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Detection of Matrix Metalloproteinases (MMPs) in the root dentin of human teeth

Introduction: Matrix metalloproteinases (MMPs) play an important role in the metabolism of dental hard tissue. They are proteolytic enzymes whose function is the degradation of extracellular matrix proteins in the oral cavity. They influence dental substances and tissues and their uncontrolled activity is associated with disease processes such as the progression of carious lesions. Since the distribution of MMPs in the dentin has not previously been investigated, this was addressed by this study.

Material and Methods: Root dentin from 30 human teeth was used for this study, divided into 3 groups of 10 each as follows: Gp1(Group1): teeth with root canal fillings, Gp2: non-endodontically treated teeth and Gp3: unerupted teeth. 90 dentin disks were obtained by taking a coronal, medial and apical slice from each tooth. Enzyme activity was measured by a gelatinase/collagenase assay over a 2 hour period with every specimen having 4 readings recorded at time = 0 min, 30 min, 60 min and 120 min. The mean values of MMP activity over the 2 hours in µU/mg dentin were tested for normal distribution by using the Shapiro-Wilk test. The coronal, medial and apical values within the groups were compared with the paired T-test, while the differences in mean values between the study groups were checked with the unpaired T-test. The significance level was set at $p \leq 0.05$.

Results: All the dentin disks exhibited evidence of enzyme activity. Mean values calculated for the entire roots were 4.8×10^{-1} µU/mg for Gp1 (root canal filled), 4.7×10^{-1} µU/mg for Gp2 (non- endodontically treated) and lastly for Gp3 (unerupted), 4.8×10^{-1} µU/mg. These differences in enzyme activity between the 3 groups were not statistically significant. However, all 3 groups displayed statistically significant increases in enzyme activities in the apical direction i.e. moving from coronal to apical. For Gp1, 4.4×10^{-1} µU/mg coronally, 4.7×10^{-1} µU/mg medially and 5.4×10^{-1} µU/mg apically were measured. For Gp2, 4.2×10^{-1} µU/mg coronally, 4.7×10^{-1} µU/mg medially and 5.1×10^{-1} µU/mg apically were measured. Lastly Gp3, 3.8×10^{-1} µU/mg coronally, 4.5×10^{-1} µU/mg medially and 6.0×10^{-1} µU/mg apically were measured.

Conclusion: The results of this study demonstrated for the first time MMP activity within mineralized hard dentin. In all 3 groups, MMP activity increased from the coronal to apical regions of the roots. MMPs which were

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originally synthesized and secreted during dentinogenesis and became incorporated into the mineralized dentin matrix, retain their reactive potential. The study demonstrated the extraordinary stability and longevity of these enzymes.

Keywords: matrix metalloproteinases; proteolytic activity; gelatinase/collagenase assay; root dentin; dentin disk/slice

Introduction

Matrix metalloproteinases (MMPs) are members of the large family of calcium-dependent, zinc-containing endopeptidases [1, 2, 17, 26, 33, 36]. Being proteolytic enzymes they play a role in the synthesis and degradation of proteins, predominantly in the extracellular matrix (ECM) [1, 11]. In the oral cavity these modifying and decomposing mechanisms have consequences with respect to dental hard tissues [5, 8].

MMPs play a key role in the normal physiology of connective tissues, during development, morphogenesis and wound healing [4]. However, their unregulated activity has general medical implications, such as in the pathogenesis of arthritis, tumor metastases, atherosclerosis [4], cardiovascular diseases, nephritis, neurological diseases, blood-brain barrier failure, skin and gastric ulcer formation, liver fibrosis, pulmonary emphysema, and, in periodontal disease [21]. For some time, the involvement of MMPs in the progression of carious lesions has also been discussed [25].

