Original Article

An *in vitro* evaluation of Icon resin infiltrant penetration into demineralized enamel lesions using an indirect staining technique with confocal laser scanning microscope analysis in dual fluorescence mode

G. P. V. Srikumar, Megha Ghosh, A. Arvind Kumar[1], Siddharth Bardia[2], Akansha Wasule, J. S. Beautlin[1]

Department of Conservartive Dentistry and Endodontics, Triveni Institute of Dental Sciences, Hospital and Research Centre, Bilaspur, Chhattisgarh, [1]Department of Conservative Dentistry and Endodontics, Rajas Dental College and Hospital, Tirunelveli, Tamil Nadu, [2]Department of Conservative Dentistry and Endodontics, Bhabha College of Dental Sciences, Bhopal, Madhya Pradesh, India

Abstract

Context: White spot lesion is the first clinical sign of a caries lesion and represents mineral loss from the enamel subsurface.

Aim: The aim of this study was to evaluate the penetration depth (PD) of Icon resin infiltrant into artificially demineralized enamel lesions using confocal laser scanning microscope (CLSM) analysis in dual fluorescence mode.

Settings and Designs: The design of the study was an *in vitro* study.

Materials and Methods: 22 extracted human permanent maxillary central incisor teeth were collected, and enamel sections were obtained from the coronal middle third. All enamel specimens were exposed to demineralization and remineralization solutions for 14 days. On positive confirmation of enamel demineralization by scanning electron microscope analysis, 20 specimens were then subjected to Icon resin infiltration following manufacturer instructions. Specimens were processed with indirect staining technique using rhodamine B and sodium fluorescein dyes and examined under CLSM at ×10 magnification in dual fluorescence mode using ImageJ software to evaluate PD of resin infiltrant into demineralized enamel lesions.

Statistical Analysis Used: Obtained data were analyzed using an independent *t*-test. $P \leq 0.05$ was considered statistically significant.

Results: The maximum depth of demineralized enamel lesion was 590 μm, and the mean depth was 290.78 ± 14.80 μm. The maximum depth of resin infiltrant penetration was 580 μm, and the mean depth was 279.08 ± 13.88 μm; $P = 0.006$. The percentage penetration of resin infiltrant was 95.99%.

Conclusion: Icon resin infiltrant was highly effective in its depth of penetration into demineralized enamel lesions. The use of indirect staining and CLSM analysis in dual fluorescence mode is more reliable and accurate technique to evaluate the PD of resin infiltrant.

Keywords: Demineralized lesions; fluorescence; penetration depth; resin infiltrant

Address for correspondence:
Dr. J. S. Beautlin,
CSI Bishop Bungalow, Palace Road, Nagercoil,
Kanyakumari - 629 001, Tamil Nadu, India.
E-mail: beautlin.js@gmail.com

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INTRODUCTION

Dental caries is defined as the destruction of tooth structure by acids generated as by-products of bacterial metabolism in dental plaque. The first clinical sign of enamel caries is a white spot lesion (WSL), which precedes cavitation.[1] WSL or incipient enamel caries are the initial enamel caries resulting in the subsurface demineralization beneath an intact surface layer of enamel and are considered reversible if they are detected early.[2] WSLs are generally reversed by the process of remineralization with fluoride application. The goal of caries management is, therefore, to stop and arrest the progression of the lesion.[3]

A new microinvasive treatment method was suggested for the management of WSLs/demineralized enamel lesions with the infiltration of resin. Paris and Meyer-Lueckel in the University of Kiel, Germany, modified the composition of conventional adhesive resin and introduced Icon Resin infiltrant (DMG, Hamburg, Germany) containing triethylene glycol dimethacrylate (TEGDMA 99%), initiators and stabilizers in 2009, with the aim of improving its depth of penetration designed specifically for the treatment of WSLs. It also helps in preventing further progression of initial enamel caries by occluding the microporosities within the enamel lesion by means of its low viscosity with minimal intervention of the demineralized enamel.[4] Icon resin infiltration technique aims to create a diffusion barrier inside the body of the lesion and not on the lesion surface.[5]

Rhodamine-B and sodium fluorescein dyes are the most commonly used fluorochromes with conventional light microscopy and confocal microscopic analysis.[6] Watson and Boyde were the first to advocate fluorescent confocal microscopy for analysis of the interface between the restorative materials and tooth surface using fluorescent dyes mixed into adhesive agents to highlight the bonded interfaces.[7] Confocal laser scanning microscope (CLSM) was developed by Marvin Minsky in 1955, it uses optical imaging to create a virtual slice or plane many micrometers deep within the tissue and provides very high-quality images with finer details, increased contrast, and is used to examine the penetration depth (PD) of resin infiltrant.[8]

