental caries in clinical practice.

2. Materials and methods

2.1 Bacterial culturing

The main cariogenic bacterium *S. mutans* was purchased from the China Center for Type Culture Collection (CCTCC-AB99010; Wuhan, China). This bacteriumwas cultured in brain-heart infusion (BHI) medium at 37 °C under an anaerobic environment (80% nitrogen (N_2), 10% carbon dioxide (CO_2), and 10% hydrogen (H_2)). After positive identification using an automatic rapid microbial mass spectrometry detection system (BD Bruker MALDI Biotyper, USA), *S. mutans* was passaged to the third generation. Colonies were inoculated into blood agar medium and cultured for 24 h. After the bacterial concentration was adjusted to 1.5 × 10^8 colony forming units (CFU)/mL, the bacterial fluids were centrifuged at 2000 × g for 5 min. *S. mutans* resuspended in BHI medium was used for animal inoculation.

2.2 Animals

Forty-eight weaned Sprague Dawley female rats with a body weight of 38 ± 5 g and no specific pathogen at 14 days of age were purchased from Pengyue experimental animal breeding Co., Ltd. (Jinan, China). Rats were fed in a standard condition (23–25 °C, 60% humidity, 12 h light/dark cycle) with free access to food and ultrapure water. Iron pollution in the environment was strictly controlled during the experiment. A flowchart of the experimental design of the present study is shown in Fig. 1.

2.3 Establishment of rat models

Rats were randomly divided into four groups (N = 12 per group) and fed different iron contents as follows: (1) IDA group fed with 8 ppm low-iron fodder; (2) positive control (PC) group fed with 45 ppm normal-iron fodder; (3) high-iron (HI) group fed with 360 ppm high-iron fodder; and (4) negative control (NC) group fed with AIN-93G standard fodder. Customized fodders were purchased from Trophic Animal Feed High-tech Co., Ltd. (Nantong, China). All rats were given ultrapure water for drinking.

Except for the those in the NC group, rats were inoculated with *S. mutans* (200 L/rat) twice daily for 5 days. No feeding or drinking was allowed within 30 min after inoculation. Five days later, two rats were randomly selected from each group and the oral plaque was scraped with a sterile bamboo stick. The bacteria in the plaque were inoculated on BHI solid medium and cultured overnight to test for *Streptococcus mutans* colonization. The rats were fed cariogenic high-sugar fodder and ultrapure water containing 5% sucrose.

2.4 Measurement of hemoglobin (HGB) and serum iron (SI) level

After modeling for approximately 1 month (day 43), blood samples were collected *via* the inner canthus. HGB levels were directly measured using a blood analyzer (XN-1000, Sysmex Medical Electronics, Kobe, Japan). Serum samples were separated by centrifugation at 2000 × g for 15 min. SI was measured using an Iron Colorimetric Assay Kit (E-BC-K772-M, Elabscience, Wuhan, China) according to the manufacturer's instructions.

2.5 Evaluation of carious lesions

After modeling for approximately 3 months (day 109), the rats were euthanized by CO_2asphyxiation and the jaws with molars were resected. The jaw samples were dried at 25 °C after sterilization and stained with 0.4% murexide solution for 18 h. After washing, the carious lesions on the smooth surface were evaluated according to Keyes scores. In addition, the jaw samples were further embedded in epoxy and hemi-sectioned in the mesiodistal sagittal plane using a hard-tissue microtome (EXAKT, E400cs, Norderstedt, SH, Germany). The carious lesions at the sulcal surface were then scored under stereomicroscope (Olympus SZX16, Tokyo, Japan) [15, 16].

2.6 Scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS)

Microstructural injuries to the caries were observed using SEM [17]. Briefly, mandibular molars were fixed with 2.5% glutaraldehyde for 2 h and post-fixed in 1% osmic acid for another 2 h. Subsequently, the fixed samples were dehydrated using graded ethanol, dried using the critical point drying method, and sprayed with gold *via* vacuum ion sputtering. The prepared samples were observed using a scanning electron microscope (SEM, VEGA3, TESCAN, Kohoutovice, Czech Republic). The elemental compositions of the enamel and dentin in the molars were determined using an energy spectrometer (X-Flash Detector 610M, BRUKER Nano GmbH, Berlin, BE, Germany).

