

Effects of Zinc, Iron, and Copper on Bovine Enamel Erosion Evaluated Using Transverse Microradiography

Yukiko Yamada, Koji Watanabe* , Shiika Hara, Katsura Saeki, Kenshi Maki

Division of Stomatognathic Function Science, Department of Health Promotion, School of Dentistry, Kyushu Dental University, Kitakyushu, Japan

Email: *r17watanabe2@fa.kyu-dent.ac.jp

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Abstract

Objective: This study aimed to clarify the effect of trace elements on enamel erosion due to citric acid eliminating other factors that can affect enamel erosion. **Method:** Forty enamel specimens were obtained by embedding bovine enamel blocks into a quick cure resin. Half of the enamel surface of the specimens was covered with nail varnish. The specimens were randomly divided into control (Ct), zinc (Zn), iron (Fe), and copper (Cu) groups and immersed in 1% citric acid solution, 1% citric acid solution with 10 mmol/L zinc, 1% citric acid solution with 10 mmol/L iron, or 1% citric acid solution with 10 mmol/L copper, respectively, at 37°C for 5 h. After immersion, the demineralized lesion depth was measured using transverse microradiography (TMR) and compared between groups using one-way analysis of variance and Tukey's test. **Results:** The lesion depth was significantly higher in the Zn ($218.9 \pm 88.5 \mu\text{m}$) group than that in the Ct group ($116.3 \pm 22.1 \mu\text{m}$) ($p < 0.01$). **Conclusion:** It was suggested that zinc has decalcification properties when consumed with citric acid, while iron and copper don't have such properties.

Keywords

Erosion, Bovine Enamel, Citric Acid, Trace Element, Transverse Microradiography

1. Introduction

Dental caries and dental erosion are two major pathological demineralized lesions in teeth. Dental caries is caused by tooth dissolution derived from bacterial acid production. On the other hand, dental erosion is determined by tooth dis-

solution derived from non-bacterial acid exposure [1] [2]. The Ministry of Health, Labor, and Welfare, Japan reported that the prevalence of dental caries in six-year-old Japanese children in 2016 decreased from 89% to 45.5% since 1993. While the prevalence of dental caries has improved, that of dental erosion occurrence is now increasing and garnering increasing attention from dentists. The etiology of dental erosion can be endogenous (e.g. gastric acid) or exogenous (e.g. frequent intake of acidic food and drink). The latter cause of dental erosion is closely related to dietary habits, especially the frequent intake of some refreshments containing acid [2]. It was reported that the contact of enamel and acidic food is less than 1 min in normal eating; however, acidic substances linger around lingual papillae, leading to prolonged acid exposure of the enamel surface [3].

Citric acid is found in citrus fruits and is used in some candies or soft drinks as one of their ingredients. It contributes to energy production through the citric acid cycle, increasing the availability of some minerals when consumed simultaneously [4], and hence has a good effect on recovery from physical exhaustion in human bodies, while it is also known to possess strong decalcifying properties [5]. Due to global warming, heatstroke is becoming a serious health problem worldwide, thus, it is encouraged to consume soft drinks with citric acid or foods containing citric acid, in addition to water. As the need for appropriate hydration is recognized and performed, the consumption of citric acid will increase, worsening the prevalence of dental erosion.

Trace elements are essential for life and are maintained at very low levels in organisms. Zinc, iron, and copper are trace elements consumed as food additives or supplements, whose high concentrations have been associated with the prevalence of dental caries [6]-[15]. Regarding dental erosion, Pereira *et al.* [16] reported that 10 mmol/L of zinc inhibited the dissolution of bovine enamel powder in some carbonated drinks. Kato *et al.* [17] reported that 10 mmol/L of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ also inhibited the demineralization of human enamel with Coca-Cola *in vitro*. Moreover, Brookes *et al.* reported in their *in vitro* study that 10 mmol/L of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ inhibited the elution of calcium and phosphate by acetic acid [18]. Although these previous reports are important, carbonated drinks contain other ingredients, besides trace elements, capable of causing dental erosion or protecting from dental erosion; as a result, cycles of de- and remineralization could have affected the mineral density of the samples. Consequently, the effect of trace elements on dental erosion due to citric acid after minimizing the effects of other factors has not yet been reported. In this study, the effects of zinc, iron, and copper on dental erosion due to citric acid were evaluated under conditions devoid of other confounding factors.