Paris and Meyer-Lueckel and Meyer-Lueckel and Paris, in their research studies, mainly focused on the clinical success and outcome of the resin infiltrant.[4,9] However, the PD of resin infiltrant into demineralized enamel could be a key determining factor in the formation of a diffusion barrier for the management of incipient caries lesions. Hence, the aim and objective of our study was to evaluate the depth of penetration of Icon resin infiltrant into artificially demineralized enamel lesions and its efficiency in completely occluding them with minimal intervention using an indirect staining technique with CLSM analysis in dual fluorescence mode.

MATERIALS AND METHODS

The present *in vitro* study was conducted in the department of conservative dentistry and endodontics after obtaining an Institutional Ethical Committee clearance certificate: TIDSHRC/IEC/2023/D0019. The study sample consisted of 22 periodontally compromised freshly extracted human permanent maxillary central incisor teeth. All teeth were examined under a fully automatic fluorescence stereomicroscope (Leica M205 FA, Leica Microsystems, Germany) to ensure that they were intact and contained no caries or noncaries lesions, devoid of restorations, clinically detectable fractures, stains, WSLs, and enamel hypoplasia following strict inclusion criteria. The collected teeth were cleaned off superficial debris, calculus, and residual tissue tags using ultrasonic instruments. The Occupational Safety and Health Administration and Centers for Disease Control and Prevention recommendations and guidelines were followed during the collection, sterilization, and handling of extracted teeth and were stored in 0.5% thymol at room temperature until used.

All specimens were then sectioned using a diamond disk (DFS, Germany) attached to a slow-speed micromotor straight handpiece (NSK, Japan) on the coronal middle third of teeth to obtain 22 enamel specimens/sections with approximate dimensions of 6 mm × 6 mm × 3 mm. They were completely coated with two layers of acid-resistant nail varnish (Maybelline, Maybelline New York, USA) except on the labial enamel surfaces. Artificial demineralization of enamel was done by immersing specimens in demineralizing and remineralizing solutions.[10,11]

Composition and preparation of demineralization solution: 66.58 mg of $CaCl_2$ powder (12 mM calcium chloride), 68.04 mg of KH_2PO_4 powder (10 mM monopotassium phosphate), and 292.20 mg of NaCl powder (100 mM sodium chloride) were taken in a glass beaker (IndoSurgicals Pvt. Ltd., New Delhi, India) and mixed with 0.186 ml of $C_3H_6O_3$ (50 mM lactic acid) and 50 ml of distilled water to obtain 50 ml of demineralizing solution.[10,11] Composition and preparation of remineralization solution: 6.32 mg of $CaCl_2$ powder (1.5 mM calcium chloride), 34.02 mg of KH_2PO_4 powder (5 mM monopotassium phosphate), and 292.20 mg of NaCl powder (100 mM sodium chloride) were taken in a glass beaker (IndoSurgicals Pvt. Ltd., New Delhi, India) and mixed with 0.285 ml of CH_2COOH (100 mM acetic acid) and 50 ml of distilled to obtain 50 ml of remineralizing solution.[10,11] The pH of demineralization and remineralization solutions was maintained at 4.5 and 6.5, respectively, measured using a digital pH meter (Shapure, Delhi, India), and any variation in pH was

corrected by adding lactic acid or potassium hydroxide. All the enamel specimens were first immersed in a glass beaker containing the prepared demineralization solution and incubated at 37°C for 6 h, followed by immersion in the prepared remineralization solution and incubated at 37°C for 18 h; this cycle of exposure of enamel specimens to demineralization and remineralization solutions was repeated daily for 14 consecutive days.[10,11]

To confirm the enamel demineralization, two specimens were randomly selected and examined under a scanning electron microscope (Carl Zeiss, Sigma 300 VP, Germany) at ×200 magnification, and only on positive confirmation of demineralized enamel, the remaining 20 specimens were subjected to resin infiltration. To facilitate ease of handling of enamel specimens during resin infiltration technique, each specimen was embedded in self-cure acrylic resin (DPI-RR Cold Cure, Mumbai, India) blocks using a standardized mold in dimensions of 8 cm × 8 cm × 4 cm with the labial enamel surfaces exposed.

