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Effects of toothbrushing on eroded dentine

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It is an established assumption that eroded dental hard tissues are particularly prone to toothbrush abrasion. Only a few studies have aimed to show this for dentine and, if so, disregarded the complex histological structure of this tissue. Therefore, the present study sought (i) to investigate the effects of toothbrushing on eroded dentine and (ii) to analyze how the organic matrix influences the outcome of established methods for quantifying dental hard tissue loss. The effects of brushing were investigated by optical (P-O) and mechanical (P-M) profilometry, by longitudinal microradiography (LMR), and by scanning electron microscopy (SEM). The SEM images showed that a demineralized organic layer had developed, which was unaffected by brushing. For substance loss, there was no significant difference between eroded and eroded/abraded samples. Considerable differences occurred, however, when results from the different methods were compared. P-O yielded the lowest (7.0 ± 3.4 μm) and LMR the highest (109.8 ± 10.7 μm) substance loss values. When the organic material was removed enzymatically, all methods gave comparable results. The results of this study do not lend support to the notion that brushing increases substance loss of eroded dentine. Profilometry was not suitable for measuring mineral loss, unless the organic material was removed.

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Oral tissue wear caused by hygiene procedures is a significant clinical feature (1), and it is a common clinical observation that toothbrushing causes some degree of dental abrasion over a lifetime. Wear of enamel and dentine can be increased if toothbrushing is excessive or, in particular, if it is related to chronic erosive challenges. This has been quantified for enamel (2–4) and, in a few studies, for dentine (5–8).

The underlying mechanism of the synergistic effect of erosion and abrasion is the loss of mineral, resulting in a decrease of surface hardness, thus making the tooth surface more susceptible to physical impacts (2). In the case of enamel, erosion is a surface-controlled process defined by a bulk mineral loss, leaving a partially demineralized softened surface. The mineral content of the remaining enamel increases with depth, which means that there is no subsurface lesion with a more or less intact surface layer, as is the case in the initial caries lesion. In dentine, however, chronic exposure to acids leads to an increasing loss of mineral and to a progressive exposure of the organic dentine matrix. With progressing erosive demineralization, the entire process becomes more diffusion controlled and the mineral loss will tend to decrease with time because of 'barrier effects' of the developing organic surface layer. So far, however, the effects of this organic layer have not yet been discussed. Dentine abrasion studies are currently performed either disregarding the complex histological structure of eroded dentine or under the tacit assumption that organic material is easily brushed away. This point is of particular importance with respect to the methods used for measuring brushing effects.

Profilometric and microradiographic methods have primarily been used for quantifying erosive or combined abrasive/erosive mineral loss. Profilometry is based on the movement of an optical or a mechanical stylus across a given polished or natural sample surface, with its vertical movement representing changes in the surface geometry. Microradiography is the X-ray projection of a given sample, from which the total mineral content per unit tooth area is determined densitometrically. In the case of enamel, both methods give similar results, which is conceivable when the histological structure of eroded enamel is regarded (9). In the case of dentine, however, profilometry might have shortcomings by assuming that organic material is present in eroded dentine and also in samples that are exposed to both erosion and abrasion. An optical stylus would just scan the surface of the organic layer, which is certainly not related to the respective mineral status, and a mechanical device would cave in the organic material to an unknown extent. Nevertheless, in the overwhelming number of erosion and erosion/abrasion studies, profilometry has been used to quantify dentine mineral loss. Regarding the addressed histological structure of eroded dentine, however, it appears necessary to re-evaluate the problem

of brushing abrasion of eroded dentine with a multimethodological approach.

The present study therefore sought (i) to investigate the effects of toothbrushing on eroded dentine and (ii) to analyze how the organic matrix influences the outcome of established methods for quantifying dental hard tissue loss. The following three substantially different methods were used: (i) profilometry, which determines the surface geometry; (ii) longitudinal microradiography (LMR), which is a method used to determine the total mineral content of a given sample; and (iii) scanning electron microscopy (SEM) for the visualization of the histological structure of both eroded and eroded/abraded dentine. To study a possible effect of the organic matrix, samples were immersed in collagenase at the end of the experiment and analyzed again.