2. Materials and Methods

2.1. Preparation of Enamel Samples

Bovine teeth were obtained randomly from cows sacrificed for consumption and

provided by the Meat Inspection and Control Center, Public Health and Sanitation Department, Public Health and Welfare Bureau, Kitakyushu City (**Table 1**). First, 4 mm × 6 mm × 3 mm cuboidal tooth blocks were cut out of the labial surface of the crowns.

Second, enamel samples of 10 mm × 12 mm × 7 mm were prepared by embedding the cuboidal tooth blocks into a quick cure resin, leaving the enamel surface uncovered. Third, the enamel surface of the sample was ground flat and polished using 400-, 600-, and 800-grit silicon carbide abrasive papers to remove the extraneous matter from the surface [19]. Finally, approximately half of the enamel surface was covered with nail varnish (**Figure 1**).

2.2. Preparation of Citric Acid Solutions

Four different citric acid solutions, *i.e.* 1% citric acid solution, 1% citric acid solution with zinc (10 mmol/L), 1% citric acid solution with iron (10 mmol/L), and 1% citric acid solution with copper (10 mmol/L), were prepared. The pH of the solutions was adjusted to 2.3 by adding potassium hydrate. All chemicals were manufactured by FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan.

Table 1. Primary information of the bovine teeth.

Extraction date	Cow breed	Sex	Age	Birthday	Tooth species
June 13 th 2018	Holstein	castrated	21 months	August 19 th 2016	central incisors
			22 months	August 30 th 2016	central incisors
			23 months	August 31 st 2016	central incisors
			20 months	September 22 nd 2016	central incisors
			21 months	August 20 th 2016	central incisors
			22 months	August 20 th 2016	central incisors
June 21 st 2018	Holstein	castrated	22 months	August 3 rd 2016	central incisors
			22 months	August 6 th 2016	central incisors
			22 months	August 20 th 2016	central incisors
			22 months	August 20 th 2016	central incisors
June 27 th 2018	Holstein	castrated	22 months	August 23 rd 2016	central incisors
			22 months	August 1 st 2016	central incisors
			22 months	August 25 th 2016	central incisors

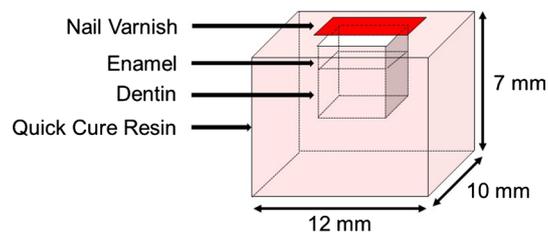


Figure 1. Enamel specimens: Enamel specimens were prepared by embedding cuboidal tooth blocks in quick cure resin, leaving the enamel surface naked.

2.3. Acid Exposure of Enamel Samples

The prepared enamel samples were randomly divided into four groups, each consisting of ten samples: control (Ct), zinc addition (Zn), iron addition (Fe), and copper addition (Cu) groups. In the Ct, Zn, Fe, and Cu groups, the enamel samples were individually immersed in 40 mL of 1% citric acid solution, 1% citric acid solution with zinc (10 mmol/L), 1% citric acid solution with iron (10 mmol/L), or 1% citric acid solution with copper (10 mmol/L), respectively, in sterilized test tubes for 5 h at 37°C. After 5 h of immersion, the enamel samples were removed from the solution. The samples were dried in room air, followed by removal of the nail varnish, resulting in two regions on the enamel surface; one region in contact with the citric acid solution during immersion and the other not in contact.