The Icon resin infiltrant (ICON, DMG, Hamburg, Germany) [Figure 1] technique was used on artificially demineralized enamel specimens following manufacturer instructions. Icon-Etch gel (15% hydrochloric acid) was first applied on enamel for 2 min with the gel stirred from time to time during application using a microbrush and then thoroughly rinsed off with water for 30 s, dried using oil and water-free air stream. Icon Dry (99% ethanol) was then applied on the enamel surface and left for 30 s, followed by drying with oil and a water-free air stream.[10]

All enamel specimens were then subjected to indirect staining technique as described by S. Paris *et al*., by immersing in a glass beaker (IndoSurgicals Pvt. Ltd., New Delhi, India) containing ethanolic solution of 0.1% rhodamine B isothiocyanate for 12 h (solution prepared by dissolving 4.79 g of rhodamine B red fluorophore powder [HiMedia Laboratories Pvt. Ltd., Nashik, India] in 100 ml of 99.9% ethanol [Changshu Hongsheng Fine Chemical Co. Ltd., China]), allowing the red fluorescent dye to fully mark the microporosities of the demineralized enamel.[12]

Figure 1: Icon resin infiltrant kit; Icon-Etch, Icon-Dry, Icon-Resin

Subsequently, all enamel specimens were again dried using an oil and water-free air stream for 10 s to maintain complete dryness. Icon Resin was then dispensed on the enamel surface using an applicator tip provided by the manufacturer for its controlled extrusion, allowed to sit for 3 min, the excess resin was wiped away using a sterile cotton pellet, and the surface was light cured for 40 s using Light Emitting Diode (SAAB, China) dental curing light.[10,11] The resin infiltrant was reapplied for a second time and allowed to sit for 1 min, the excess resin was removed and light cured for an additional 40 s according to the manufacturer's instructions to completely infiltrate any missed microporosities of demineralized enamel during the first application and to compensate for the polymerization shrinkage of resin.

The specimens were then placed in a glass beaker containing 30% hydrogen peroxide solution (Molychem laboratory reagents, Mumbai, India) and incubated at 37°C for 12 h to bleach all unbound red fluorophore dye (rhodamine B) from the demineralized enamel microporosities not enclosed or sealed by resin infiltrant; subsequently, specimens were washed with deionized water (Stanbio Reagents Pvt. Ltd., Kolkata, India) for 60 s.[12] All specimens were then carefully removed from acrylic resin blocks along with the removal of nail varnish using a surgical blade (Glass Van, Medinatal Healthcare Limited, India) visually inspected under a dental operating light, and each specimen was longitudinally sectioned into two equal halves using a Precision Saw (IsoMet 1000, Buehler, USA). We chose the most representative half of each specimen, thus 20 samples were selected.

To mark the demineralized enamel microporosities not sealed by resin infiltrant, the selected samples were immersed in a glass beaker containing 50% ethanolic solution of 100 μM sodium fluorescein for 3 min (solution prepared by dissolving 4 mg of sodium fluorescein green fluorophore powder [HiMedia Laboratories Pvt. Ltd., Nashik, India] in 50 ml of 99.99% ethanol and 50 ml of distilled water) and subsequently washed with deionized water for 10 s.[12]

From each specimen, 300 μm thick slices were obtained using a hard-tissue microtome (SP 1600, Leica Microsystems, Germany) and examined with CLSM (Arquimed, FV1000 Olympus FluoView, Japan), approximately 10 μm underneath the tooth surface at ×10 magnification in dual fluorescence mode.[12-14] The emitted argon/krypton laser light was made to pass through a long pass filter to ensure only the fluorescent light was detected and the

reflected light was suppressed. Rhodamine B (red color) and sodium fluorescein (green color) dyes were detected simultaneously. The excitation and emission wavelength of rhodamine B dye were 568 nm and 590 nm and sodium fluorescein dye was 488 nm and 525 nm, respectively. Images were recorded with a size of 1000 μm × 1000 μm and a resolution of 1024 × 1024 pixels.

