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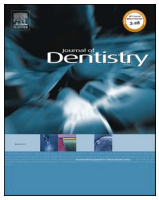

Infiltration of salivary proteins into dentin during erosive processes

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ABSTRACT

Objective: Ultrastructural analyses showed that during erosion under oral cavity conditions, dentin is infiltrated by a substrate morphologically similar to salivary proteins. This in-situ study aimed to investigate the presence of salivary proteins in demineralized dentin.
Methods: Bovine dentin specimens were attached to individual maxillary splints ($n = 1$ per subject and condition) and worn intraorally by four subjects for 1 min. During intraoral exposure, the subjects rinsed with 10 ml of either sterile water or 1 % citric acid. Additional specimens were eroded in-vitro as controls ($n = 2$). The specimens were then fixed, embedded in acrylic resin, and ultrathin sections were prepared. The salivary protein lysozyme was labelled with immunogold staining and examined using transmission electron microscopy.
Results: Lysozyme was absent in the in-vitro controls but was detected on the dentin surface after rinsing with water, indicating its role in the initial pellicle formation on dentin. After rinsing with citric acid, lysozyme infiltrated deeper layers of the dentin.
Conclusions: These findings support the initial morphological observations that lysozyme, representing other salivary proteins, is deposited in demineralized dentin during erosion with citric acid.
Clinical significance: Infiltration of salivary proteins into dentin during erosive processes can be a factor to consider in remineralization approaches under oral cavity conditions.

1. Introduction

Saliva is considered the most important biological factor influencing dental erosion. It has protective properties as it can dilute, clear, neutralize, and buffer erosive substances. Saliva is rich in calcium and phosphate, and its proteins adsorb to the dental surface to form the protective pellicle [1,2]. During erosion, hydroxyapatite, the mineral of dental hard tissue, is dissolved by acids and/or chelators. Frequent exposure to erosive substances leads to loss of enamel and, eventually, dentin [3]. Unlike enamel, dentin contains a high proportion of organic material, primarily type I collagen [4]. During dentin erosion, hydroxyapatite is dissolved, exposing the collagen network. This transforms the initial surface process into a diffusion phenomenon [5]. As erosion progresses, the intra- and extrafibrillar hydroxyapatite is replaced by water. During remineralization, these compartments can be refilled with minerals by saliva or remineralizing agents [6,7]. In recent decades, an increasing number of remineralizing agents with different mechanisms were introduced to the market [8]. Although these agents are successful under laboratory conditions, there are factors in the oral cavity that limit remineralization.

Remineralization is a complex process influenced by various environmental factors. Saliva is unable to fully remineralize enamel, even after prolonged exposure, a limitation often attributed to salivary proteins that inhibit the precipitation of calcium and phosphate [9,10]. These proteins may also interact with other remineralizing agents or hinder the process by forming the pellicle on the dental surface. Additionally, in dentin, the collagen network is a crucial structure for remineralization but is susceptible to enzymatic degradation in the oral cavity [11]. A recently published study made another observation that could represent a further limitation for remineralization. In the study by Schestakow et al. (2023), dentin was eroded under laboratory and oral cavity conditions, and it was found that upon contact with saliva, at least the extrafibrillar cavities were infiltrated with an organic network showing ultrastructural similarities to salivary proteins [12].

Therefore, the aim of the present study was to detect and localize the infiltration of salivary proteins in demineralized dentin using immunogold labelling. Lysozyme was selected as a representative salivary protein due to its small size of 14,3 kDa, which falls below the size exclusion threshold of collagen [13], as well as its abundance in both saliva and the initial pellicle [14–17]. If infiltration occurs, we suggest that

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extrafibrillar spaces may be occupied by these proteins during erosion, replacing dissolved minerals. This could impede the complete remineralization of the exposed collagen network, prompting a re-evaluation of current remineralization concepts and therapies.

The hypothesis is that lysozyme infiltration into dentin will be higher in eroded dentin under oral cavity conditions, compared to either non-eroded dentin in oral cavity conditions or eroded dentin under laboratory conditions.