Material and methods

Coplanar, 750-μm-thick longitudinal slices of coronal dentine were prepared from freshly extracted, previously impacted human third molars (Exact Trennschleifsystem and Exact Mikroschleifsystem; Exakt-Apparatebau, Norderstedt, Germany) and polished up to P1200 (Leco, St Joseph, MI, USA). All grinding and polishing procedures were performed under sufficient water flow. The experimental area was covered with tape (2×2 mm), and the four edges were marked with small holes produced by means of a cylindrical diamond bur (ISO no. 806,314111514; Komet, Lemgo, Germany) to ensure a well-defined area for microradiography. The sample was covered with a light-curing acrylate (Technovit 7230 VLC; Kulzer-Exakt, Wehrheim, Germany) to achieve a reference area for profilometry. Afterwards, the tape was removed. Finally, the experimental and reference areas were thoroughly checked with a stereo-microscope for any remnants or imperfections. The sample design is shown in Fig. 1. Samples were kept in 100% humidity at room temperature until used for experimental procedures; a sample size of 23 was used in each group.

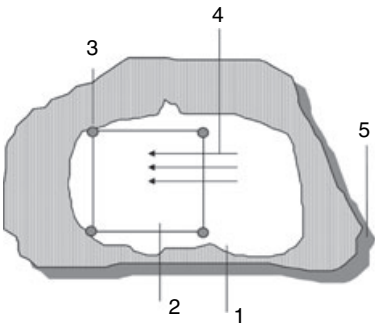

Fig. 1. Sample design and area of measurements: 1, flat undisturbed area, covered with acrylic resin during the erosion and abrasion/erosion procedures (reference area for profilometric measurements); 2, experimental area; 3, drilled holes marking the experimental area for identification during longitudinal microradiography (LMR) measurements; 4, profilometric tracings; and 5, sample thickness 750 μm, as required for LMR procedures.

Experimental protocol

The samples were cycled through a de- and remineralization procedure with erosion in hydrochloric acid for 6×2 min per day. In one group, samples were eroded only, and in a second group samples were eroded and brushed. Standardized brushing was performed twice daily after the first and the last erosion procedure for 15 s per sample using fluoride-free toothpaste (aronal, RDA 60, pH 6.9 for 10% in water; Gebro Pharma, Fieberbrunn, Austria, in licence of GABA International, Münchenstein, Switzerland) using a powered toothbrush (Oral-B Plak Control Ultra; Braun, Frankfurt, Germany) and a brushing force of 2 N. For brushing force control, the samples were mounted on a scale and the toothbrush was lowered until the required value was achieved. After each erosion or erosion/brushing procedure, the samples were thoroughly rinsed with distilled water for at least 1 min and stored in remineralization solution.

The total experimental period was 9 d. At the end of the experiments, optical and mechanical profilometry, as well as microradiography, were performed. The organic material was then enzymatically removed with collagenase (10) for 36 h at room temperature in 15 of the samples of each group and again analyzed profilometrically and microradiographically. The remaining samples were prepared for SEM.

Solution preparation

The remineralization solution was supersaturated with respect to hydroxyapatite and was prepared by combining 40 ml of 0.1 M $H3PO_4$, 100 ml of 0.2 M KCl and 100 ml of 0.12 M $NaHCO_3$ and adjusting the volume to 600 ml with distilled water. Thereafter, 100 ml of 0.02 M $CaCl_2$ was added and the total volume was adjusted to 1 l (11).

The demineralization solution was prepared from de-ionized water with 0.5 wt% NaCl adjusted to pH 1.6 with hydrochloric acid. All chemicals were obtained from Merck (Darmstadt, Germany).