To allow reproducible measurement of demineralized enamel lesions depth and PD of resin infiltrant, 10 random markers per image were set, and the measurement points were marked with a 50 μm grid. Three CLSM images of each lesion were made with different detector sensitivities by adjusting photomultiplier amplification (PMT). The first image was taken with a small detector sensitivity of PMT-300, thus depicting only the beginning of the lesion in good quality. The second image was taken with PMT-500 used to configure the lesion body. The third image taken with PMT-700 showed the progressive demineralization underneath the lesion body. The three images were then combined using ImageJ software (LOCI, Wisconsin, USA), resulting in one computed picture and assessed. The lesion depth (LD) was defined as the distance from the specimen surface to the base of prism cores, and PD was defined as the distance from the specimen surface to the deepest point of resin infiltration, seen in red and green fluorescence, respectively.[15,16]

CLSM images of specimens with double-fluorescence staining showed: "Red areas" (labeled by rhodamine B dye) representing the depth of acid-etched demineralized enamel penetrated by resin infiltrant, as the dye accumulated at the progressing lesion front or beneath the resin layer, fluorescence was noticed. The "Green areas" (labeled by sodium fluorescein dye) represent demineralized enamel not sealed by resin infiltrant. In contrast, sound enamel without etch and demineralized enamel microporosities completely infiltrated with resin showed no fluorescence, displayed as "Black areas" [Figure 2a and b].

The mean resin infiltrant PD per image of each sample was obtained, by adding the depths of penetration of resin infiltrant at the 10 pre-selected random marker points, and the summation was divided by 10. The mean demineralized enamel LD per image of each sample was obtained, by adding the demineralized enamel lesions depth at the 10 preselected random marker points, and the summation was divided by 10.

Using the mean depth penetration of rhodamine B dye (red areas) into the base or progressing lesion front of demineralized enamel and the mean depth penetration of sodium fluorescein dye (green areas) not infiltrated by resin, we calculated the difference between the depth of demineralized enamel lesions and PD of resin infiltrant in all samples. Percentage penetration was calculated using the formula:[17] PP = PDmax/LDmax × 100, where PDmax is the maximum PD of resin infiltrant and LDmax is the maximum demineralized enamel LD. The collected data were recorded and tabulated.

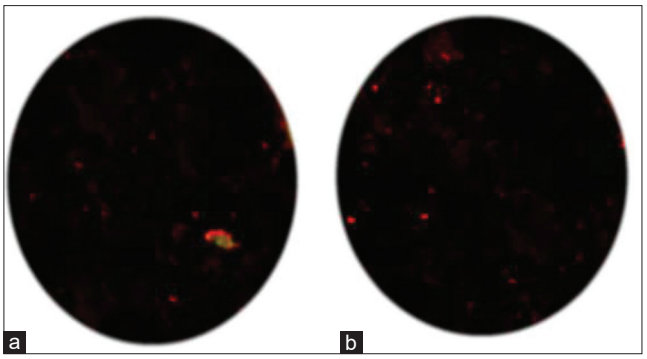


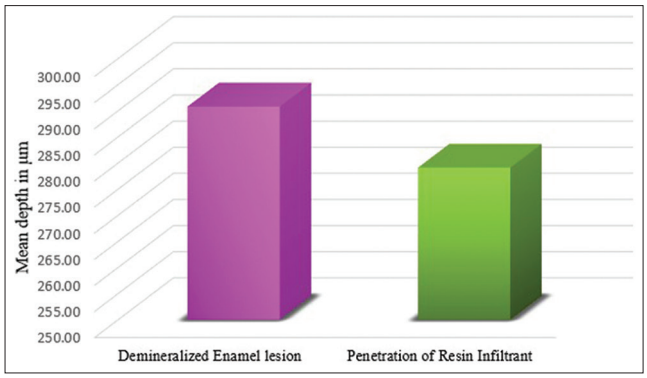
Figure 2: (a) Confocal laser scanning microscope (CLSM) images of specimens after dual fluorescence staining; Red area represents the depth of acid-etched demineralized enamel penetrated by resin infiltrant, as the dye accumulated at the progressing lesion front or beneath the resin layer. Green area represents demineralized enamel not sealed by resin infiltrant. Black area represents sound enamel and also demineralized enamel completely infiltrated with resin showed no fluorescence (b) CLSM images of specimens after dual fluorescence staining; Red area represents the depth of acid-etched demineralized enamel penetrated by resin infiltrant, as the dye accumulated at the progressing lesion front or beneath the resin layer. Green area represents demineralized enamel not sealed by resin infiltrant. Black area represents sound enamel and also demineralized enamel completely infiltrated with resin showed no fluorescence

RESULTS

Data analysis was carried out using SPSS (Statistical Package for the Social Sciences) software (Version 18.0, SPSS Inc., Chicago, IL, USA). An independent *t*-test was used for statistical analysis. $P \leq 0.05$ was considered statistically significant. Among the specimens, the maximum depth of demineralized enamel lesion was 590 μm, and the mean depth was 290.78 μm. The maximum depth of resin infiltrant penetration was 580 μm, and the mean depth was 279.08 μm [Graph 1 and Table 1]. Independent *t*-test showed a statistically significant difference, as $P = 0.006$. The percentage penetration of the Icon resin infiltrant was 95.99% [Graph 2 and Table 2]. The results of our study showed that resin infiltrant was highly effective in its depth of penetration into the demineralized enamel lesions.