2. Materials and methods

2.1. Specimens

Dentin specimens ($n = 10$) were prepared from the labial surfaces of bovine incisor teeth by cutting with a circular saw and polishing with abrasive paper up to 4000 grit using a wet grinding machine. The smear layer was removed by immersing the specimens in 3 % sodium hypochlorite for 30 s, followed by washing with distilled water and an ultrasonic bath with distilled water for 2 min. After disinfection in 70 % isopropyl alcohol for 15 min, the specimens were stored in sterile water at 4 °C for at least 6 h before use.

2.2. Subjects

Four healthy volunteers (3 males, 1 female, 27–42 years) participated in this study, which was approved by the Medical Ethics Committee of the Medical Association of Saarland (54/21, 2021). All volunteers provided written informed consent. None of the participants had cavitated carious lesions, clinical signs of gingivitis or periodontitis, nor had they taken antibiotics in the past 3 months. Exclusion criteria included systemic diseases, smoking, and pregnancy.

2.3. Erosion

One specimen per trial was attached to the buccal side of individual upper right splints made from methacrylate using silicone impression material. Immediately after the splints were inserted into the oral cavity, subjects rinsed with either 10 ml of sterile water (pH 4.41) (Ampuwa, B. Braun Melsungen, Melsungen, Germany) or 10 ml of 1 % citric acid (pH 2.5). As an additional control without exposure to saliva, specimens were immersed in 10 ml of 1 % citric acid for 1 min under agitation. For the in-vitro trial, the in-situ trial with water, and the in-situ trial with citric acid, $n = 2$, 4, and 4 specimens were used, respectively. The in-situ trials included 4 subjects and consequently more specimens to account for interindividual variability.

2.4. Fixation and embedding

Immediately after rinsing the oral cavity or immersion in citric acid, the specimens were rinsed with water and then fixed in a solution containing 0.05 % glutaraldehyde and 2 % paraformaldehyde in 0.1 M cacodylate buffer. Following fixation, the specimens were washed in 0.5 M cacodylate buffer (4 times for 10 min each), dehydrated through an ascending ethanol series, and embedded in acrylic resin (LR White middle catalysed, Science Services, Munich, Germany). The enamel and portions of the dentin were removed using 1200-grit abrasive paper on a wet grinding machine, leaving 0.5 mm of the superficial dentin. The specimens were then re-embedded in acrylic resin. Cross-sectional ultrathin sections were cut using the ultramicrotome Leica EM UC7 (Leica Microsystems, Wetzlar, Germany) with a diamond knife (Diatome, Nidau, Switzerland), and mounted on nickel slot grids (Plano, Wetzlar, Germany).

2.5. Immunogold labelling

Immunogold labelling of lysozyme was performed using a two-step labelling process. First, the ultrathin sections were treated with 50 mM ammonium chloride for 5 min, followed by washing in phosphate-buffered saline (PBS) (5 times for 3 min each). The sections were then treated with 2 % bovine serum albumin (BSA) for 10 min and labelled with the primary antibody at a 1:10 dilution in 2 % BSA at 4 °C overnight. The specificity of the primary antibody (anti-lysozyme antibody produced in rabbit, Sigma-Adrich, Taufkirchen, Germany) was tested by western blot in human saliva (Fig. A.1). After washing in PBS (5 times for 5 min each), the sections were labelled with gold-conjugated (10 nm) secondary antibodies (Goat-anti-Rabbit IgG, Aurion, Wageningen, The Netherlands) at a 1:200 dilution in 2 % BSA at RT for 2 h Following final fixation in 2 % glutaraldehyde for 30 min, the sections were washed in sterile water (3 times for 5 min each), contrasted with 2 % uranyl acetate (Science Services, Munich, Germany), and washed again in sterile water (3 times for 5 min each).

2.6. Transmission electron microscopy

The ultrathin sections were examined using TEM (FEI Tecnai 12 BioTwin, FEI Company, Hillsboro, OR, USA). From each section, approximately 50 images at 30,000x magnification were acquired, covering the entire dentin surface of each section. This magnification was chosen to observe the dentin surface to a depth of 1 μm, which represents the demineralization depth of the erosive protocol used [12], and to precisely count the gold particles.