2.7 Histopathological examination of salivary gland

Hematoxylin and eosin (HE) staining was performed to reveal histopathological changes in the salivary glands [18, 19]. Briefly, the parotid and submandibular gland tissues were fixed in 10% formaldehyde, dehydrated with graded ethanol, trans-

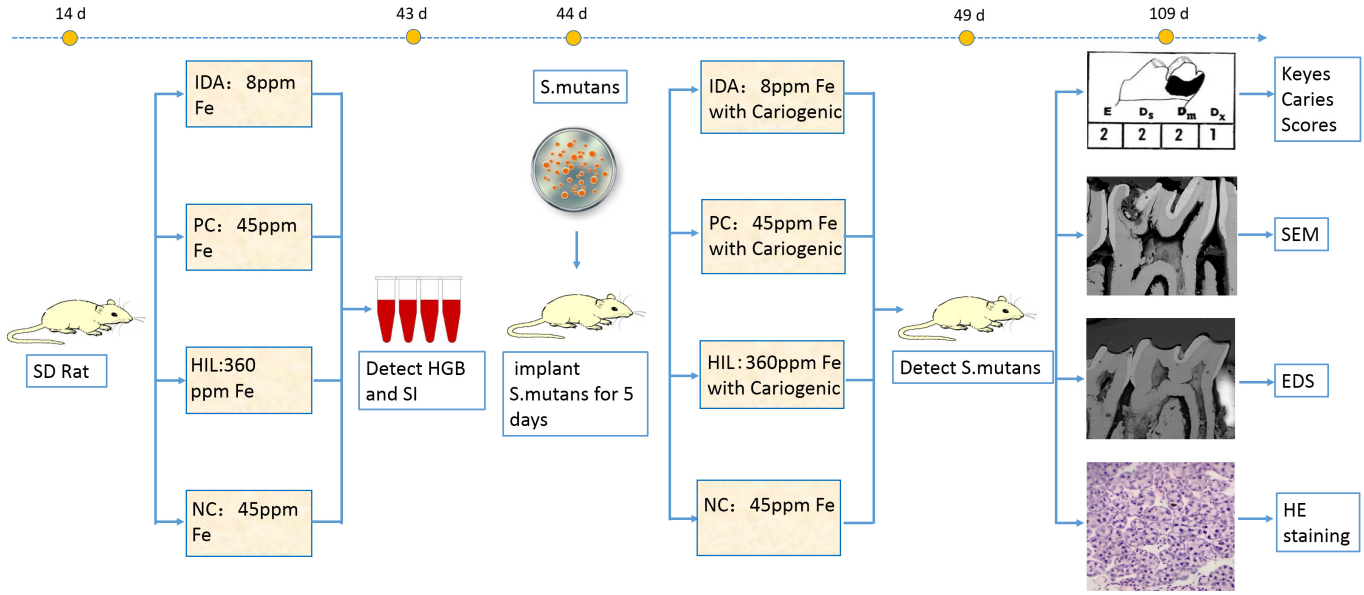

FIGURE 1. A flowchart of the experimental design of this study. SD: Sprague Dawley; IDA: Iron-deficiency anemia; PC: Positive control; HIL: high iron level; NC: Negative control; HGB: hemoglobin; SI: serum iron; SEM: Scanning electron microscopy; EDS: Energy-dispersive spectroscopy; HE: Hematoxylin and eosin.

parented with xylene, embedded in paraffin, and sectioned into 4 μm slices. Tissue sections were dewaxed with xylene, rehydrated with graded ethanol, and stained with HE. The stained sections were observed under a microscope (Olympus).

2.8 Statistical analysis

GraphPad Prism 7.0 (GraphPad, San Diego, CA, USA) was used for statistical analysis. The data were expressed as mean ± standard deviation. The differences among diverse groups were determined by one-way analysis of variance followed by Tukey's test. A p value < 0.05 was considered statistically significant.

3. Results

3.1 Manifestations of IDA and HI models in rats

Two weeks after modeling, rats in the IDA group began to show symptoms of IDA, including pale skin, ears, and paws, as well as rough and sparse fur. The HGB level is an important parameter in the diagnosis of anemia. After modeling for one month, HGB levels were significantly lower in the IDA group than in the control groups (PC and NC; $p < 0.05$), and these significant changes lasted until the third month after modeling (Table 1). In addition, the SI is a crucial indicator of iron levels. From the first to the third month after modeling, significantly lower SI levels were observed in the IDA group than in the control group (PC and NC; $p < 0.05$). In contrast, the HI group exhibited a significantly higher SI than the control group (PC and NC; $p < 0.05$) (Table 2). These results indicate that the IDA and HI models were successfully established.