DISCUSSION

Icon resin is a newer group of light curable dental resins often known as "infiltrants." Icon resin infiltration is a therapeutic approach bridging the gap between preventive and operative dentistry.[18] Arresting incipient enamel caries



Graph 1: Vertical bar graph: Depth of demineralized enamel lesions and penetration depth of resin infiltrant

Table 1: Independent *t*-test: Mean depth of demineralized enamel lesions and penetration depth of resin infiltrant (µm)

Number of specimens	Depth	Mean±SD	*P*
20	Demineralized enamel lesions	290.78±14.80	0.006*
	Penetration of Icon resin infiltrant	279.08±13.88	

*$P<0.05$, considered statistically significant. *P*: Probability, SD: Standard deviation, Icon resin infiltrant showed higher mean depth of penetration into demineralized enamel lesions

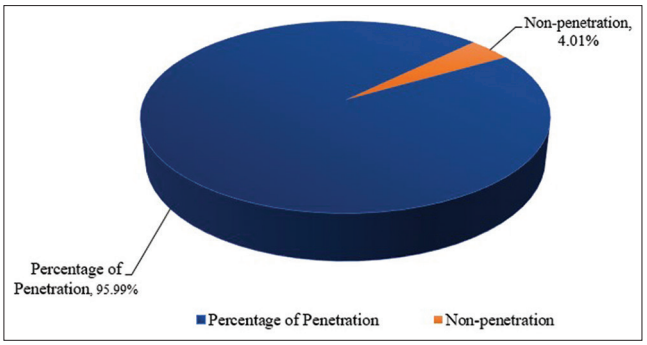
Table 2: Percentage penetration and nonpenetration of Icon resin infiltrant

Number of specimens	Depth	Percentage
20	Percentage of demineralized enamel lesions	100.00
	Percentage penetration of Icon resin infiltrant	95.99
	Nonpenetration of Icon resin infiltrant into demineralized enamel lesions	4.01

lesions with resin infiltration is a promising treatment approach.[13] The resin infiltrant for this purpose must have high surface tension, lower viscosity, and contact angle with enamel, all of which are important properties for penetration of resin into the depth of demineralized enamel lesions.[19]

The use of HCl gel as an etchant demonstrated to be superior to 37% phosphoric acid gel in removing the surface layer of enamel lesions, and Meyer-Lueckel *et al*. reported that etching with 15% HCl gel is more suitable compared to 37% phosphoric acid gel for enamel pretreatment for resin infiltration and 15% HCl produces greater PD of 58 µm, twice that of phosphoric acid gel of 25 µm enabling deeper penetration of resin.[20] In our study, the maximum and mean depth of demineralized enamel lesions were 590 µm and 290.78 µm, respectively. Mueller *et al*., in a previous study, reported 237 µm as the mean depth of artificially demineralized enamel lesions.[13]

In our study, we observed nonuniformity in the depth of demineralized enamel lesions among specimens. Meyer-Lueckel *et al*., in a previous *in vitro* study, also reported



Graph 2: Pie chart: Percentage penetration of resin infiltrant

nonuniformity in the depth of artificially demineralized enamel lesions and attributed them to the structural differences in terms of pore volume, pore diameter, and surface porosities of enamel after acid etching.[15]

The ethanol wet-bonding technique was used to desiccate the etched surface by applying 99% ethanol (Icon Dry) for 30 s followed by air drying. The use of ethanol slowly replaces moisture within the demineralized collagen matrix without causing shrinkage of interfibrillar spaces, thus preventing phase separation of the hydrophobic resin monomers.[21]

