

ORIGINAL RESEARCH

Visualization of etching cycles efficacy at the resin infiltration into artificial enamel caries: *in-vitro* study on bovine teeth

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(Mehmet Çiloğlu)**Abstract**

This study evaluated the effect of repeated etching cycles on resin infiltrant penetration. Enamel samples measuring $4 \times 4 \times 3 \text{ mm}^3$ were obtained from the facial aspect of 50 extracted bovine teeth. Samples were immersed in a demineralization solution for 21 days to create artificial lesions and divided into five equal groups ($n = 10$). A 15% hydrochloric acid gel was administered to each group. The acid etching application time differed between groups: Group 1; 2 min, Group 2; 2×2 min, Group 3; 3×2 min, Group 4; 4×2 min, and Group 5; 5×2 min. Resin infiltration was visualized using a confocal laser scanning microscopy. The lesion, penetration and erosion depth (μm) were calculated, and data were statistically analyzed. The highest penetration depth ($75.59 \pm 9.42 \mu\text{m}$) was seen in Group 5, followed by Groups 4, 3, 2 and 1. However, there were no statistically significant differences in the penetration depths between Groups 4 and 5 and between Groups 2, 3 and 4 ($p > 0.05$). In conclusion, a repeated etching cycle enhanced resin infiltrant penetration.

Keywords

Confocal laser scanning microscopy; Resin infiltration; Enamel caries; Acid etching

1. Introduction

Dental caries, defined as a dynamic disparity between alternating dissolution periods and mineral loss, is a multifactorial disease [1]. The first clinically visible stage of this disease is characterized by enamel demineralization without cavitation (white spot lesion; WSL) [2]. Diseased enamel typically maintains its outer morphology with an area of high subsurface porosity, which alters the enamel's optical properties and light scattering [3]. Resin infiltration is used for WSL management; it involves filling enamel pores with cured resin, thereby reducing the enamel's light scattering and permeability [4]. The infiltrants used for this method are liquids with low viscosity and high penetrating ability, developed with the aim of clogging the subsurface spaces of WSLs with capillary forces, filling the pores in the mineral deficient areas on the surface and preserving the structural integrity of Hydroxyapatite (HA) crystals [5]. Icon (DMG Chemisch-Pharmazeutische Fabrik GmbH, Hamburg, Germany), a resin used for resin infiltration, comprises bisphenol A ethyl 4-(dimethylamino) benzoate, glycerolate dimethacrylate, camphorquinone, triethylene-glycol-dimethacrylate-based resin and ethanol. Icon facilitates deeper penetration as it has an extremely high penetration coefficient [6].

The penetration of a liquid (e.g., resin infiltrant) into a porous solid structure (e.g., enamel tissue) can be explained by Washburn's equation. A study based on this equation

investigated the effect of penetration coefficients on penetration rate and depth by changing the viscosity and surface tension properties of the resin infiltrant. A positive relationship between the penetration coefficient and penetration depth was reported [7]. Other studies have evaluated the efficacy of different preconditioning parameters on the penetration depth of infiltrants and noted that the etching procedure might affect penetration depth [8–10].

Acid etching on the less porous and highly mineralized outer surface of enamel lesions is often done for infiltration, as it increases the permeability of this pseudo-intact by promoting demineralization and wear of some micrometers, allowing deeper infiltrant penetration [11]. A previous study revealed that treatment with 15% hydrochloric acid (HCl) gel results in the virtually complete removal of the surface layer and may be more suitable than 37% phosphoric acid (H_3PO_4) gel for the pretreatment of enamel lesions before resin infiltration [12]. *In vitro* studies have recommended an HCl application time of 90–120 s for effective surface layer erosion, which is similar to the 2 min application time suggested by the manufacturer [12–14]. However, the manufacturer also proposed repeating the etching step until the whitish-opaque coloration of enamel lesions is diminished. Clinical studies have indicated that the etching time must be greater than 120 s to achieve improved aesthetic results for WSLs [15]. Arnold *et al.* [16] investigated whether erosion depth increased with repeated 15% HCl conditioning of the enamel surface; however, the overall

erosion depth remained relatively shallow. There is insufficient data regarding the effect of repeated HCl application on resin penetration depth in the literature. This study aimed to determine the effect of repeated etching cycles on resin infiltrant penetration. The null hypothesis was that there would be no difference between the groups regarding resin infiltrant penetration depth.