TABLE 1. HGB levels in rats of different groups.

Group	HGB (g/L, mean ± SD)		
	1 month	2 months	3 months
IDA	89.0 ± 2.6^a	79.3 ± 11.2^a	79.0 ± 13.2^a
PC	122.3 ± 3.5^b	134.0 ± 11.1^b	131.0 ± 7.8^b
HI	140.3 ± 8.5^b	144.3 ± 13.6^b	147.0 ± 11.1^b
NC	122.7 ± 12^b	129.0 ± 11.3^b	135.7 ± 7.5^b

Note: Different lowercase letters present statistical significance ($p < 0.05$; One-Way ANOVA, followed by Tukey's HSD post hoc test). HGB: Hemoglobin; IDA: Iron-deficiency anemia; PC: Positive control; HI: High-iron; NC: Negative control; SD: standard deviation.

TABLE 2. SI levels in rats of different groups.

Group	SI (mg/L, mean ± SD)		
	1 month	2 months	3 months
IDA	1.6 ± 0.4^a	1.5 ± 0.3^a	1.7 ± 0.2^a
PC	2.9 ± 0.7^b	2.7 ± 0.3^b	2.8 ± 0.4^b
HC	5.2 ± 0.6^c	5.3 ± 0.7^c	5.0 ± 0.4^c
NC	3.0 ± 0.2^b	2.9 ± 0.2^b	2.6 ± 0.2^b

Note: Different lowercase letters present statistical significance ($p < 0.05$; One-Way ANOVA, followed by Tukey's HSD post hoc test). HGB: Hemoglobin; IDA: Iron-deficiency anemia; PC: Positive control; HC: High-iron; NC: Negative control; SI: serum iron; SD: standard deviation.

3.2 Severity of molar caries in rats

To reveal the effects of different iron concentrations on caries, they were induced in rats by inoculating their molars with *S. mutans* and feeding them cariogenic high-sugar fodder. The severity of molar caries was evaluated using the keywords score. As shown in Fig. 2a1–d3,3a–f, rats in the PC group exhibited obvious carious lesions (enamel and slight dentinal lesions at both smooth and sulcal surfaces, and moderate dentinal lesions at the sulcal surface) compared with rats in the NC group ($p < 0.01$). Among rats with caries, enamel lesions on the smooth surface and enamel and slight/moderate dentinal lesions on the sulcal surface were significantly higher in the IDA group than in the PC group ($p < 0.05$). In contrast, rats in the HI group showed significantly fewer enamel lesions on the smooth surface and fewer enamel and slight dentinal lesions on the sulcal surface than rats in the PC group ($p < 0.05$). Notably, significantly more extensive dentinal lesions at the sulcal surface were observed in the IDA group than in the HI and NC groups ($p < 0.05$) (Fig. 2a1–d3,3a–f). These results indicate that carious lesions were enhanced by IDA but relieved by HI *in vivo*.

3.3 Microscopic observation and elemental compositions of molar caries in rats

The micro-morphologies of the hemi-incisor molars were observed using SEM. In the IDA group, the enamel was completely destroyed, and the damage reached the middle dentin (Fig. 4a). In the PC group, obvious pit and fissure caries were observed (Fig. 4b). In addition, the molars in the HI group exhibited some degree of enamel demineralization, but the underlying dentin was almost intact (Fig. 4c). No caries were observed in the NC group (Fig. 4d). Subsequently, the elemental compositions of the molars were determined by EDS. Calcium (Ca), phosphorus (P), sodium (Na), magnesium (Mg), carbon (C), and oxygen (O) were detected in the enamel and dentin of molars in all groups (Fig. 4e–h,k,l). Notably, Fe was also detected in the HI group (Fig. 4i–j).

3.4 Morphological structure of salivary glands in rats

The effects of different iron concentrations on the salivary glands were further explored using HE staining. In the NC group, the acinus filled the parotid and submandibular glands, presenting a pyramidal shape, neat arrangement, round nucleus, and slightly stained cytoplasm. No acinar atrophy, connective tissue hyperplasia, or inflammatory infiltration of the salivary glands were observed. Induction of caries and changes in iron concentration (IDA and HI) did not influence the morphological structure of the salivary glands in rats (Fig. 5).

