Acta Odontologica Scandinavica, 2007; 65: 298–305

informa
healthcare

ORIGINAL ARTICLE

Effect of pepsin on erosive tissue loss and the efficacy of fluoridation measures in dentine *in vitro*

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Abstract

Objectives. In dentine, erosive lesion progression and efficacy of fluoridation measures for symptomatic therapy of dental erosion are both dependent on the presence of the organic matrix. In patients with eating disorders in combination with chronic vomiting, the demineralized organic matrix can be degraded by gastric enzymes. The aim of this study was to investigate the effect of pepsin on erosion progression and the efficacy of fluoride in dentine. ***Material and methods.*** Human dentine specimens were prepared and randomly divided into 4 groups of 20 specimens each. They were subjected to a cyclic de- and remineralization procedure for 9 days. For demineralization (6×2 min per day), an HCl solution (pH 1.6) was used in all groups. In two groups, pepsin (1.5 mg mL^{-1}) was added to the demineralization solution. Fluoridation was performed in two groups 6×1 min per day with a mouth rinse (Olaflur/SnF_2; 250 ppm F^-) after demineralization with both the HCl solution and the pepsin containing solution. Degradation of collagen was quantified by analyzing hydroxyproline and tissue loss was determined microradiographically. SEM images were taken in addition. ***Results.*** In the pepsin group, 1.72 (0.26) μg mL^{-1} (mean (SD)) hydroxyproline per day was detected, and in the pepsin-fluoride group 1.95 (0.50) μg mL^{-1}. Tissue loss after 9 days in the control group was similar to that in the pepsin group (122.2 (53.4) μm and 122.2 (38.0) μm, n.s., respectively). Fluoridation reduced tissue loss after demineralization (98.8 (30.2) μm) but not after pepsin treatment (125.2 (34.2) μm; $p \leq 0.05$). ***Conclusion.*** Under the conditions used, pepsin had no influence on tissue loss, but altered the efficacy of fluoridation measures.

Key Words: *Collagen, dentine erosion, eating disorder, fluoride, gastric juice*

Introduction

Dental erosion is defined as substance loss caused by exogenous or endogenous acids on tooth surfaces without bacterial involvement. Erosive substance losses can be serious in some risk groups. Besides vegetarians and those who frequently consume acidic beverages, patients with chronic reflux [1,2] or with eating disorders in combination with vomiting [3–5] number among these risk groups. In particular, patients with endogenous erosions often show severe and generalized defects that expose the dentine.

The reasons for such distinct endogenous dental substance losses are not completely known. Gastric juice is very acidic with a pH of 1–3 [6] and the hydrochloric acid found within it is strong with high erosive potential [7]. *In vitro* studies have shown that erosive mineral loss in dentine is 25–30% higher after HCl-treatment (2%, pH 1.22) than after immersion in a higher concentrated citric acid (5%, pH 1.76) [8]. Enamel erosion is a surface phenomenon which leads to centripetal mineral loss [9–11]. Therefore, rapid progression of endogenously caused erosion in enamel could be explained by the erosive potential of hydrochloric acid.

In dentine, however, erosion is not a simple surface process. After acid-induced mineral dissolution, the organic matrix, mainly composed of collagens [12], is exposed [13]. A thicker matrix slows erosive mineral loss, because the organic components act as a diffusion barrier [14,15]. Thus, in contrast to enamel, dentine erosion progression is not predominantly a surface phenomenon; rather, it is diffusion controlled. An *in vitro* study has shown that after enzymatic removal of the organic matrix,

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(Received 24 April 2007; accepted 12 September 2007)

ISSN 0001-6357 print/ISSN 1502-3850 online
DOI: 10.1080/00016350701678733

erosion progression is increased significantly [14], indicating that the presence of the organic matrix affects erosion progression in dentine.

Gastric juice also contains proteolytic enzymes, such as pepsin, which can reach the oral cavity during reflux or vomiting, and can pose a threat to the exposed organic matrix. It is well known that pepsin is capable of degrading collagen in several tissues such as intervertebral disks [16] or tendons [17]. However, very little is known about its effect on the collagenous matrix in eroded dentine. Previous studies have shown that pepsin is capable of degrading the completely demineralized organic matrix of dentine when immersed in the enzyme for more than 3 days [18,19]. From these results, and also from clinical experience with bulimic patients, it can be assumed that pepsin contributes to erosion progression of endogenously caused dentine erosions.