2. Materials and methods

2.1 Sample preparation

The experimental design is schematized in Fig. 1. Ethical approval was obtained from the Local Ethics Committee of Animal Experiments to extract 50 bovine teeth. The rinsed and cleaned specimens were stored in a 0.1% Thymol solution until use. Enamel specimens ($4 \times 4 \times 3 \text{ mm}^3$) were sectioned from the facial aspect of bovine incisors using a cutting machine (IsoMet 1000 Precision Sectioning Saw machine, Buehler Ltd, Lake Bluff, IL, USA) and subsequently embedded in acrylic resin (Imicryl SC; Imicryl Dental Materials, Inc, Konya, Turkey). Enamel surfaces were polished using up to 4000 grit abrasive paper and a polishing machine (Ecomet 3 grinder-polisher, Buehler Ltd, IL, USA). Half of the enamel surfaces were then covered with acid-resistant nail varnish (sound control). To create enamel lesions, the specimens were exposed to 5 L of an artificial caries solution for 21 days. The solution contained 50 mM acetic acid, 3 mM Calcium Chloride (CaCl_2) + water ($2\text{H}_2\text{O}$), 3 mM Potassium dihydrogen phosphate (KH_2PO_4), 3 mM Acetic Acid (CH_3COOH), 6 mM thymol, H_2O , 10 mM methylhydroxy-diphosphonate, and 50 mM Potassium Hydroxide (KOH). The solution was kept at a temperature of 37.8°C and had a pH of 4.95 [17]. The samples were divided into five equal groups ($n = 10$ that underwent different etching cycles:

Group 1: 1×2 min HCl etching application.

Group 2: 2×2 min HCl etching application.

Group 3: 3×2 min HCl etching application.

Group 4: 4×2 min HCl etching application.

Group 5: 5×2 min HCl etching application.

The etching (Icon-Etch) and drying (Icon-Dry) steps in each cycle were performed according to the manufacturer's instructions. First, 15% HCl (Icon-Etch) was gently applied to the sample surfaces with a micro brush for 2 minutes. The samples were then rinsed with water for 30 s and dried. Icon-Dry was then applied for 30 s and carefully dried using oil- and water-free air. The number of etching applications was determined based on a sample's assigned group.

2.2 Determining staining technique

Direct or indirect staining techniques were performed to obtain confocal laser scanning microscopy (CLSM) images. A pilot study of three specimens was conducted for each staining technique to determine which technique was appropriate for resin penetration visualization.

2.2.1 Direct staining technique

The rhodamine B isothiocyanate red fluorophore (0.1% RITC; Merck, Darmstadt, Germany) was used to label the infiltrant

(Icon). The dye-labeled infiltrant was applied to the lesion surface and left to penetrate for 3 min. The excess material was subsequently removed with a cotton pellet. A light-emitting diode (LED) light source (Valo Cordless, Ultradent, South Jordan, UT, USA) was applied for 40 s using 1000 mW/cm^2 standard power to light-cure the infiltrant [18].

2.2.2 Indirect staining technique

Before infiltration, the lesion was labeled with a fluorescent dye. The specimens were stored in an ethanolic solution of 0.1% RITC for 12 h to label all accessible porosities with the red fluorophore. Subsequently, the infiltrant (Icon) was applied onto the dried lesion surfaces, and the excess was removed with a cotton pellet after 3 min. An LED light source (Valo Cordless) was applied for 40 s using 1000 mW/cm^2 standard power to light-cure the infiltrant [18]. This technique was selected for use in the study to allow adequate visualization of the resin (Fig. 2).