MMPs are secreted by a variety of connective tissue- and proinflammatory cells, including fibroblasts, osteoblasts and odontoblasts [5] as well as endothelial cells, macrophages and neutrophils [33]. They have diverse genetic origins, but as a structurally related group these enzymes are responsible for the degradation of many extracellular matrix proteins (ECM) together with the components of basement membranes [25]. Gross and Lapiere firstly reported MMPs in 1962 as a part of their studies about tadpole development [10].

Research issues for consideration

It is established that MMPs are proteolytic enzymes that are crucial in the synthesis and degradation of extracellular matrix proteins [22, 32–35]. The extracellular matrix comprises fibrillar collagens [7], and this in particular includes a major constituent of dentin, namely 90 % of Type I collagen [6]. If during dentin demineralization the exposure of collagen fibers occurs simultaneously with the activation of pro-MMPs then the discharged collagenases and gelatinases could decompose the exposed collagen fibers [20]. In dentistry, the literature highlights various areas where this could be of crucial importance, but it is especially relevant with respect to adhesive restorative dentistry. Some studies have demonstrated that collagenolytic and gelatinolytic activity in dentin can occur in the coronal root dentin. This becomes particularly crucial for endocrowns which have no post reinforcement but just rely on preparation within the pulp chamber and the adhesive microtags of the bonding resin. Therefore, the question of the stability of this hybrid layer is imperative.

Similarly, the literature does not assume that root fractures occur purely for mechanical reasons, but also, because of a weakening of the collagen matrix in the dentin, caused as a result of its decomposition by MMP activity. An in vitro study examined endodontically treated teeth that had post-retained restorations. These had been clinically monitored over a suitable time period and upon restoration failure electron microscopy techniques demonstrated that the structural integrity of the collagen matrix in the root dentin had been compromised [9]. The experimental protocol prevented the detection of any definitive causality for why this weakening had occurred. However, two central hypotheses were postulated. Either the decomposition of the collagen matrix was caused by bacteria and their virulence factors as collagenases, or else it was because the host's own MMPs had been activated.

On the basis of previous study results, consideration is currently given to the enzyme activity found in the root dentin of teeth both of those that have had endodontic treatment and of those that haven't. The following questions were formulated:

1. Is there any MMPs activity in the root dentin of teeth either with or without endodontic therapy, and can this be detected directly on the dentin surfaces?
2. Can any differences in the distribution of MMPs activity be demonstrated in the dentin, when moving along a root from coronally to apically?
3. Can any differences in the amount or distribution of MMPs activity be demonstrated between specific groups of teeth: a) endodontically treated b) no endodontic therapy c) unerupted?

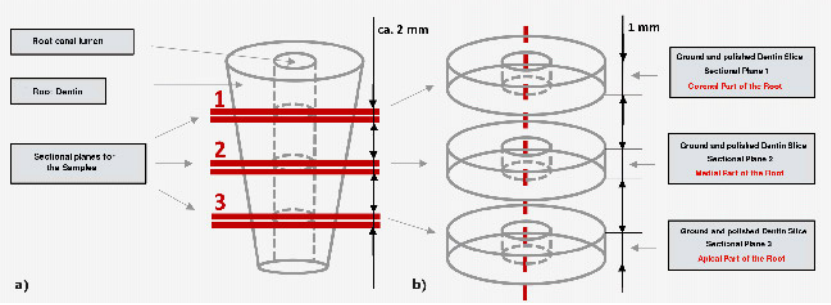

Figure 1 Schematic illustration of the cuts in the roots (**a**) and the corresponding prepared dentin slices (**b**).

Materials and method

Extracted teeth (n = 30) were assigned to 3 groups (Gp) with n = 10 each: Gp1 (root canal filled or endodontically treated teeth), Gp2 (non-endodontically treated teeth) and Gp3 (unerupted teeth). There was no root caries affecting any of these teeth. Teeth from Gp2 and Gp3 did not have any restorations. The teeth of Gp1 had by definition a completed root filling which was confirmed radiographically. They also had extensive coronal restorations, such as extensive amalgam/composite fillings or metal crowns. The age of the root fillings could not be determined because the teeth had been collected anonymously. All teeth had completely formed root apices. Gp1 and Gp2 comprised all tooth types (i.e. anteriors, premolars and molars), whilst Gp3 contained exclusively surgically removed unerupted third molars (upper and lower).