2.7. Quantification and statistical analysis

Quantification and statistical analysis of lysozyme labelling were performed using the Chi-square test as described by Mayhew (2011). First, compartments were defined by overlaying the images with a line-typed and random-offset grid in ImageJ 1.54i (NIH, Bethesda, MD, USA), aligned parallel to the dentin surface, creating layers at 0.1 μm increments up to a depth of 1 μm, corresponding to 10 layers (Fig. 1). Irregular surfaces were excluded from the analysis. The number of gold particles per layer (G_o) and the fields per layer (P) were counted, considering that the layers varied in size due to artifacts. The expected number of gold particles per layer (G_e) was calculated using the formula $G_e = P_{partial} \times G_{o,total} / P_{total}$. The relative labelling intensity (RLI) was then calculated as $RLI = G_o / G_e$. The partial Chi-square value for each

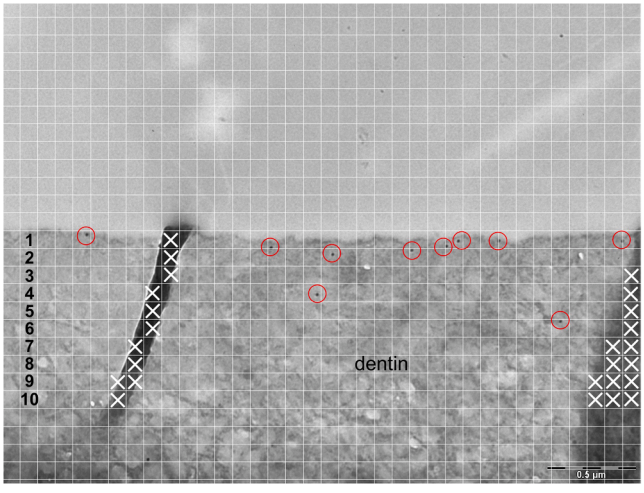

Fig. 1. Illustration of image processing for quantitative analysis. Transmission electron micrographs were overlaid with a grid parallel to the dentin surface, creating layers at 0.1 μm increments up to a depth of 1 μm, corresponding to 10 layers. The number of gold particles (circled in red) per layer and the fields per layer were counted, taking into account that the layers varied in size due to artifacts, such as folds formed during the ultrathin sectioning of hard tissues, which appeared as electron-dense artifacts.

layer was calculated by $\chi^2_{partial} = (G_o - G_e)^2 / G_e$, and the sum of these values provided χ^2_{total}. With df = 9 and $p = 0.05$, the critical χ^2 value was 16.919. A χ^2_{total} value <16.919 indicated a random distribution, suggesting that the gold particles were present due to nonspecific binding. Conversely, χ^2_{total} greater than 16.919 indicated a non-uniform distribution, where the RLI and $\chi^2_{partial}$ values in the contingency table highlighted the locations of the preferred labelled layers. An RLI > 1 and a $\chi^2_{partial}$ >10 % of the total were chosen as the cut-off criteria [18].

3. Results

Western blot analysis demonstrates high specificity of the primary antibody against lysozyme in human saliva, indicated by a distinct band at approximately 14 kDa (Fig. A.1). TEM images illustrate the distribution of gold particles within the dentin, which are visible as electron-dense spheres 10 nm in diameter (Fig. 2). The cross-sections of the embedded specimens reveal the superficial dentin to a depth of up to 2 µm, with its surface bordering the oral cavity. Collagen, depending on its orientation within the dentin, appear as a round or elongated structure when cut transversely or longitudinally, respectively. Due to the specialized fixation and embedding methods used for immunogold labelling, the contrast in the ultra-thin sections is low, making it difficult to clearly distinguish demineralized and exposed dentin from native dentin. Additionally, during ultra-thin sectioning, folds may form, appearing as electron-dense artifacts. Chi-square analysis reveals that gold particles are randomly distributed in dentin treated with citric acid in-vitro ($\chi^2 < 16.919$), suggesting non-specific binding and the absence of lysozyme (Table 1). Conversely, during intraoral exposure, an increased number of gold particles were observed (G_e), with preferentially labelled layers identified, indicating specific binding to lysozyme ($\chi^2 > 16.919$). When subjects rinsed with water, gold particles predominantly accumulated in the superficial first layer (RLI > 1 and χ^2 value > 10 % of total) (Table 2). After rinsing with citric acid, gold particles were enriched not only in the superficial layer but also to a greater extent in the deeper second layer (RLI > 1 and χ^2 value > 10 % of total) (Table 3). Occasionally, a thick pellicle layer was observed during the 1-min intraoral exposure, appearing as a loose granular network on the dentin surface, where gold particles accumulated, indicating that lysozyme is involved in the initial pellicle formation on dentin (Fig. 3).