4. Discussion

Dental caries are among of the most prevalent dental diseases in children [20]. Increasing evidence has demonstrated that ID is closely associated with ECC [9, 21]. In this study, we explored the effects of iron levels on the pathological characteristics of dental caries in a rat model. The results showed that ID enhanced pathological damage in caries (enamel demineralization and dentin damage), whereas HI weakened carious damage. The morphological structures of the salivary glands were not influenced by ID or HI. Our findings indicate that iron is involved in the pathological damage of dental caries in infant rats by affecting enamel mineralization.

Tooth development is affected by genetic and environmental factors [22]. Adverse factors affecting tooth development not only affect the number of teeth but also damage the structure and quality of tooth enamel and the formation of dentin [23]. Caries are the most important disease of the oral cavity and one of the most common diseases in humans [24]. Developmental mineralization of teeth is closely related to susceptibility to caries [9, 21]. Iron is indispensable in most biophysiological and biochemical processes [25]. Iron is associated with the incidence of caries and can reduce the sensitivity of the body to caries. IDA is a common type of anemia, and although its incidence decreases with increasing economic levels, it is more often observed in preschool children (<5 years) and pregnant women [26, 27]. An epidemiological investigation by Clarke *et al*. [5] found that severe caries in young children may be a risk factor for IDA. Another prospective cohort study evaluated the impact of anemia during pregnancy on the risk of caries in pregnant women and revealed that anemia during pregnancy is a potentially independent risk factor for dental caries [28]. Martinhon *et al*. [29] demonstrated that ferrous sulfate reduces the demineralization of tooth enamel mass and changes the ion composition of tooth biofilms formed *in situ*. Similarly, Buzalaf *et al*. [30] showed that Fe^{2+} can effectively inhibit the dissolution of tooth enamel, and this effect may be long-lasting; however, a continuous increase in the concentration of Fe^{2+} does not have an additional effect on the dissolution of enamel. Considering the available literature and previous epidemiological investigations, IDA is more prevalent in children with ECC. We hypothesized that a low iron level in the body may not be conducive to enamel mineralization and thereby promote the progression of dental caries, whereas high iron levels may exert contrary effects. We designed an experiment to test this hypothesis.

A large number of epidemiological and clinical studies have shown that there is a link between ID and dental caries, especially in children and pregnant women [12, 31–34]. However, few researchers have conducted experimental studies on the response of dental caries to different iron levels at the pathological level. To address this, a rat model of dental caries was established and treated with different levels of iron. The results showed that HI decreased the carious score and relieved pathological damage to the enamel and dentin in the rat model. Our results are consistent with those of previous studies. Eshghi *et al*. [35] found that the intake of iron supplements could inhibit the development of dental caries. Martinhon *et al*. [29] indicated that ferrous sulfate reduces demineralization of the enamel mass and changes the dental biology formed *in situ*. Similarly, some scholars have found that Fe^{2+} can effectively inhibit the dissolution of tooth enamel and promote remineralization [30, 36–39]. Therefore, HI may block the progression of dental caries by inhibiting enamel demineralization. In addition, evidence has shown that iron