2.4. Measurement of the Lesion Depth Using Transverse Microradiography (TMR)

One section, approximately 300 μm thick, was sliced from the center of the enamel samples (**Figure 2**). Each section and an aluminum wedge were placed on the X-ray films. Radiographs were taken using PW3830 (Panalytical, The Netherlands) at a tube voltage of 25 kV, tube current of 25 mA, a focus-film distance of 350 mm, and exposure time of 15 s. The virtual surface line (VSL) was determined on the radiographs by stretching the enamel surface in the sound region toward the acid-exposed region, followed by the measurement of the lesion depth using TMR2000 (Inspektor Research System, The Netherlands) (**Figure 3**).

2.5. Statistical Analyses

Values in the text are shown as mean \pm standard deviation (SD). Six available data in the Ct group, nine in the Zn group, seven in the Fe group, and eight in the Cu group were obtained through measurement using TMR. One-way analysis of variance and Tukey's test were performed for multiple comparisons between the five independent groups. A p-value < 0.05 was considered significant.

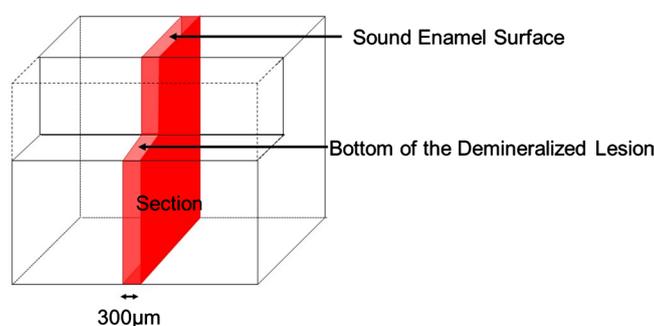


Figure 2. Schematic illustration of enamel sections: Enamel specimens were cut into 300- μm -thick sections as shown in red in the illustration to be analyzed using TMR.

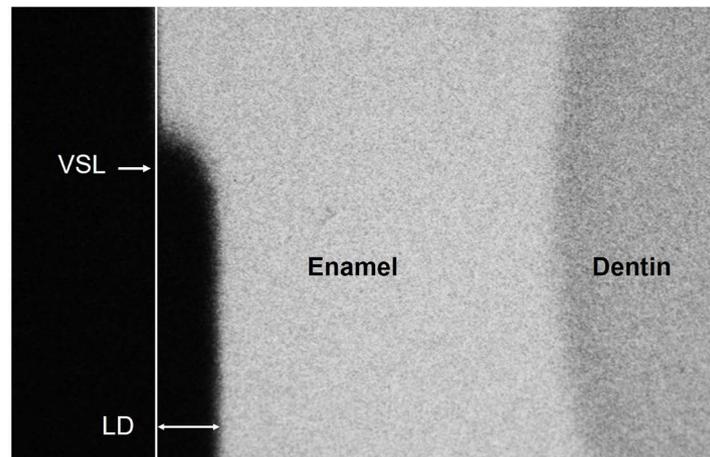


Figure 3. Measurement of the lesion depth using TMR: The virtual surface line (VSL) was determined on the radiographs. The border between sound and demineralized enamel was defined as pixels whose X-ray opacity was 5% lower than that of sound enamel. Lesion depth (LD) was obtained as the maximum value of the distance between the VSL and each pixel on the bottom border of the lesion.

3. Results

Measurement of the Lesion Depth Using TMR

As shown in **Figure 4**, the value of the lesion depth in the Zn group (218.9 ± 88.5) was significantly higher than that in the Ct ($116.3 \pm 22.1 \mu\text{m}$) ($p < 0.01$) and Cu ($133.1 \pm 37.9 \mu\text{m}$) ($p < 0.05$) groups.

4. Discussion

The enamel samples were immersed in citric acid solution for 5 h in this report. In previous *in vitro* studies on enamel demineralization by soft drinks, immersion time was settled between 1 to 48 h [20] [21] [22] [23] [24]. Taking these reports and intervals between meals in our daily lives into consideration, duration of acid immersion in this study is reasonable. Generally, solubility of hydroxyapatite crystal increases as pH in oral cavity reduces [3]. In this study, intake of citric acid was intended, so the pH of citric acid solution was determined as 2.3, referring to pH of ordinary acid soft drinks.