2.3 CLSM analysis

To obtain $700 \mu\text{m}$ thick sections, specimens were cut perpendicular to the enamel lesion surfaces. The surfaces were polished with up to 4000 grit abrasive paper. To remove unbound RITC from non-infiltrated porous structures, all specimens were bleached in 30% hydrogen peroxide at 37°C for 24 h. The samples were then immersed in a sodium fluorescein (NaFl) dye (50% ethanol solution of 100 M NaFl; Merck, Darmstadt, Germany) for 3 min to label the porous structures. The NaFl was removed *via* flushing with distilled water for 10 s. The specimens were visualized using CLSM (Leica TCS SPE, Leica Microsystems GmbH, Mannheim, Germany) on a $5 \times$ objective. This objective was used to collect single-plane 526×526 -pixel images with a $1.6 \text{ mm} \times 1.6 \text{ mm}$ field of view and the dual fluorescence mode. The red RITC dye demonstrated optical excitation (Oex) at a wavelength of 561 nm and optical emission (Oem) at 572–682 nm. The green NaFl dye demonstrated Oex at 488 nm and Oem at 551 nm. In the CLSM images, due to the imbibition of NaFl within the pores, the lesions appeared green, whereas the infiltrated areas appeared red due to the fixation of RITC by photopolymerized resin. The distances from the enamel surface to the deepest point of the red and green fluorescence were defined as the penetration and lesion depth, respectively. Erosion depth was defined as the distance from the sound enamel surface to the base of the defective area. The Image J software (v1.53, National Institutes of Health; Bethesda, MD, USA) was used to analyze the two-dimensional CLSM images. For each CLSM image, the resin penetration depth was measured at three selected points in microns, with the average depth then calculated.

2.4 Statistical analysis

The normal distribution of the data was checked using the Shapiro-Wilk test. Based on this result, a parametric statistical analysis was selected. The descriptive statistics are presented in the form of mean \pm std. Statistical significance was considered when $p < 0.05$. The data were analyzed using one-way Analysis of Variance (ANOVA) tests followed by Tukey's