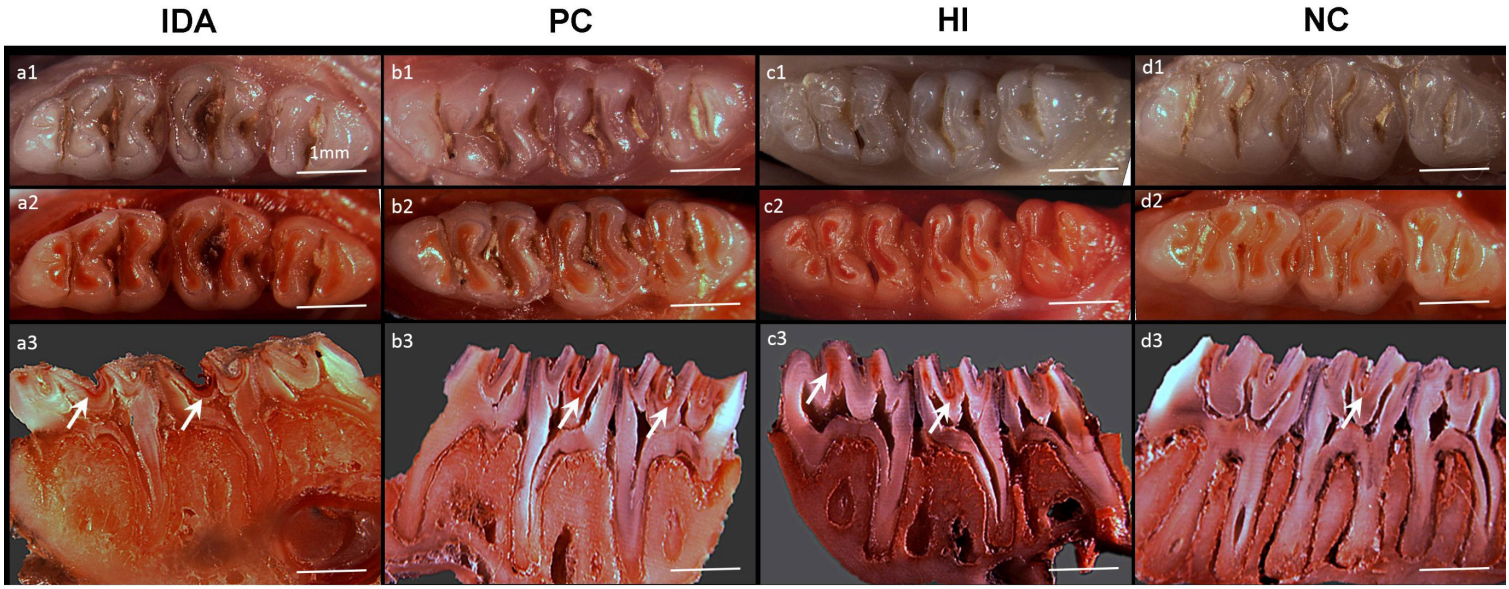

FIGURE 2. The morphology of molar caries. a, b, c and d represent the molars in the IDA, PC, HI, and NC groups, respectively. 1, 2 and 3 represent stereomicroscopic images of molars before staining, after staining, and after hemisection, respectively. White arrows indicate positive sites of carious lesions. IDA: Iron-deficiency anemia; PC: Positive control; HI: High-iron; NC: Negative control.