The enzyme solution was the remineralization solution with collagenase (100 U ml^{-1}; collagenase from *Clostridium histolyticum* type VII (Sigma Aldrich, St Louis, MO, USA), with a collagen digestion activity of 1680 U μg^{-1} of solid at 25°C at pH 7.5 in the presence of calcium ions.

Optical and mechanical profilometry

The profilometric procedure has been described previously (9). A Perthometer S8P (Perthen-Mahr, Göttingen, Germany), equipped with a mechanical (FRW-750) or an optical (Focodyn, Rodenstock, Germany) pick-up, was used. To ensure that all tracings were made at the same sample areas, the pick-up was moved in a defined way. First, it was placed in one of the drilling holes and then positioned 0.7 mm apart from the hole on the reference area. Three tracings (1.75 mm length), with a distance of 300 μm, were made, first with the optical device (P-O) and then using the mechanical pick-up (P-M).

The traces were interpreted with special software (PERTHOMETER CONCEPT 4.0; Perthen-Mahr). On the flat reference area, a regression line of 0.4 mm length was constructed to allow the orientation of the profile tracing into the *xy* coordinates. On the eroded area, a second regression line of 0.4 mm length was constructed. On both regression lines, the midpoints were determined and their

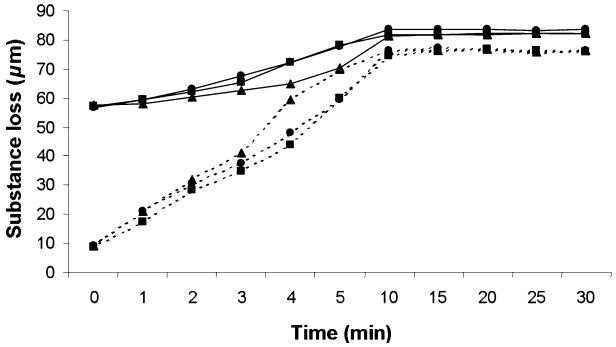

Fig. 2. Values for erosive substance loss of the same tracing area of a given sample obtained from optical (dashed lines) and mechanical (solid lines) profilometry after various periods exposed to the ambient air (circles) and rewetting for 30 s (triangles) and 1 min (quadrates). Note that the value for substance loss obtained from longitudinal microradiography (LMR) was 114.8 μm.

vertical distance (μm) was used as a measure of the erosive substance loss. The substance loss was defined as the mean of the three tracings.

The influence of the ambient air drying was analyzed by performing repeated tracings after various waiting and rewetting procedures. Three cycles were performed with the same sample (Fig. 2), which was not removed from the profilometer system during the whole procedure to ensure that the same area was traced. Initially, samples were contactlessly dabbed off with absorbent tissue and a profile tracing was then performed without further drying or wetting, every min ($n = 10$) and every 5 min ($n = 4$). The sample was then covered with distilled water for 30 s or 1 min, dabbed off and traced again as described above. Distinct drying effects were already obvious after 1 min and reached a plateau after 10 min, but were completely reversible after rewetting for at least 30 s (Fig. 2). Throughout the experiment, samples were therefore rewetted for 30 s before each tracing.

Longitudinal microradiography

The total mineral content of the bulk samples was determined microradiographically (12). At baseline and after the end of treatment, an X-ray projection (CuKα X-ray radiation operated at 20 kV, 50 mA, exposure time 2.5 min) was made on high-resolution films (high-speed holographic film, Kodak SO-253; Kodak, Stuttgart, Germany), which were developed under standardized conditions according to the manufacturer. From the resulting images, the mineral content (scan area 1 × 1 mm) was calculated automatically using a computer-controlled microdensitometer (Leitz MPV compact Ortholux II; Leitz, Wetzlar, Germany) using the drilling holes as reference points to ensure that the scan area was situated within the experimental area.