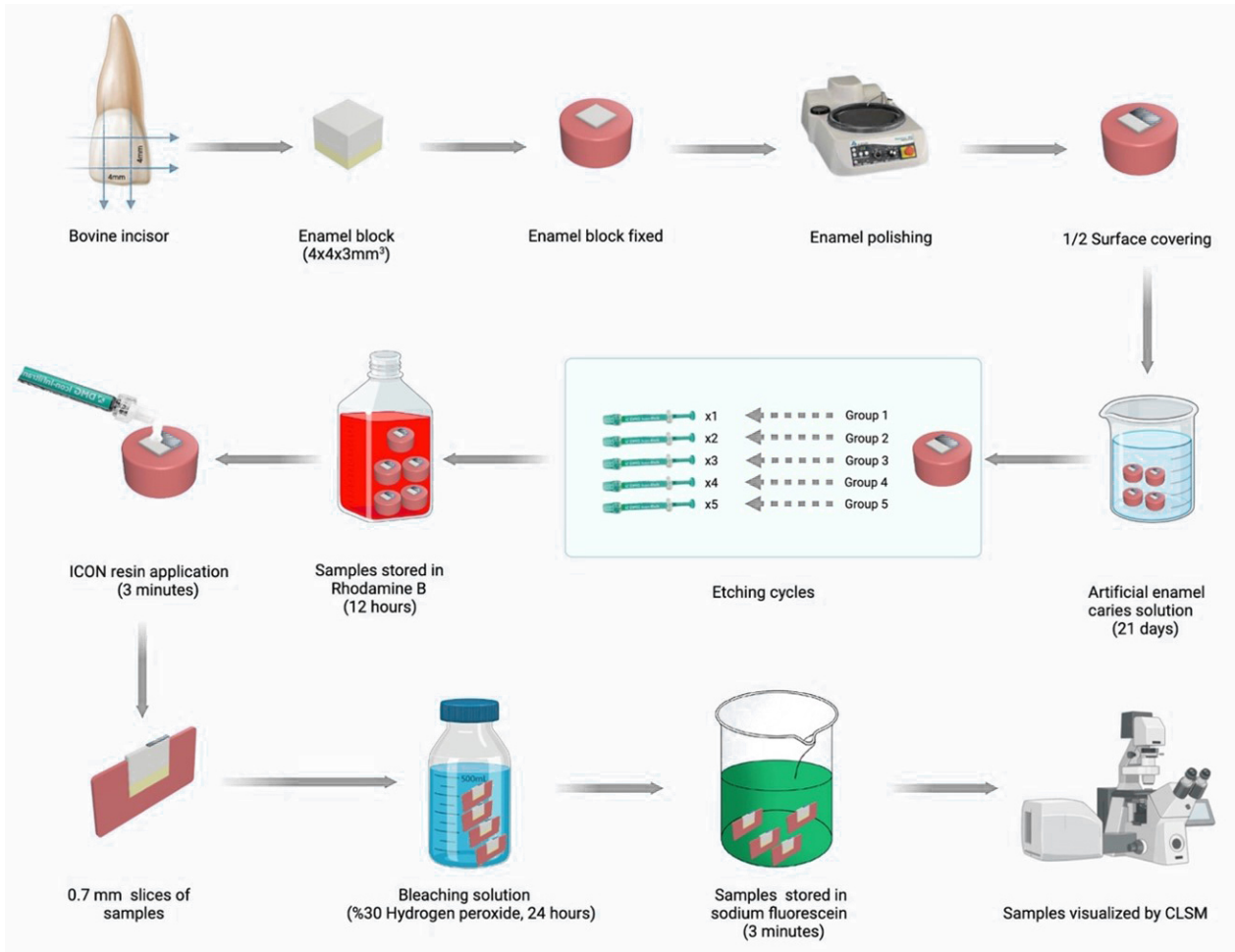


FIGURE 1. A schematic drawing of the experimental design. CLSM: confocal laser scanning microscopy.

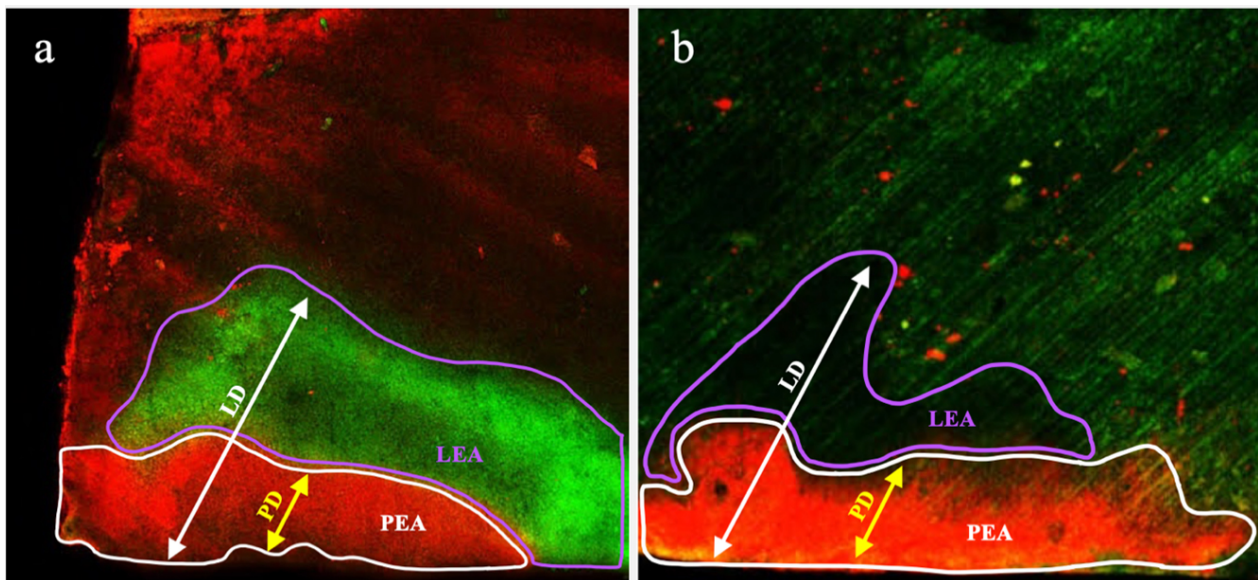


FIGURE 2. Representative CLSM images of the different staining techniques. (a) Representative CLSM images of the indirect staining technique. Non-infiltrated demineralized enamel appeared green (sodium fluorescein dye), while the resin-infiltrated areas appeared red (RITC dye). Sound enamel appeared black. (b) Representative CLSM images of the direct staining technique. Non-infiltrated demineralized enamel appeared black, the resin-infiltrated areas appeared red (RITC dye), and the sound enamel appeared green (sodium fluorescein dye). CLSM: confocal laser scanning microscopy, LD: lesion depth, PD: penetration depth, LEA: lesion area, PEA: penetration area.

test for multiple comparisons. All tests were performed using SPSS v22.0 (SPSS, Inc., Chicago, IL, USA).