Fluoride preparations used for symptomatic therapy of dental erosion can reduce or even stop erosion progression in dentine if a layer of organic matrix is present [20,21]. However, after enzymatic removal of the matrix, fluorides do not show any efficacy [14]. This means that the organic matrix is essential not only for erosion progression, but also for the efficacy of fluoride measures. Therefore, enzymatic degradation by pepsin can hamper the efficacy of symptomatic therapeutic approaches, especially fluoridation measures.

The aim of the present study was therefore to investigate the effect of a pepsin-hydrochloric acid solution on erosive dental substance losses. We hypothesize that erosive tissue loss increases in pepsin-treated dentine. Furthermore, we investigated whether pepsin treatment has any influence on the efficacy of fluorides. The second hypothesis is that the therapeutic effect of fluoride is diminished after pepsin treatment.

Material and methods

Sample preparation

Previously impacted and freshly extracted human 3rd molars without cracks, as inspected under a stereo-microscope (magnification 10 ×, SMZ-1 Zoom Stereomicroscope; Nikon GmbH, Düsseldorf, Germany), were used to prepare coplanar longitudinal dentine slices (Exakt Trennschleifsystem; Exakt-Apparatebau, Norderstedt, Germany), which were ground flat to a thickness of 750 μm and polished under sufficient water flow (Exakt Mikroschleifgerät; Exakt-Apparatebau, Norderstedt, Germany; P800 and P1200 silicon carbide abrasive paper; Leco, St. Joseph, USA). Circular specimens with a defined area of 10.6 mm^2 were prepared using a hollow drill with an outer diameter of 5 mm (Rio Grande, Albuquerque, NM, USA). Samples were embedded in an acid-resistant, light-curing acrylate (Technovit 7230 VLC; Kulzer-Exakt, Wehrheim Germany), leaving the experimental area undisturbed, and fixed with the acrylate on microradiography holders. Specimens were checked for enamel remnants, cracks and contamination of acrylate under a stereo-microscope (magnification 10 ×). They were randomly divided into 4 groups ($n = 20$ each) and stored in 100% humidity until use.

Treatment

All specimens were subjected to a cyclic de- and remineralization procedure for 9 days with 6 demineralization periods per day, 2 min each. Two groups were additionally treated for 6 × 1 min per day with a fluoride mouth rinse (Meridol®, Olaflur/SnF_2, 250 ppm (0.013 mol L^1) F^-, pH 4.2; GABA, Münchenstein Switzerland). The groups were defined as follows:

Control group	Demineralization with HCl solution
Pepsin group	Demineralization with pepsin-HCl solution
Fluoride group	Demineralization with HCl solution, fluoridation following demineralization
Pepsin-fluoride group	Demineralization with pepsin-HCl solution, fluoridation following demineralization

The HCl solution was prepared with 0.5 g NaCl dissolved in 99.5 mL distilled water, adjusted with 6 mol L^{-1} HCl to pH 1.6 (0.025 mol L^{-1}) [22] with a pH-electrode. For the pepsin-HCl solution, 1.5 mg mL^{-1} (4800 U ml^{-1}) pepsin (P-6887, pepsin from porcine gastric mucosa, 3200 U mg^{-1}; Sigma-Aldrich, Seelze, Germany) [6] was added to the HCl solution.

Specimens were rinsed for 1 min in tap water before being placed in different solutions. After each demineralization in the control and pepsin groups, and after each fluoridation in the fluoride and pepsin-fluoride groups, as well as overnight, specimens were stored in remineralization solution [14,23].

To achieve constant immersion times, specimens were placed on plastic sieves, which were used to transfer the samples into containers filled with 150 mL of demineralization, remineralization or fluoridation solution. Solutions were renewed at the beginning of each experimental day. All procedures were carried out under gentle agitation at 37°C. At the end of each experimental day, 10 mL of the HCl and pepsin-HCl solutions for all groups were frozen at −20°C until further analysis.