4. Discussion

The aim of this study was to investigate the assumptions made in a previous study by Schestakow et al. (2023), which suggested that dentin is infiltrated with salivary proteins during demineralization when exposed to a mixture of erosive substance and saliva [12]. Considering the low sample size, the present findings confirm these assumptions.

In this study, immunogold labelling and TEM analysis were employed to detect and localize lysozyme deposition in demineralized dentin up to 1 µm in depth. However, the specificity of immunogold labelling poses a challenge, as colloidal gold from the secondary antibody can bind non-specifically to ultrathin sections. To address this, we optimized the dilution and used a buffer containing unrelated proteins [19]. Despite confirming the primary antibody's monospecificity against lysozyme via western blot, the natural occurrence of lysozyme in dentin remains uncertain. A recent study detected lysozyme in dentin, but its exact origin - whether from dentin, cellular components, or dentinal fluid - was not determined [20]. Consequently, this study focused on intertubular dentin.

In vitro erosion of dentin without saliva contact resulted in few, evenly distributed gold particles, indicating the absence of lysozyme (Fig. 2A and Table 1). However, exposure to saliva led to a distinct increase in gold particles, which were unevenly distributed in the layers. When subjects rinsed with water, gold particles were found on the dentin surface, suggesting their role in the initial pellicle formation on dentin (Fig. 2B and Table 2). Previous studies have detected lysozyme in the initial pellicle on enamel using various methods [14,15,17,21], while for dentin, only enzyme-coupled reactions on a 30-minute pellicle have been used [16]. Lysozyme can adsorb to hydroxyapatite via electrostatic interactions with the tooth surface [22], and might interact with collagen through protein-protein interactions [23].