3. Results

The mean penetration, lesion and erosion depth values (μm) are presented in Table 1. The mean lesion depth was 108.87 ± 2.96 . The lowest penetration depth (37.27 ± 8.36) was seen in Group 1 ($p < 0.05$). The mean penetration depth of the resin infiltrant was 65.33 ± 4.64 in Group 2, 65.33 ± 6.81 in Group 3, and 69.48 ± 5.69 in Group 4. These differences were not significant ($p > 0.05$). Group 5 showed the highest penetration depth (75.59 ± 9.42); however, this was not significantly different from that of Group 4 ($p > 0.05$). The penetration depths are presented in a box-and-whisker plot in Fig. 3. All specimens were evaluated for enamel erosion. Only five specimens showed enamel erosion, with a mean depth of 22.54 ± 2.35 (Fig. 4).

4. Discussion

The visualization technique using CLSM is suitable for quantifying resin penetration, estimating lesion depth and qualitatively analyzing hard porous tissues [7, 19]. Direct or indirect staining techniques can be performed to obtain images using CLSM [20, 21]. Paris *et al.* [18] assessed the use of the direct staining technique to determine penetration depth and reported that no infiltrated lesion areas included the fluorophore, leading to an underestimation of penetration depth. Therefore, they suggested using the indirect technique. The current study used the indirect staining technique as, following a pilot study, it was found to enable adequate resin visualization.

In studies involving microscopic teeth measurements, the absence of anatomical defects and surface smoothness is essential for data reliability. Bovine teeth are preferred for dental research as the elemental composition of bovine enamel is very similar to human dental hard tissues, allows for the preparation of larger specimens, and access to intact human teeth is limited [22, 23]. In addition, specimens of the same shape, size and thickness can be prepared from bovine teeth, allowing standardization and eliminating the confounding effects of teeth anatomy and morphology on results.

Similar to the present study, Wierichs *et al.* [17] used bovine

teeth to investigate pre-demineralized artificial enamel lesions (21 days of demineralization). The mean lesion depth was approximately $130 \mu\text{m}$, and they reported that lesions at this depth could be considered clinically “subclinical.” The mean lesion depth in the present study is compatible with lesions termed “highly demineralized caries-like enamel lesions” in the literature [17, 24]. However, the clinical differences between natural caries lesions in humans and artificial demineralization lesions in bovine enamel might be a limitation of this study.

Resin infiltration is a treatment developed to prevent the progression of initial enamel caries by improving demineralization resistance and micro-hardness [25]. The volume and diameter of the pores in lesions are crucial for infiltration results due to their influence on the penetration of low-viscous resin monomers into carious lesion bodies *via* capillary forces. Therefore, to enable successful resin penetration into a lesion body, the surface layer should be perforated or removed *via* etching [26, 27]. HCl acid is a strong inorganic acid that has demonstrated efficacy for surface layer erosion [13, 28]. Using 15% HCl for 90–120 s has been found to effectively remove the relatively hypermineralized surface layers of initial enamel lesions and enhance penetration into the lesion bodies [29].

Different enamel lesion types (*e.g.*, active, inactive, shallow or deep) might require repeated etching procedures. Active lesions have larger pores and less mineralized surface layers, which could allow the resin to penetrate deeper [30]. Inactive lesions have thicker and less permeable surface layers, which could cause insufficient resin penetration [31]. For smaller and more superficially located lesions, a single etching cycle may be sufficient, whereas the aesthetic healing of larger and deeper lesions requires further etching procedures [32, 33]. A single 120 s etching cycle before infiltration has been performed in some studies [34, 35]. However, it has been argued that esthetic outcomes might be improved with increased etching duration or increased number of cycles, as this could enable greater resin penetration [36]. The improved esthetic appearance associated with resin infiltration is due to the resin’s ability to mask enamel lesions. The refractive index of a resin infiltrant is close to the index of enamel/apatite, as opposed to the indices of water and air; therefore, light scattering is reduced with increasing degrees of infiltration

TABLE 1. Mean penetration depth values of the resin infiltrant (μm).