Our study showed that Icon resin infiltrant effectively penetrated deeper and sealed the artificially demineralized enamel lesions in all specimens, as the mean depth of demineralized lesions was 290.78 µm and the mean PD of resin infiltrant was 279.08 µm. Subramaniam *et al*., Arslan *et al*., Paris *et al*., reported the superior ability of Icon resin infiltrant to penetrate deeper into demineralized enamel lesions.[10,19,22] The findings of our study were also in accordance with the earlier observations. Kielbassa *et al*. stated that the depth of resin infiltration into subsurface demineralized enamel lesions was over 100 µm.[23] Paris *et al*. reported that adding ethanol and TEGDMA to Icon resin significantly reduced its viscosity and contact angle, thereby increasing the permeability coefficient with satisfactory hardening and reducing morbidity and microleakage.[18] Torres *et al*., in their study, concluded that resin infiltration proved as an effective treatment modality for masking WSLs.[1]

The use of fluorescence dyes with CLSM analysis proved to be a valuable newer technique for the visualization of subsurface demineralized microporosities in dental hard tissues.[12,13] Fluorescence confocal microscopy is a useful tool to analyze the depth of penetration of low-viscosity resins into demineralized enamel lesions.[12,16]

The use of multiple fluorescence stains allows selective visualization of different materials and changes in the tooth substrate, thus frequently used in CLSM imaging for detailed investigation. Both rhodamine B and sodium fluorescein

dyes (fluorophores) allow for selective visualization of specimens due to their well-separated excitation and emission wavelengths, and this peak-to-peak difference between the excitation and emission spectra is called as Stokes shift. It is due to intramolecular energy transfer with part of the molecule acting as a donor, absorbing light, and another portion of the molecule acting as an acceptor, emitting light with a significant shift.[24]

The direct staining technique was conventionally used to visualize resin infiltrant penetration into demineralized enamel lesions; in this, the resin is labeled with dye (fluorophore) before its use as an infiltrant. However, the limitation of this technique is the chromatographic separation of the resin-dye mixture during penetration into demineralized lesions causing erroneous interpretation of CLSM images of false assumption that the fluorophore was not seen in all infiltrated lesions leading to underestimation of PD.[12] Therefore, the indirect staining technique was used in our study due to its reliable interpretation in CLSM image analysis.

In our study, the use of the indirect staining technique with CLSM imaging in dual fluorescence mode allowed for a well-defined analysis of the depth of demineralized enamel lesions and PD of resin infiltrant. The maximum depth of demineralized enamel lesion and PD of resin infiltrant were 590 and 580 μm, respectively. The findings of our study are in accordance with previous studies of Paris *et al*., and Paris *et al*. reported that the use of the indirect staining technique with CLSM imaging in dual fluorescence mode yielded reliable results in better visualizing the depth of demineralized lesions and infiltrant penetration.[12,17]

In our study, the percentage penetration of Icon resin infiltrant into demineralized lesions was found to be 95.99%, thus making the resin infiltration technique a valuable, clinically efficient restorative treatment option for incipient caries or WSLs. The "Washburn equation" describes the penetration of a liquid (light-cured resin) into a porous solid (enamel lesion), with the enamel as a bundle of open capillaries and the resin penetrating into these microporosities by capillary forces. Washburn equation is the penetration coefficient (PC), as it describes the surface tension, viscosity, and contact angle of the resin infiltrant to the demineralized enamel lesions. According to this equation, the application time is as important for the PD of resin infiltrant as the PC.[18] Paris *et al*. concluded that the Washburn equation was applicable to predict the effective penetration of resin infiltrant into demineralized enamel.[18] Hence, in our study, the Icon resin infiltrant was manipulated strictly following manufacturer instructions with its 1st application for 3 min on the enamel surface followed by its 2nd application for 1 min.

The limitation of our study, *in vitro* conditions differ from *in vivo* situations. In the oral environment, the initial invasion of tooth enamel by cariogenic bacteria is accompanied by simultaneous demineralization and remineralization in a continuous cycle with bacterial invasion into the deeper layers of enamel causing subsurface demineralized lesions/ incipient caries lesions with intact healthy surface layer of enamel.

CONCLUSION

The Icon resin infiltrant was highly effective in its depth of penetration (95.99%) into the demineralized enamel lesions. The use of indirect staining technique and CLSM analysis in dual fluorescence mode is a more reliable and accurate method for the evaluation of PD of resin infiltrant into demineralized lesions and thus, should be preferred. Nonetheless, further clinical studies are needed to confirm the effectiveness in the depth of penetration of resin infiltrant into incipient enamel caries lesions.

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Conflicts of interest
There are no conflicts of interest.

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