Scanning electron microscopy

Specimens were fractured into halves, in order to visualize not only the sample surface but also a transverse section of the demineralized dentine and the organic matrix. Following this, they were critical-point dried (Critical point dryer CPD 030; Baltec, Witten, Germany) and lightly gold sputtered. Samples were inspected in a scanning electron microscope (Type XL20; Philips Electron Optics, Eindhoven, the Netherlands) equipped with an LaB_6 cathode. The acceleration voltage was set to 10 kV. Images were recorded using a secondary electron detector with the voltage of the collector grid biased to +300 V in order to improve the signal-to-noise ratio and to reveal optimal topographical contrast. The settings of the SEM, including tilt angle, spot size, and scanning mode, were kept constant. The magnifications used were 100-, 500-, 2,000-, 5,000-, and 8,000-fold.

Statistics

Statistical procedures were performed with SPSS 10.0 for Windows. No data were significantly different from the normal distribution (Kolmogorov–Smirnov test). For comparison of the values obtained from different methods, *t*-tests for paired samples were used. The Bonferroni adjusted level of significance was 0.008 (0.05/6). For comparisons of groups, *t*-tests for independent samples were performed. The Bonferroni adjusted level of significance was 0.006 (0.05/9).

Results

Data for substance loss are given in Table 1. Substance loss values after erosion and brushing were ≈5–10% higher than after erosion only, which, however, for all measuring methods did not reach significance.

Significant differences, however, were seen when the measuring methods were compared. After both erosion and erosion/abrasion, P-O and P-M indicated less apparent substance loss than LMR ($P \leq 0.001$ each), whereas use of the mechanical pick-up resulted in a higher substance loss than use of the optical stylus ($P \leq 0.001$). Collagenase treatment grossly increased loss values obtained with both mechanical and optical pick-ups ($P \leq 0.001$ each), and, to a much lesser extent, also for LMR ($P \leq 0.001$). When mechanical

Table 1

Substance loss values (µm ± standard deviation) obtained from optical (P-O), mechanical (P-M) profilometry and longitudinal microradiography (LMR) after 9 experimental days and after removal of the organic material with collagenase

	P-O	P-M	LMR	P-O + collagenase	P-M + collagenase	LMR + collagenase
Erosion	7.0 ± 3.4	52.0 ± 9.7	93.6 ± 16.0	103.2 ± 2.8	111.8 ± 9.2	108.9 ± 10.7
Erosion/abrasion	7.2 ± 4.2	62.7 ± 13.8	101.7 ± 16.6	107.2 ± 7.0	118.8 ± 6.6	120.4 ± 11.5

profilometry and LMR were compared, no significant differences were found, whereas optical profilometry resulted in somewhat lower values than both mechanical profilometry ($P \leq 0.001$) and LMR ($P \leq 0.008$).

The SEM images revealed no obvious structural differences between specimens that were only eroded and samples that were eroded and brushed (Fig. 3). The cross-sections showed a distinct zone of demineralized dentine, which almost seamlessly turned into sound dentine. At higher magnification, peritubular fibres, as well as the fibrous and fluffy structure of the demineralized intertubular organic matrix, became visible, which was undisturbed by the brushing procedure. Similar results were also found for the sample surface where open tubules and the fibrous intertubular matrix were present. In some samples, the intertubular matrix was fibrous and fluffy, whereas in others a more homogenous and spongy structure was seen, although these differences occurred in both groups. Immersion in collagenase resulted in complete removal of the bulk organic material (Fig. 4). In most specimens, fibrous structures and more or less continuous amorphous coverings were present, but these layers were only a few μm in thickness, as verified in the cross-sections.