Groups	n	Mean (Standard Deviation)	Min/Max
PD			
1	10	$37.27 (8.36)^\dagger$	25.88/51.75
2	10	$65.33 (4.64)^\ddagger$	59.73/73.13
3	10	$65.33 (6.81)^\ddagger$	56.50/74.50
4	10	$69.48 (5.69)^\ddagger, \$$	61.18/81.50
5	10	$75.59 (9.42)^\$$	59.88/88.20
LD	50	$108.87 (2.96)$	101.33/115.50
ED	50	$22.54 (2.35)$	19.35/24.48

PD: penetration depth, LD: lesion depth, ED: erosion depth. The symbols ($^\dagger, \ddagger, \$$) indicate statistically significant differences ($p < 0.05$).

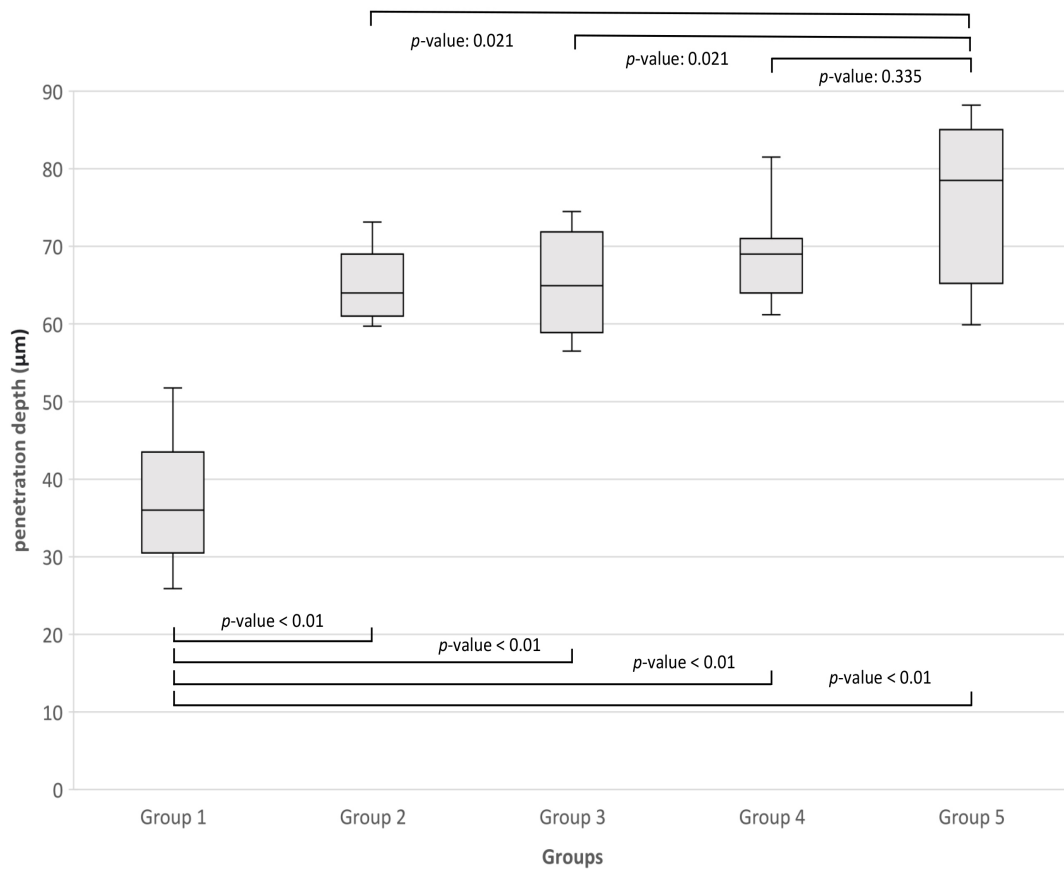


FIGURE 3. A box-and-whisker plot of the penetration depths between the groups. *p*: *p*-value. μm : micrometer.