When dentin was eroded under oral cavity conditions, lysozyme was

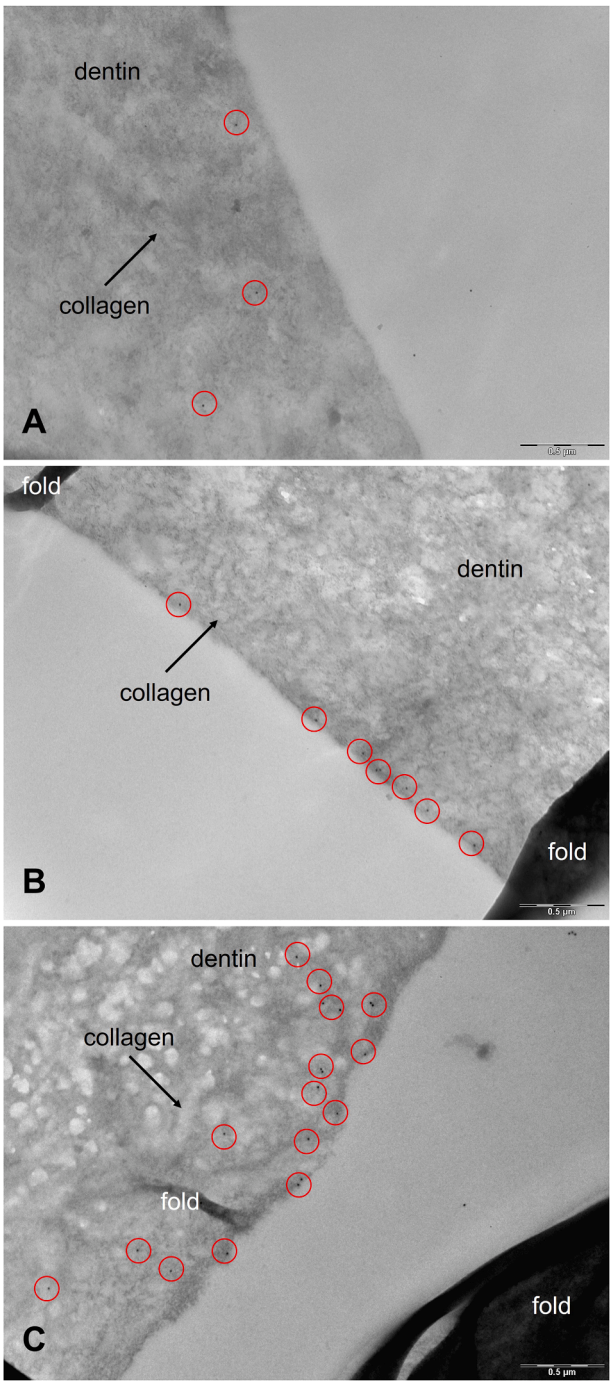

Fig. 2. Representative transmission electron micrographs of dentin surface cross-sections. Dentin was treated with citric acid in-vitro (A), water in-situ (B), or citric acid in-situ (C). Due to the specific preparation method, the distinction between demineralized and native dentin is difficult. Collagen, depending on their orientation within the dentin, appear as round structures when cut transversely and as elongated structures when cut longitudinally. Lysozyme was immunolabeled using primary antibodies and 10 nm gold-conjugated secondary antibodies (circled in red). Folds that are formed during ultrathin sectioning of hard tissues appear as electron-dense artifacts.

Table 1
Localization of gold-labelled lysozyme in layers of different depths of dentin treated with citric acid in-vitro.

Layer	G_o	P	G_e	RLI	χ^2 value	χ^2 as %
1	27	1673	32.54	0.83	0.94	8.41
2	45	1674	32.56	1.38	4.75	42.32
3	36	1671	32.50	1.11	0.38	3.35
4	31	1671	32.50	0.95	0.07	0.62
5	28	1672	32.52	0.86	0.63	5.60
6	42	1670	32.48	1.29	2.79	24.83
7	28	1670	32.48	0.86	0.62	5.51
8	28	1669	32.46	0.86	0.61	5.47
9	29	1669	32.46	0.89	0.37	3.29
10	31	1669	32.46	0.95	0.07	0.59
Column total	325	16,708	325		11.23	100

For $\chi^2 = 11.23$ and df = 9, $p > 0.05$ (Chi-square analysis). The gold labelling distribution is not significantly different from random. G_o = observed gold count, P = point count, G_e = expected gold count, RLI = relative labelling index.

Table 2
Localization of gold-labelled lysozyme in layers of different depths of dentin treated with water in-situ.

Layer	G_o	P	G_e	RLI	χ^2 value	χ^2 as %
1**	444	2663	82.12	5.41	1594.62	83.64
2	131	2663	82.12	1.60	29.09	1.53
3	64	2663	82.12	0.78	4.00	0.21
4	41	2663	82.12	0.50	20.59	1.08
5	22	2662	82.09	0.27	43.99	2.31
6	35	2655	81.88	0.43	26.84	1.41
7	27	2643	81.51	0.33	36.45	1.91
8	12	2626	80.98	0.15	58.76	3.08
9	15	2607	80.40	0.19	53.19	2.79
10	24	2583	79.66	0.30	38.89	2.04
Column total	815	26,428	815		1906.42*	100

For $\chi^2 = 1906.42$ and df = 9, $p < 0.05$ (Chi-square analysis). The gold labelling distribution is significantly different from random (*). Layer 1 is preferentially labelled (RLI >1 and χ^2 value >10 % of total, **). G_o = observed gold count, P = point count, G_e = expected gold count, RLI = relative labelling index.

Table 3
Localization of gold-labelled lysozyme in layers of different depths of dentin treated with citric acid in-situ.

Layer	G_o	P	G_e	RLI	χ^2 value	χ^2 as %
1**	190	2984	108.98	1.74	60.23	12.78
2**	285	2976	108.69	2.62	285.99	60.69
3	115	2964	108.25	1.06	0.42	0.09
4	116	2954	107.89	1.08	0.61	0.13
5	63	2945	107.56	0.59	18.46	3.92
6	83	2937	107.27	0.77	5.49	1.16
7	57	2926	106.86	0.53	23.27	4.94
8	58	2907	106.17	0.55	21.86	4.64
9	61	2862	104.53	0.58	18.13	3.85
10	42	2842	103.80	0.40	36.79	7.81
Column total	1070	29,297	1070		471.24	100