Tissue loss measurement

Tissue loss was determined by longitudinal microradiography [24]. An X-ray projection (Cu-K-radiation, 20 kV, 50 mA; duration of exposure 2.5 min) of

the sample slice, together with an aluminum calibration step-wedge, was made at baseline and after days 3, 6, and 9 on a high-resolution film (high-speed holographic film, Kodak SO-253; Kodak, Stuttgart, Germany), which was developed under standard conditions. The mineral content of specimens was calculated automatically with a computer-controlled microdensitometer (Leitz MPV compact Ortholux II; Leitz, Wetzlar, Germany). A repeat measurement (reproducibility) of one (enamel) specimen gave a standard deviation of 1.7 μm and a repeat analysis of one radiogram a standard deviation of 1.8 μm. Mineral content values output from the computer program are based on the assumption that the sample consists of pure hydroxyapatite. Bulk tissue loss of a given sample was calculated assuming that the mineral content of dentine was 47 vol% [25]. Cumulative tissue loss (μm) at days 3, 6, and 9 is defined as the difference to the values at baseline.

Detection of collagen degradation

Degradation of collagen was determined after each experimental day in the HCl and pepsin-HCl solutions from all groups with hydroxyproline analysis [26,27]. The principle of this method is to measure photometrically the content of the amino acid hydroxyproline, which is specific for collagens.

Two milliliters of the HCl or pepsin-HCl solution was put into an air-tight closable reaction vessel (Scintillation vessel 20 mL; MAGV, Rabenau, Germany) and 1 mL 6 mol L^{-1} HCl was added. The solutions were hydrolyzed in closely locked vessels for 24 h at 105°C. The pH of 0.2 mL of the hydrolysate was raised to 5.5 with 0.8 mL citrate acetate buffer (pH 6.0); 0.5 mL Chloramine-T reagent was added to oxidize the hydroxyproline and incubated for 20 min at room temperature. The oxidation was stopped by adding 0.5 mL 6 mol L^{-1} perchloric acid and incubation for 12 min at room temperature. To transfer the colorless molecule to a chromophore, the solution was incubated with 0.5 mL p-dimethyl-amino-benzaldehyde for 20 min at 60°C. Hydroxyproline content was determined photometrically within 1 h after the previous incubation period (Visible UV Spectrometer "Ultrospec 3000"; Amersham Pharmacia Biotech, Cambridge, UK; Measuring cells, no. 27111, Sarstedt, Nümbrecht, Germany) and expressed in μg mL^{-1}.

All chemicals, unless otherwise noted, were obtained from Merck, Darmstadt, Germany.

Calculation of the percentage rate of collagen degradation

The percentage rate of collagen degradation can be calculated from the cumulative hydroxyproline content in the HCl and pepsin-HCl solutions after 9 days. For this purpose, cumulative mineral loss (μm) based on pure hydroxyapatite loss after 9 days was converted into weight using the following formula:

(1) mineral loss (g) = mineral loss (μm) * density of hydroxyapatite (g cm^{-3}) * sample area (mm^2) [28].

[density of hydroxyapatite 3.15 g cm^{-3}; sample area 10.6 mm^2]

The exposed collagen can be calculated from mineral loss (g) if a collagen content of 18 wt% and a mineral content of 70 wt% in dentine is assumed [29]. The amount of actual degraded collagen was calculated from the cumulative content of hydroxyproline after 9 days in the HCl and pepsin-HCl solutions:

(2) Collagen (μg) = Hydroxyproline (μg) * 7.98 [30,31].

The values of the exposed collagen were set in relation to the actual value of degraded collagen to calculate the percentage rate of collagen degradation.

Scanning electron microscopy

SEM micrographs were taken from randomly selected specimens from each experimental group. After treatment, the moist specimens were fractured into halves, critical point dried (critical point dryer CPD 030; Baltec, Witten, Germany) and lightly gold sputtered. Sample surfaces were inspected on a scanning electron microscope (SEM/Type: XL20; Philips Electron Optics, Eindhoven, The Netherlands) equipped with a LaB_6 cathode. The acceleration voltage was set to 5 kV. Images were recorded using a secondary electron (SE) 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 for the SEM, including tilt angle, spot size, scanning mode, etc., were kept constant for all sample groups. SEM micrographs of randomly selected specimens of all groups were taken at a 500-, 2000-, and 8000-fold original magnification.