TMR has been considered the gold standard method for measuring tooth demineralization [25] [26] [27] [28]; hence, it was reasonable to measure lesion depth by TMR in the present study. Early dental caries is regarded as subsurface lesions [29], which makes it easy to measure the lesion depth based on the visible enamel surface. On the other hand, dental erosion progresses layer by layer from the enamel surface [3], so the virtual surface line was determined in the radiographs by stretching the sound enamel surface toward the acid-exposed region (**Figure 3**). The bottom of the demineralized lesion was determined based on the pixels whose X-ray opacity was decreased by 5% from that of sound enamel [27]. The measurement indicated that the lesion depth was deeper in the Zn group than in the Ct group (**Figure 4**), suggesting that dental erosion due to citric acid

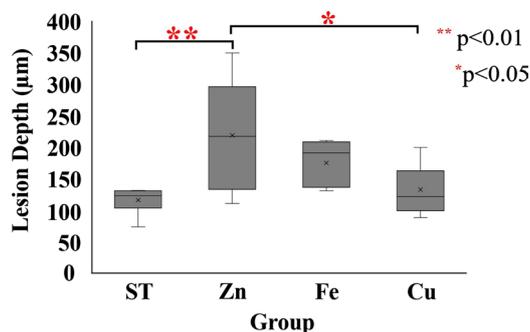


Figure 4. Box plot of LD measured using TMR: LD was first measured as the number of pixels in the radiographs, and then translated into μm . The lesion depth in the Zn group was significantly higher than that in the Ct ($p < 0.01$) and Cu ($p < 0.05$) groups. The cross marks express the mean value in each group.

was accelerated by zinc. For caries, Rahman *et al.* reported that if calcium in hydroxyapatite crystals is replaced by zinc, the solubility of hydroxyapatite crystals will be increased [7]. On the other hand, Saad *et al.* [9] reported in their *in vitro* study that zinc inhibited the formation of biofilm and dentin demineralization. Mohammed *et al.* [11] [12] also reported in their *in vitro* study that zinc inhibited demineralization and advanced remineralization. Moreover, Lynch [10] mentioned in his review article that zinc inhibits caries formation. Focusing on the stage of caries progression, Lynch reported that the addition of zinc to fluoride toothpastes did not affect their ability to reduce caries during clinical trials [10]. It could be considered that these previous reports and results in this study suggested that zinc inhibits biofilm formation and advanced remineralization but accelerates chemical dissolution of enamel hydroxyapatite crystals due to the presence of acid. For erosion, in contrast to our results, Pereira *et al.* [16] reported in their *in vitro* study using bovine enamel powder and soft drinks that demineralization was inhibited when zinc was added at 10, 15, 30, or 60 mmol/L to Coca-Cola, which contains phosphoric acid, although a significant difference was not found when zinc was added at 1.25, 2.5, or 5 mmol/L. They also reported that demineralization was inhibited only when zinc was added at 2.5 mmol/L to Sprite Zero, which contains citric acid. The present and previous studies suggested that the effect of zinc on enamel erosion may be changed by the type of acid or concentration of zinc. In this study, the zinc level was set at 10 mmol/L based on the recommended intake (11 mg/day) and maximum permissible intake (40 mg/day) of zinc for humans. Creeth *et al.* [30] and Colombo *et al.* [31] also reported in their *in vitro* study using dentifrices and bovine enamel specimens that zinc increased demineralization resistance. The discrepancy between the present study and these previous reports could be due to differences in experimental methods. Besides, Coca-Cola, Sprite Zero, and dentifrices contained some ingredients, such as sodium or potassium, which could affect the critical pH [32] of enamel. The presence of zinc could have affected the ionization state of these ingredients in the solutions through the oxidation-reduction reaction. Though Mohammed *et al.* [12] concluded that zinc reduces enamel