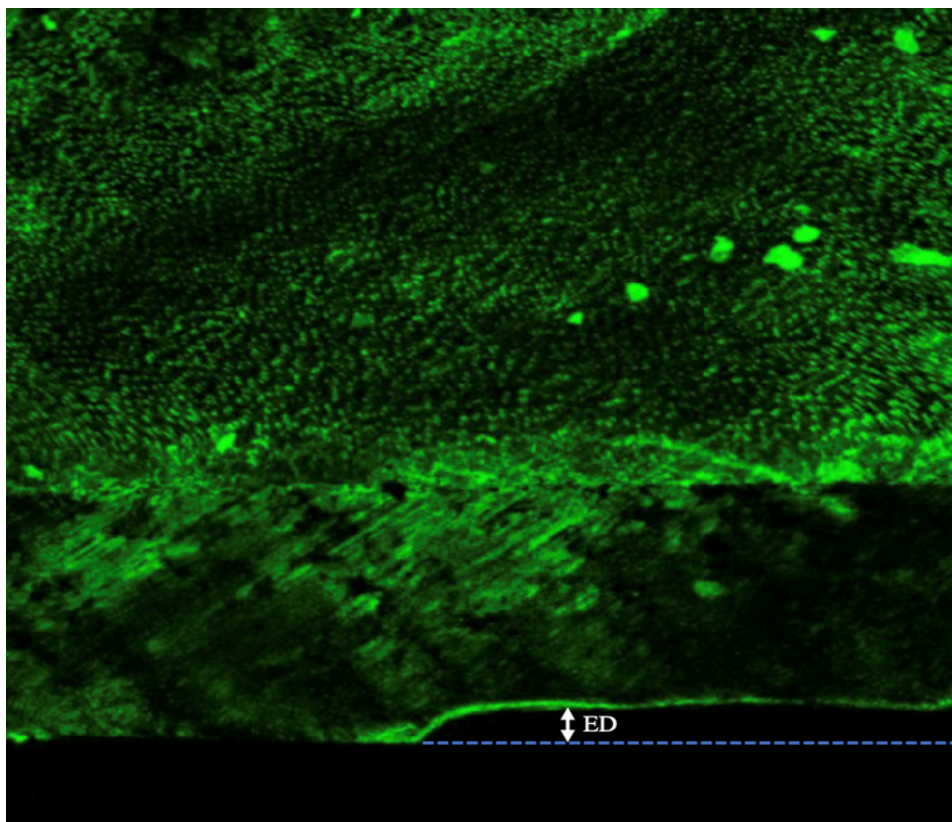


FIGURE 4. A representative CLSM image of the enamel erosion following the etching procedure. The black area marked with a white arrow indicates the erosion depth (ED). CLSM: confocal laser scanning microscopy.

[37]. However, it is not clear whether this effect is achieved by increased superficial infiltration or resin penetration into deeper tissues. Furthermore, although the improved esthetic appearance of enamel lesions has been reported as being dependent on repeated etching cycles, this is insufficient data regarding its effect on resin penetration depth.

In the present study, the null hypothesis was rejected, as repeated etching cycles enhanced the penetration depth of the resin into the artificial enamel caries. This may suggest that repeated HCl application removes the surface layer and increases porosity due to the loss of minerals in the enamel subsurface area. Askar *et al.* [38] reported that demineralized enamel in deep lesions demonstrated greater resin infiltration than in shallow lesions. This deep infiltration may be caused by extensive mineral loss, resulting in more porous enamel.

One concern with repeated etching is that it could cause enamel surface erosion. Arnold *et al.* [16] reported a median enamel substance loss of 34.02 μm following 2 min of 15% HCl application. Furthermore, erosion depth increased significantly (total median enamel surface loss = 77 μm) when the etching time increased (2×2 , 3×2 and 4×2 min). However, they concluded that the total erosion depth was negligible as it was rather shallow. Another *in vitro* study reported no significant differences regarding the erosion depths in lesions compared to sound enamel post-etching [12]. In the present study, enamel erosion was only observed in five samples, which constituted a very small fraction of the overall sample size.

5. Conclusions

Resin penetration depth into artificial enamel lesions was enhanced following repeated acid etching cycles in this study. However, the total erosion depth of the enamel surface was rather shallow and was only observed in a small proportion of the overall sample. Therefore, repeated acid etching cycles might be beneficial in clinical practice to enhance the penetration of resin infiltrants.

AVAILABILITY OF DATA AND MATERIALS

The data presented in this study are available on reasonable request from the corresponding author.

AUTHOR CONTRIBUTIONS

GK—conceived the ideas; wrote the manuscript. GK and MÇ—designed the experiments and acquired the data; interpreted the data; revised and improved the manuscript. All the authors have read and approved the final version for publication.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethical approval was obtained by the Local Ethics Committee of Animal Experiments of Gaziantep University in Turkey (220/3;131).

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Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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