Statistics

Statistics were performed for data concerning tissue loss. Statistical procedures were performed with SPSS 10.0 for Windows (SPSS, Chicago, Ill., USA). The Kolmogorov-Smirnov test was used to check for normal distribution and *t*-tests for comparison of groups. Level of significance was set at 0.05. Data are given as mean (SD).

Results

Tissue loss

The data are presented in Table I. After demineralization with HCl (control group), mean tissue loss was similar to that of the pepsin group. The fluoridation measures (fluoride group) did not reduce tissue loss

Table I. Tissue loss (μm, mean (SD)) in all groups after 3, 6, and 9 days cyclic de- and remineralization. Statistical significance ($p \leq 0.05$, *t*-test) of differences between groups is indicated by corresponding indices.

Group	Day 3	Day 6	Day 9
Group 1 (HCl)	66.0 (42.2)	96.2 (48.4)[a]	122.2 (53.4)
Group 2 (HCl+pepsin)	65.6 (39.0)	80.2 (45.0)	122.2 (38.0)
Group 3 (HCl+fluoride)	69.2 (37.8)	67.4 (30.0)[a,b]	98.8 (30.2)[c]
Group 4 (HCl+pepsin+ fluoride)	64.6 (36.4)	103.2 (34.2)[b]	125.2 (34.2)[c]

after 3 days compared to the control group. After fluoridation treatment for 6 days, however, tissue loss was reduced by 30% and after 9 days by 19%. After treatment with pepsin, fluoridation was without effect under the prevailing conditions. Tissue loss in the pepsin-fluoride group was similar to that of the control and pepsin groups.

Hydroxyproline content

No hydroxyproline was found after erosive demineralization with HCl (control group), and after erosive demineralization with HCl and following fluoridation (fluoride group). In both the pepsin group and the pepsin-fluoride group, considerable amounts of hydroxyproline were found after each experimental day. The averaged hydroxyproline content in the pepsin-HCl solutions per day was 1.72 μg mL^{-1} in the pepsin group and 1.95 μg mL^{-1} in the pepsin fluoride group.

In the pepsin-fluoride group, the cumulative hydroxyproline content after 9 days was slightly higher (17.57 μg mL^{-1}) than in the pepsin group (15.46 μg mL^{-1}). Calculation of the percentage rate of collagen degradation showed that in the pepsin group 23.5%, and in the pepsin-fluoride group 25.9%, of the exposed collagen was degraded by pepsin.

Scanning electron microscopy

In all treated specimens, the organic matrix was visible with considerable thickness. The structural differences between the groups were negligible (Figure 1a, b). After pepsin treatment, the organic matrix was sharply demarcated against the sound dentine (Figure 1b), whereas after HCl treatment a zone of partially demineralized dentine was visible (Figure 1a). The transverse sections of the fluoridated specimens (not shown) were identical to the non-fluoridated ones. Differences caused by fluoridation were only found on the surfaces of specimens. After HCl treatment, the typical structures of an eroded dentine surface were visible with wide dentine tubules surrounded by the demineralized organic matrix (Figure 2a). Fluoridation without pepsin treatment led to precipitates on the surface (Figure 2b) which were not visible on the surfaces of combined fluoridated and pepsin-treated specimens (Figure 2c).

Discussion

The impact of gastric enzyme pepsin on eroded dentine matrix was investigated in the present study. The experimental design simulated repeated acid impacts, which can occur in patients with eating disorders in combination with vomiting. The demineralization solution corresponded to the physiological pH (1.6) of gastric juice after a complete meal [6]. In healthy subjects, mean pepsin activity of 712 μg mL^{-1} was found, but considerably higher values up to 2200 μg mL^{-1} were also reported [6]. Therefore, the chosen pepsin concentration seemed to be adequate to simulate the gastric juice of a bulimic patient after an eating attack with ingestion of large masses of high caloric foods. Experiences with bulimic patients show that vomiting 6–10 times per day occurs regularly, which made six experimental demineralization phases per day meaningful. The demineralization period (2 min) conformed to the length of the pH decline in saliva after an acid attack [32,33]. The duration of fluoridation was performed in accordance with the manufacturer's instructions.