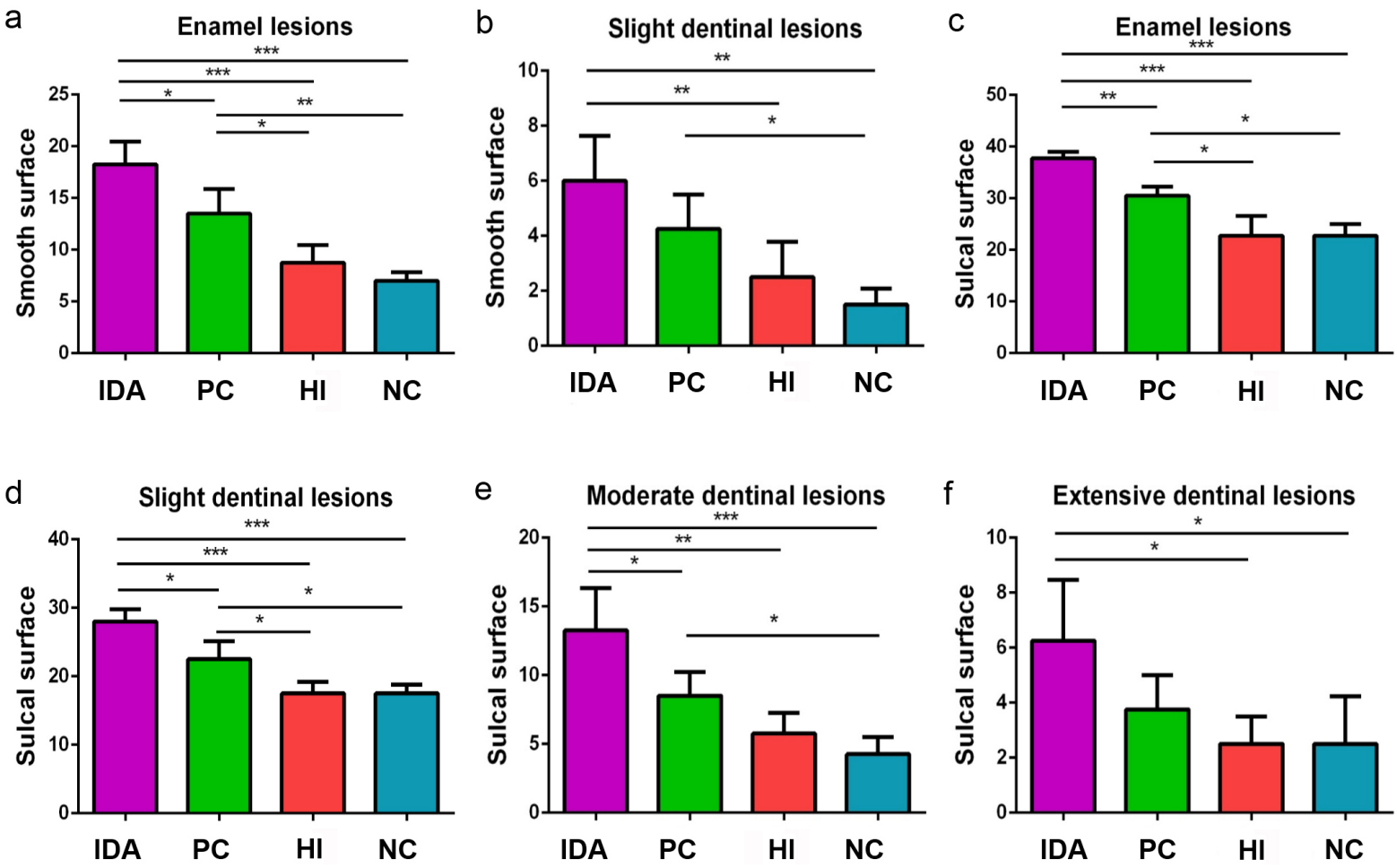

FIGURE 3. The severity of molar caries referring to Keyes scores. a,b represent a grade of E and Ds in the smooth surface, respectively; c–f represent a grade of E, Ds, Dm, and Dx in the sulcal surface, respectively. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. IDA: Iron-deficiency anemia; PC: Positive control; HI: High-iron; NC: Negative control.