Discussion

Erosive mineral loss becomes manifest in clinically visible lesions when the erosive impact occurs regularly and for a period of at least 1–2 yr (13). The present experiment was therefore designed to simulate clinical conditions in risk groups for dental erosion, in particular, patients with eating disorders combined with chronic vomiting. The erosive demineralization was performed with HCl at pH 1.6, which corresponds to the pH of gastric juice. Experiences with bulimic patients from our special consultation hour show that vomiting 6–10 times per day occurs regularly, supporting our use of 6 experimental demineralization phases. The demineralization period of 2 min conforms to the length of a pH decline in saliva after an acid attack (14,15). Brushing abrasion per sample was performed twice daily for 15 s with a brushing force of 2 N. This is somewhat prolonged compared with actual common hygiene habits, but refers to common values for brushing force (16). In brief, the experimental setting reflects clinical conditions with frequent exposure to acid, as present in risk groups, and lifelike tooth brushing procedures.

The most striking finding of the study was that the organic matrix was still present after brushing abrasion

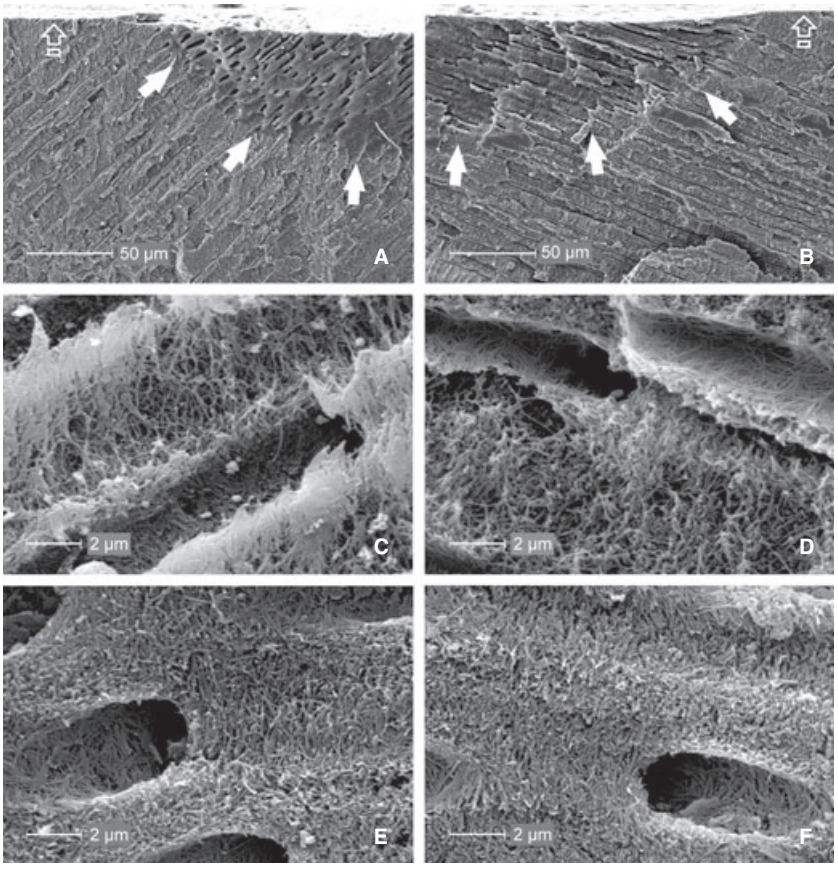

Fig. 3. Scanning electron microscopy images of typical dentine samples after erosion (left column) and after erosion/abrasion (right column). Cross-sections from both groups show a distinct demineralized surface zone (A,B), which is clearly demarcated from the reference area (dashed arrows) as well as from the underlying sound dentine (arrows). At higher magnification (C,D) it is evident that brushing had no structural effects on the organic matrix. Also, the sample surface appears similar in both groups (E,F), and the demineralized collagen with its fibrous structures is evident.