The control group showed the expected results. With increasing erosion time, tissue loss decreased (66 μm after the first 3 days, compared to 30 μm and 26 μm after the second and third 3 days, respectively), which correlated to previous studies [14,15,31]. These findings were in agreement with the broad zone of demineralized organic matrix visible in the SEM pictures, which corresponded to those of a study by Meurman et al. [34] dealing with experimental erosions in dentine.

In contrast to our hypothesis, pepsin treatment did not increase mineral or tissue loss. This is explained by the SEM findings. The organic material was still present as a broad zone (Figure 1b) despite the fact that high amounts of hydroxyproline were found in the pepsin-HCl solution. The results of the SEM micrographs can also be confirmed by calculating the percentage of collagen degradation in relation to mineral loss. Degradation was not sufficient to remove the matrix completely. Therefore, it can be assumed that the remaining layer of the organic matrix still acted as a diffusion barrier and was adequate to reduce the progression of dentine erosion *in vitro*.

Transferring the present results into the *in vivo* situation, it must be remembered that erosion progression is slower *in situ* than *in vitro* [35], possibly due to the protective effect of the pellicle and the influence of saliva. Although it has been shown that the pellicle is not protective in dentine erosions with prolonged

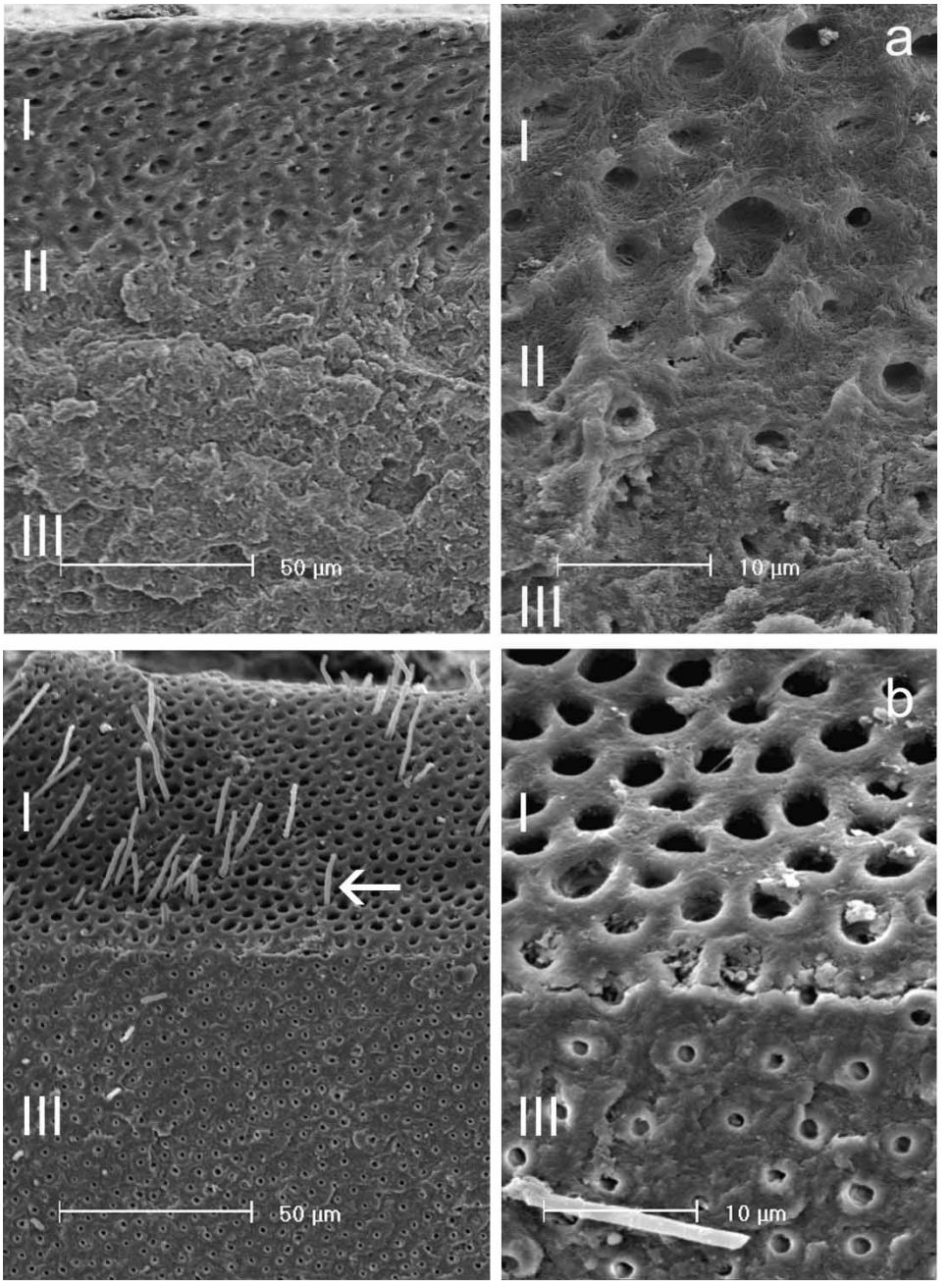