For $\chi^2 = 1906.42$ and df = 9, $p < 0.05$ (Chi-square analysis). The gold labelling distribution is significantly different from random (*). Layer 1 and 2 are preferentially labelled (RLI >1 and χ^2 value >10 % of total, **). G_o = observed gold count, P = point count, G_e = expected gold count, RLI = relative labelling index.

detected not only on the surface but also in deeper layers (Fig. 2C and Table 3). During the erosion process, the hydroxyapatite in the intra- and extrafibrillar spaces demineralizes [6], creating water compartments that we hypothesize are rapidly infiltrated by small salivary proteins. Similar observations were made with enamel, where subsurface pellicles form below the enamel surface after erosion [24]. Although only lysozyme was examined in the present study, it is possible that other small proteins from saliva also infiltrate the demineralized dentin, with lysozyme being representative. Given that the

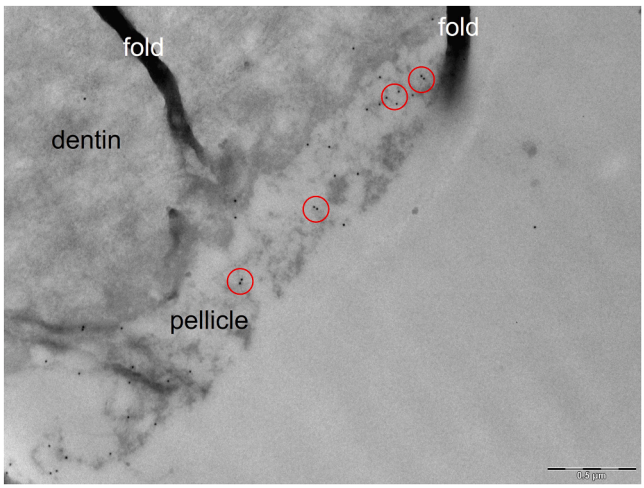

Fig. 3. Transmission electron micrograph of dentin treated with citric acid in-situ. Lysozyme was immunolabeled using primary antibodies and 10 nm gold-conjugated secondary antibodies (circled in red). Cross-sections occasionally show the dentin surface covered by a thick loosely and granularly structured pellicle layer. Folds that are formed during ultrathin sectioning of hard tissues appear as electron-dense artifacts.

demineralization with our protocol reaches approximately 1 μm deep [12], the dentin was not completely infiltrated. The demineralization front is not demarcated due to the special preparation of specimens for immunogold labelling. It remains unclear to what extent the collagen matrix is blocked with salivary proteins, potentially preventing deeper penetration of these proteins.

These results highlight that the de- and remineralization of dentin under oral cavity conditions are more complex than under laboratory conditions. The intra- and extrafibrillar spaces are crucial for the application of adhesives and remineralizing agents. In adhesive techniques, demineralization is deeper [25], saliva contact is shorter, and the impact of saliva contamination can be reduced by decontamination [26]. However, the infiltration of salivary proteins can hinder remineralization after erosion. In the case of the remineralizing agent casein phosphopeptide-amorphous calcium phosphate, remineralization usually takes days to weeks under laboratory conditions, but longer times are observed in the oral cavity. Various factors, such as nutrition, saliva, pellicle, and plaque, are often discussed as influencing this process [7, 27]. The present findings suggests that the infiltration of salivary proteins may be an additional limiting factor. More research is needed to determine the extent to which salivary protein infiltration actually impedes remineralization. The specimens in this study were exposed to the oral cavity for only 1 min, specifically during the erosive challenge. Investigating how demineralized dentin is affected by salivary proteins during prolonged intraoral exposure after erosion is also necessary.

5. Conclusion

These findings support the initial morphological observations that lysozyme, representing other salivary proteins, is deposited in demineralized dentin during erosion with citric acid, a factor that must be considered in remineralization approaches under oral cavity conditions.

CRediT authorship contribution statement

Anton Schestakow: Writing – original draft, Visualization, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Vanessa Schmitt:** Writing – review & editing, Investigation. **João Victor Frazão Câmara:** Writing – review & editing, Investigation. **Matthias Hannig:** Writing – review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jdent.2024.105457.

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