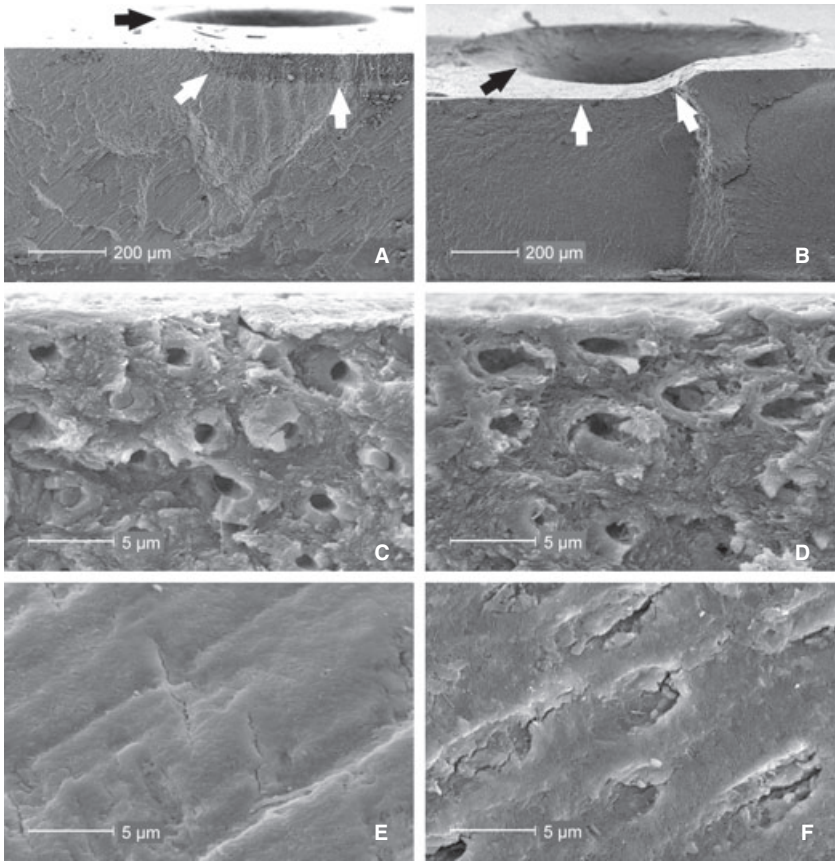

Fig. 4. Cross-sections of a typical sample after erosion (A) with a distinct demineralized zone (arrows) and another sample after erosion and immersion in collagenase (B). The removal of the bulk organic material is obvious. At higher magnification, the typical structure of sound dentine with small tubules surrounded by peri- and intertubular dentine is present up to the surface in the untreated reference (C) area. In the in the experimental area (D), a small superficial zone with signs of demineralization is apparent. After collagenase treatment, the sample surface of the reference area (E) appears smooth, whereas in the eroded area (F) tubules are present, which appear mostly mineralized. In panels A and B the drilling wholes for the identification of the experimental area are seen (black arrows).

and, in addition, appeared unaltered with respect to its histological structure. The presence of this organic layer after acid etching alone has been described previously. After only 30 s of immersion in low-pH citric acid, a small zone of a dense, fibrous collagen network becomes present (17). This zone increases with increasing erosion time and, depending on the demineralization agent and the demineralization time, a fully demineralized zone develops followed by partially demineralized dentine until the sound dentine is reached (18). In the present study, the exposed collagen had reached considerable thickness but there was, if any, only a small partially demineralized zone visible. At 8,000-fold magnification, there was a distinct demarcation between typical sound dentine and fibrous matrix structures, without peritubular dentine or any apparent remnants of mineral. The upper edge of the demineralized zone was on the same level as the adjacent reference area. This was not unexpected because dentinal collagen is not degraded by the demineralization solution under conditions used in the present study (C. Ganss, unpublished results). These findings were the same for the eroded, as well as for the eroded/abraded, samples. The abrasion resistance of the organic material is, in part, explained by its considerable tensile strength. Experiments with completely demineralized coronal dentine revealed values between 9 and 17 MPa, depending on the shear direction relative to the direction of the tubules (19). Nevertheless, one could expect that in the upper part of the lesion, or at least at the surface, a certain dissociation of the normal structure could occur – the absence of which is one of the most interesting findings of the study inspiring further investigations.