Figure 1. Transverse section of an HCl-treated specimen (a) and a pepsin-treated specimen (b). (a) The HCl-treated specimen can be divided into three zones: totally demineralized dentine (I), followed by partially demineralized (II), and sound dentine (III). On the right side, the region of partially demineralized dentine is displayed at a higher magnification. Clearly visible is the enlargement of the dentine tubules compared with the sound dentine (zone III on left side). (b) The organic matrix in the pepsin-treated specimen is still visible as a broad zone (I), but the partially demineralized dentine is missing. The organic matrix is directly followed by sound dentine (III). The border zone is shown on the right side at a higher magnification. The arrow (→) points to an odontoblast process.

acid impact (10 min) [36], it is possible that the pellicle is protective if a less severe erosive challenge, such as the protocol in the present study, is used. The organic matrix must be much thinner *in situ* and, therefore, the degradation and the demineralization front are more closely related. In this case, however, pepsin could have an influence on erosion progression, because the organic matrix could be more completely degraded, thus reducing the protective potential of the organic matrix. Furthermore, it is conceivable that other enzymes in the vomit, such as trypsin, or in saliva, such as matrix metalloproteinases [37], have an influence on collagen degradation and render the process more complex. Patients with eating disorders in combination with vomiting often show a higher frequency of toothbrushing [5]. Thus, abrasive processes should be regarded, as these too could have an influence on the enzymatically modified matrix. It is possible that the enzymatic degradation destabilizes the matrix, which could lead to increased susceptibility to mechanical impacts. Therefore, further studies dealing with different