demineralization, they also showed the formula on the interaction of zinc with hydroxyapatite; $3\text{Zn}^{2+} + 2\text{H}_2\text{PO}_4^- + 4\text{H}_2\text{O} \rightarrow \text{Zn}_3(\text{PO}_4)_2 \cdot 4\text{H}_2\text{O} + 4\text{H}^+$. Generally, critical pH is decreased by increase of the ratio of calcium ions to phosphate ions in the solution. In the formula, zinc makes the amount of phosphate ions decrease in the solution, resulting in decrease of critical pH. At the same time, hydrogen ions that decrease pH of the solution are also released into the solution through the chemical reaction. So, it could be suggested that the degree of undersaturated condition of the solution with respect to hydroxyapatite crystal could be changed based on 1) the original pH of citric acid solution, 2) amount of produced hydrogen ions, and 3) amount of phosphate ions remaining in the solution through the chemical reaction in the formula. Although the reason why zinc accelerated enamel erosion is unclear, the experimental method of the present study was based on eliminating other factors that may affect enamel mineral density. Iron has been considered to have cariostatic effects by suppressing biofilm formation and aggregation [13] [14]. Iron has been considered to reduce enamel erosion as suggested by *in vivo* [15] and *in vitro* studies [17] [33]. On the other hand, Pereira *et al.* [16] found that erosion of bovine enamel by Coca-Cola was inhibited when iron was added at 1 mmol/L, while erosion was accelerated when iron was added at 10 mmol/L. It was also reported that iron inhibited the dissolution of enamel powder in Coca-Cola which contains phosphoric acid, but accelerated dissolution in Sprite Zero, which contains citric acid [34]. Although there was a possibility that the action of iron on enamel demineralization may be changed by the concentration of iron, iron levels in this study were set at 10 mmol/L based on the recommended intake (11 mg/day) and maximum permissible intake (45 mg/day) of iron for humans. In the present study, no significant difference was found in the lesion depth between the Ct and Cu groups, suggesting that 10 mmol/L of copper did not affect dental erosion due to citric acid. Pereira *et al.* [16] reported that 10 mmol/L of copper did not inhibit the dissolution of human enamel powder due to Sprite Zero, while inhibition of enamel dissolution was found when the copper level was set at 15 or 30 mmol/L. If the copper concentration in this study was set at 15 or 30 mmol/L, the amount of copper would exceed the maximum permissible intake for humans (10 mg/day). Therefore, the copper concentration in this study was determined based on a previous study that reported that copper inhibited mineral dissolution of human enamel powder due to acetic acid [18]. As a result, the previous and present studies suggest that copper, at normal intake levels, does not affect dental erosion due to citric acid.

The present study evaluated the eroded lesion depth by TMR. While zinc was found to accelerate erosion due to citric acid, iron and copper were found not to affect erosion under conditions of the present study; however, citric acid is consumed with different concentrations of trace elements in our daily life. Therefore, the present study suggests that further studies are needed with different concentrations of trace elements to clarify which condition makes these trace elements protective or pathological factors in the progression of dental erosion

from a chemical point of view.

5. Conclusion

The effect of zinc, iron, and copper on bovine enamel erosion due to citric acid was evaluated using TMR with minimum number of other factors that could affect the mineral density of enamel samples other than trace elements. The results of the TMR evaluation, in contrast to previous reports, indicated that eroded lesion depth in the Zn group was significantly deeper than that in the other groups, suggesting that zinc accelerates dental erosion when consumed with citric acid. However, further studies are needed to clarify which conditions make zinc a protective or pathological factor in the progression of dental erosion from a chemical point of view because citric acid is consumed with various ingredients in food and beverages in daily life. On the other hand, under the conditions of this study, the presence of iron or copper in citric acid did not affect lesion depth.

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Not applicable.

Ethical Approval

The study protocol of the present study was reviewed and approved by the ethical review committee of Kyushu Dental University (approval number; 17-31).

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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