Taking the considerable thickness of the organic material into account, it is evident that shrinkage from drying will occur. It was, however, remarkable that the dimensional changes were so extensive, even after exposure in ambient air for only a few minutes. This problem appears most relevant when non-contacting profilometry is used, but also for mechanical profilometry the need for a defined moisture control must be emphasized. The results from the various measurements mirror the histological structure of eroded dentine. Only a few μm of substance loss was found with optical profilometry, which probably includes a certain drying effect as focusing the laser beam takes $\approx$30 s. Mechanical

profilometry resulted in about seven-fold higher values, implying that the stylus indeed caves in the organic material but obviously does not reach the mineralization front indicated by microradiography. These results clearly show that profilometry does not measure mineral loss in eroded or eroded/abraded dentine.

This was particularly evident when profilometry was used in collagenase-treated samples, where all methods revealed similar results. Collagenase removes the bulk of demineralized dentine. Collagen from completely demineralized areas is most easily degraded, but partly demineralized collagen can be also removed to a certain extent. The amount of degradable collagen, however, reaches a plateau when a certain degree of mineralization is present and fully mineralized dentine is degraded only very slowly (20). In the present study, the scanning electron micrographs of surface areas after collagenase treatment showed remnants of demineralized collagen fibres or amorphous debris, which, however, were only a few microns in thickness, as measured in the cross-sections. These organic remnants could explain the somewhat lower values for substance loss measured with optical profilometry, whereas the mechanical stylus might have caved in the material, thus explaining the converging LMR results. In the experimental area, the cross-sections revealed fully demineralized dentine up to the upper edge in most samples. In some samples (for example see Fig. 4D), however, a small zone with minor signs of erosive demineralization was present. Both the surface and the cross-sectional aspects of the samples show that collagenase treatment removed most of the organic material without degrading the fully mineralized dentine, confirming results published previously (10). The increased LMR values after collagenase treatment, however, indicate that the remaining mineral might be present on or between the collagen fibrils also in the extensively demineralized dentine, thus confirming suggestions from Klont & ten Cate (20). The good agreement of the profilometric and microradiographic measurements in collagenase-treated samples opens new perspectives for an improved methodology for quantifying erosive mineral loss in dentine.

No significant differences in substance/mineral loss were found between the eroded and the eroded/abraded group, consistent with the SEM findings. Nevertheless, there was a continuous trend for somewhat higher mineral loss values in the brushed samples. It is possible that structural changes which had caused differences in the amount of residual mineral in the matrix structure had occurred at a level that was not detectable at the magnifications used here. In contrast to the present results, significantly increased substance loss values after brushing abrasion of eroded dentine were reported in experimental settings with stronger abrasive impacts (5,6,8). In these studies, total erosion times between 20 and 63 min, and total brushing abrasion times between 10 and 40 min, were used, compared with a total erosion time of 108 min and a total brushing abrasion time of 4.5 min in the present study. While the organic material was persistent under the present experimental setting, it is, of course, conceivable that it might be affected under more severe physical impacts. Further studies on the wear resistance of the organic matrix could clarify this. It must, however, be emphasized that the cited studies had used profilometry and that they should be re-interpreted in the light of results presented in this paper. Even if the organic matrix is totally or partially removed by the bushing procedure, it will be still present in eroded, but unbrushed, controls. Thus, profilometry will overestimate the overall impact of brushing abrasion. From these considerations, the established assumption that eroded dentine is more prone to brushing abrasion appears open for debate.

In conclusion, it has been shown that the organic portion of dentine is exposed during the erosive demineralization and appears unaffected from clinically relevant brushing regimes. In addition, brushing abrasion after erosion had only a small and non-significant impact on mineral loss. Furthermore, the results indicate that, in the case of dentine erosion, profilometry is not related to mineral loss unless the organic material is removed. Studies using this method must be re-interpreted.

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