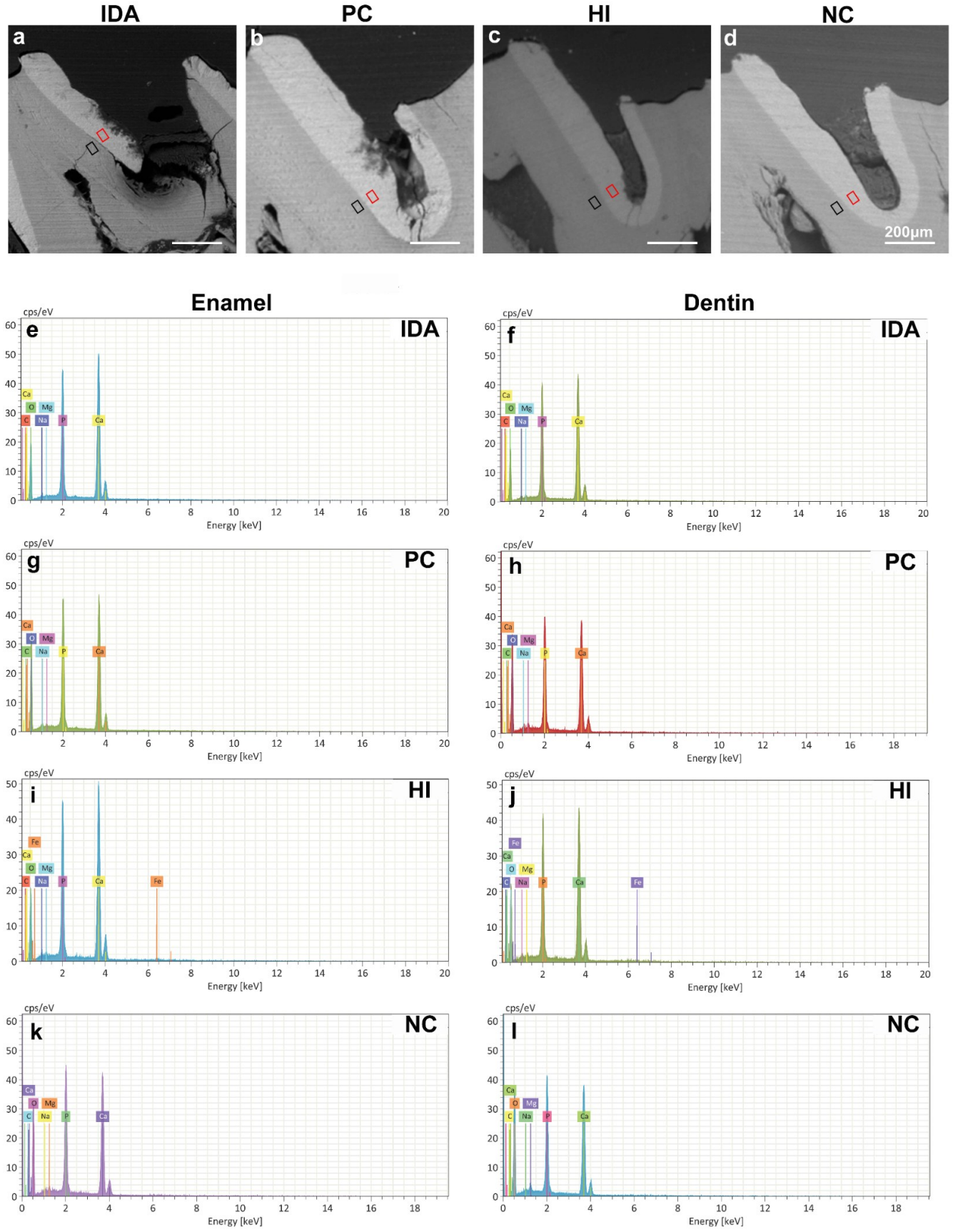

FIGURE 4. The micromorphology and elemental compositions of molar caries. a–d represent the SEM images of the molars in the IDA, PC, HI, and NC groups; e–l represent the EDS of enamel and dentin in different groups. IDA: Iron-deficiency anemia; PC: Positive control; HI: High-iron; NC: Negative control.