The teeth of Gp2 and Gp3 were subjected to root canal preparations following standard clinical protocols. The pulp chambers were opened, the pulp tissues removed and the root canal orifices identified. The canals were then mechanically prepared using files of ISO size 25/06 and 25/07 from the Mtwo rotary file System (VDW, Munich, Germany), and also Hedström files of ISO sizes 40 and 45. Gp1 teeth had their existing root fillings removed by means of Mtwo revision files of ISO size 15/05 and 25/05 followed by the same methodology as the other two groups. 1 % physiological saline was used for irrigating the root canals.

Next, the crowns of the teeth, the apical 2 mm of the roots and the complete root cementum were removed using diamond instruments. To prepare dentin slices for measuring enzyme activity the roots were first sectioned into thirds (coronal, medial, apical) and then segments of 2 mm in thickness were cut horizontally from each piece (Figure 1). Slices were prepared using a sawing machine with water cooling (Conrad Apparatebau GmbH, Clausthal-Zellerfeld, Germany, diamond-cut blade, Ø 100 × 0.6, Scott Diamant Tools GmbH, Stadtoldendorf, Germany). Finally, the 2 mm thick dentin slices were reduced to a thickness of 1 mm by polishing (Polisher Metkon GRiPO 2V, Buehler, Lake Bluff, USA, Silicon Carbide Grinding Paper P 1200, P 2500, Buehler). The completed specimens were then stored in a 0.1 % thymol solution.

Before testing for enzyme activity the slices were stored in water for 24 hours. Next, to expose the collagen and to activate the MMPs incorporated in the dentin, the slices were placed in a 0.5 M EDTA demineralizing solution for 10 min at room temperature, supported by constant agitation with a mechanical shaker set at 10 rpm. Following demineralization, the dentin slices were placed in the wells of a laboratory microplate (Greiner 96 Well Flat Bottomed Black/Polystyrene), covered with 80 µl buffering reagent solution together with 20 µl DQ gelatin solution from an EnzChek Gelatinase/Collagenase Assay Kit E12055 (Fisher Scientific GmbH, Schwerte, Germany).

The fluorescence-labeled cleavage products of DQ gelatinase have an absorption maximum of 495 nm and a fluorescence emission maximum of 515 nm. An electronic fluorescence detector (GENios, Tecan, Salzburg, Austria) was used (Firmware V4.62–07/01 GENios, software Tecan-i-control 1.10.4.0). Fluorescence measurements (10 x i.e. once for each sample tooth within every study group) were recorded during a 2 hour incubation period (at 37°C) after t = 0 min, t = 30 min, t = 60 min and t = 120 min.

Levels of enzyme activity were then calculated by converting the measured fluorescence values by comparison with corresponding standard curves of standard collagenase type IV from Clostridium histolyticum. Preliminary tests had determined that the expected measuring range would be 0 to 25 µU per ml and therefore serial dilutions were necessary to adjust the original concentration of 1 U per ml. Based on the weight of the dentin slices and the calculated enzyme concentration, the mean enzyme activity per mg of dentin was calculated for every slice at each of the 4 measurement intervals. The enzyme activity values in µU/mg dentin were tested for conformation to a normal distribution using the Shapiro-Wilk test. To compare the enzyme activity values between the 3 root areas (coronal, medial and apical) a paired T-test was performed. The differences in the mean values of the respective study groups as related to the total dentin were checked with the unpaired T-test. The significance level was set at $p < 0.05$.

Results

For each of the three groups, Gp1 (endodontically treated teeth), Gp2 (non-endodontically treated teeth) and Gp3 (unerupted teeth), enzyme activity was detected at every measurement interval. It declined according to the enzyme kinetics, both continuously and in parallel in the 3 study groups.