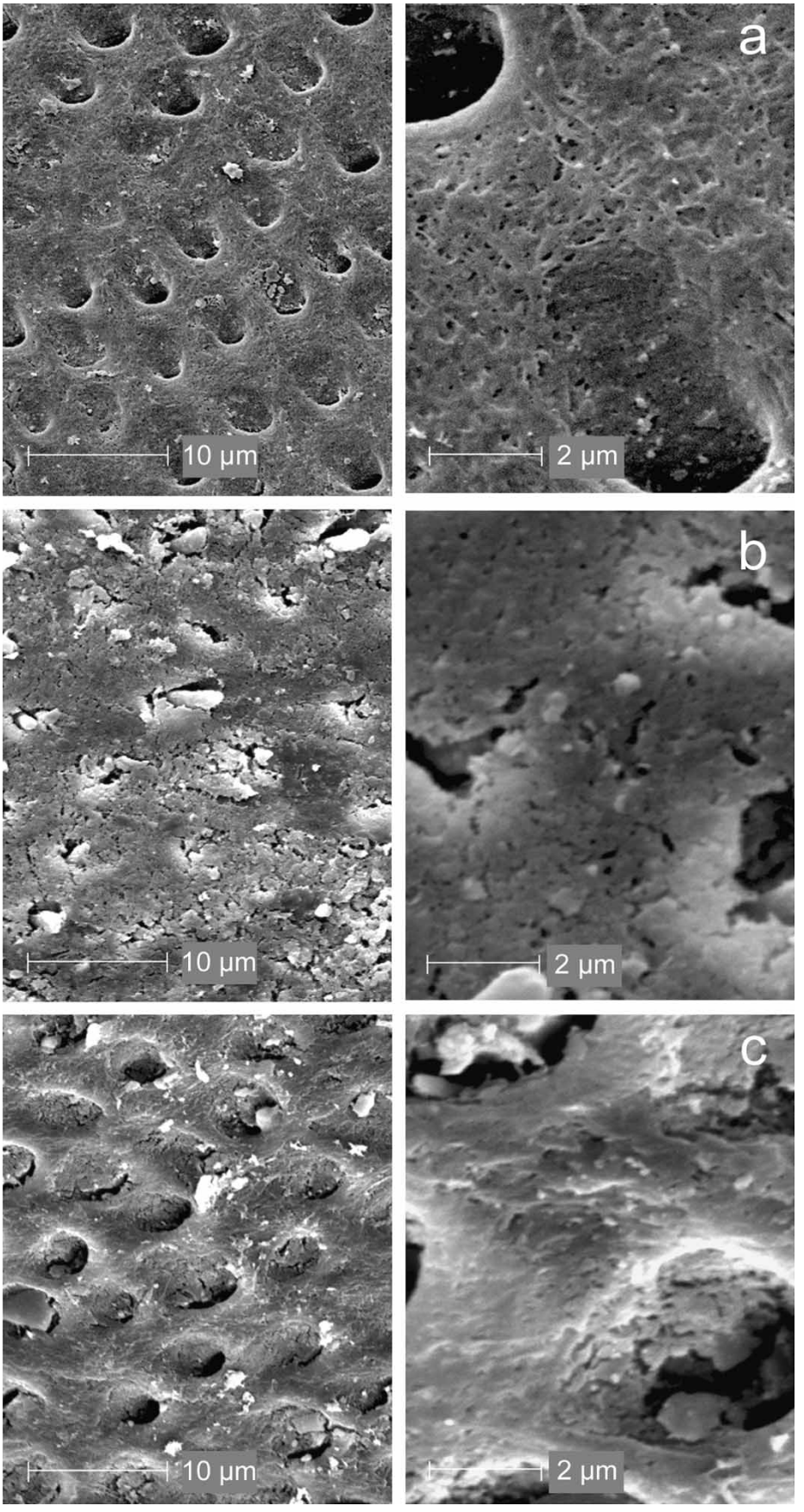

Figure 2. (a) Surface of an HCl-treated specimen. The typical structure of demineralized organic matrix with enlarged dentine tubules is visible. (b) Surface of an HCl-treated specimen which was fluoridated following each demineralization. The whole surface and the dentine tubules are coated with precipitates. The structure of the organic matrix is no longer visible. (c) Surface of a pepsin-treated specimen that was fluoridated following each demineralization. Precipitates can only be found isolated and the organic matrix is uncovered.

combinations of several enzymes or investigating the impact of abrasions after treatment with different enzymes could yield more information.

The second part of the study was to investigate the effect of fluorides after HCl and pepsin-HCl treatment. The efficacy of fluoridation [21] is assumed to be the effect of precipitation of CaF_2-like material, which can be retained by dentine [38]. CaF_2-like precipitates are readily dissolvable in acids [21] and thus will be dissolved during an acid attack rather than the dental mineral. The present study showed that erosion progression was reduced by fluoridation, but not inhibited. This could be explained by the low pH of the demineralization solution in combination with short fluoridation periods. These circumstances can lead to a fast and complete dissolution of the CaF_2 and to a more distinct progression of erosive substance loss.

After degradation of the organic matrix by pepsin, fluoride was not effective. These results conformed to earlier findings from an investigation into the efficacy of fluorides after complete removal of the organic matrix by collagenase. Without the organic structures, fluorides showed no beneficial effects [14]. This seemed to apply even in the case of incomplete removal of the matrix. To what extent these *in vitro* results are transferable to the *in vivo* situation cannot be answered at this time. However, more studies dealing with the efficacy of diverse fluoride preparations with different fluoride doses, or fluoride compounds after pepsin degradation of the dentine matrix *in situ* or *in vivo*, could yield more information. In the event of general inefficacy of fluorides after enzymatic degradation, other preventive strategies must be developed.

In conclusion, pepsin was capable of partially degrading the organic matrix in eroded dentin. The degradation had no influence on erosive tissue loss, but it was shown that low-dose fluoridation measures have a reduced efficacy at least *in vitro* after pepsin impact.

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