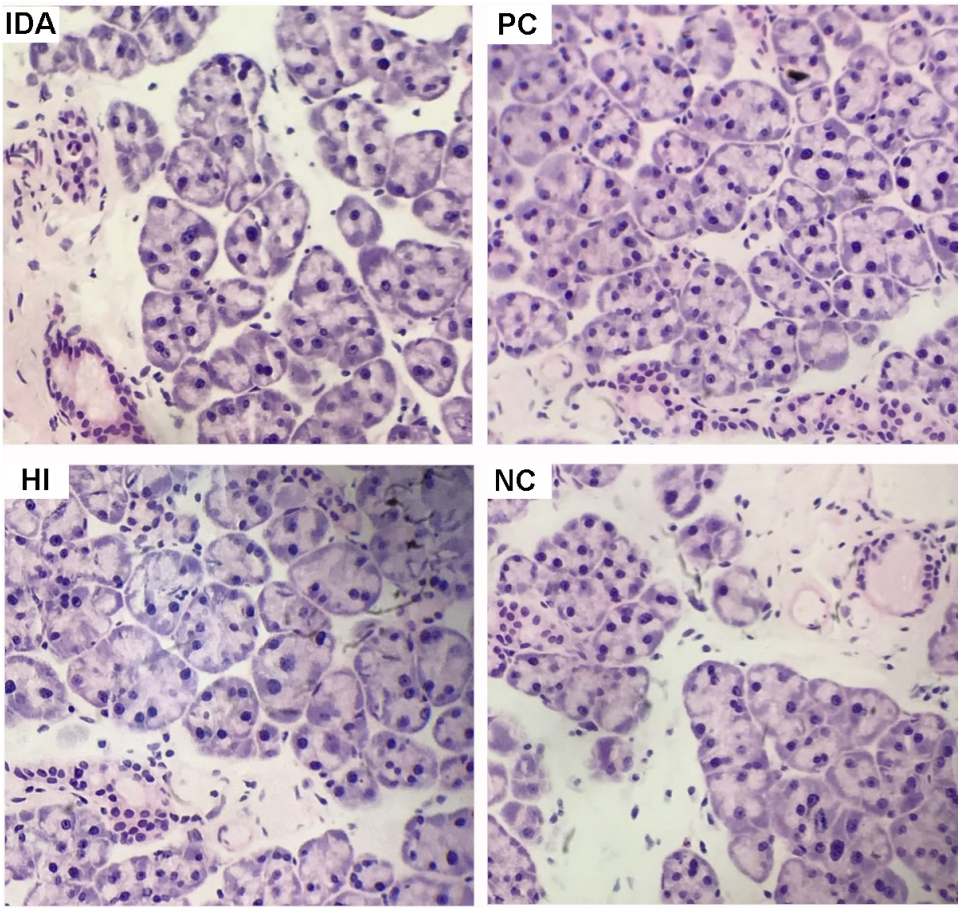

FIGURE 5. Morphological structure of the salivary glands was observed by HE staining (×400). IDA: Iron-deficiency anemia; PC: Positive control; HI: High-iron; NC: Negative control.

interferes with the implantation of *S. mutans.* in the oral cavity of rats [40–42]. Iron can inhibit the growth of *S. mutans* by interfering with biofilm formation [13]. Therefore, the mechanism of action of iron in dental caries may be closely associated with the inhibition of *S. mutans*. Furthermore, iron-binding proteins play important roles in host defense in animal and human models [43, 44]. The aggravated caries in the IDA group may also have been caused by the blocking effects of IDA on oral defenses. Further studies on the underlying mechanism of action of iron in dental caries are required.

The currently recognized causes of dental caries involve four principal factors: host sensitivity, specific pathogenic flora, suitable substrates, and sufficient time [45]. Saliva secreted by the salivary glands is an important factor affecting the host [46]. According to statistics, 49.3% of adult patients with IDA have dry mouth [47]. Effective treatment of IDA in children can significantly improve the pH and buffering capacity of the saliva [48]. Evidence has also shown that the function of the salivary glands in patients with ID is often impaired, resulting in decreased salivation secretion and reduced buffering capacity [49]. To further explore the effects of iron levels on the salivary glands, a histopathological examination was conducted. However, HE staining showed that the parotid and submandibular glands were full of acinar glands in rats in each group without pathological damage (such as acinar atrophy, connective tissue hyperplasia, and inflammatory infiltration). These results suggest that differences in iron content may not cause damage to the salivary glands. The relevant mechanisms involved in saliva reduction in dental caries should be explored in future research.

5. Conclusions

In conclusion, IDA enhanced the pathological damage caused by dental caries in infant rats, as evidenced by aggravated enamel demineralization. In contrast, HI weakens carious damage by inhibiting enamel demineralization. Different iron levels did not affect the morphological structure of the salivary glands. These findings provide new insights into the pathogenesis of dental caries and valuable guidance for clinical prevention. Furthermore, iron levels can be included as a potential risk factor for dental caries in clinical evaluations. The use of iron supplements to treat IDA may also be beneficial for preventing caries. However, the detailed mechanism of action of iron on caries requires further investigation.

AVAILABILITY OF DATA AND MATERIALS

The data that supports the findings of this study cannot be made publicly available as required consent to publish data were not given. However, the corresponding author can make deidentified data available on reasonable request.

AUTHOR CONTRIBUTIONS

LX, JW and LM—designed the research study. LX, JW, RH, YW and JY—performed the research. LX, JW and RH—analyzed the data. LX and JW—drafted the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All animal experiments were approved by the Ethics Committee of the Affiliated Hospital of Qingdao University in accordance with the Guide for the Care and Use of Laboratory Animals (QYFYWZLL 25820).

ACKNOWLEDGMENT

Not applicable.

FUNDING

This study was supported by Qingdao Key Health Discipline Development Fund (No. 2022-2024), Natural Science Foundation of Shandong Province (No. 2018GSF118167), and Qingdao Clinical Research Center for Oral Diseases (No. 22-3-7-lczx-7-nsh).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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How to cite this article: Libin Xu, Jiaying Wang, Rui Han, Yongliang Wang, Jin Yue, Lei Ma. Iron level participates in the pathological damages of dental caries in infant rats by affecting enamel mineralization. Journal of Clinical Pediatric Dentistry. 2023; 47(4): 86-94. doi: 10.22514/jocpd.2023.039.