Gp1 (endodontically treated teeth)

The dentin discs of Gp1 had an average weight of 35.2 mg (± 8.9 mg) per sample. Figure 2a shows enzyme activity values for the 3 root areas over the 2 hour measurement period at

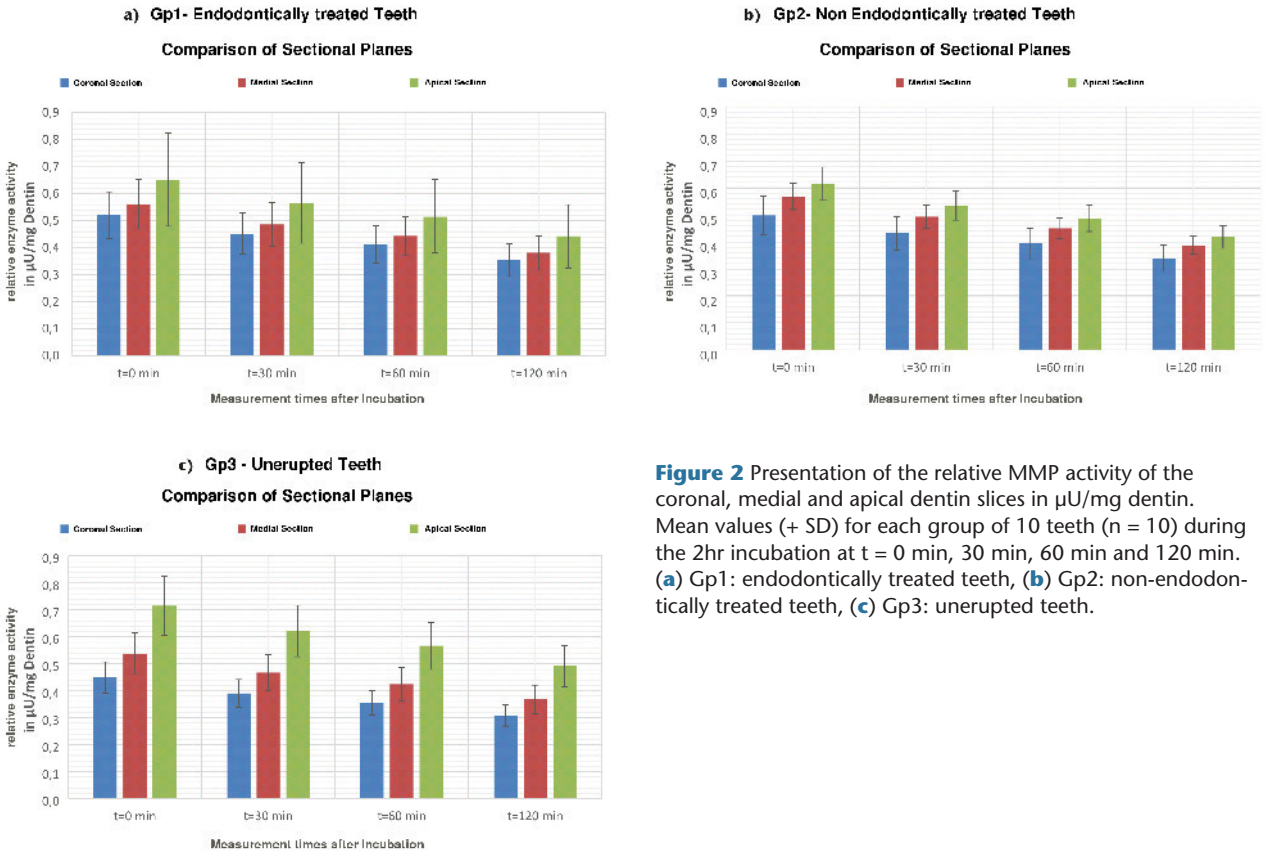

Figure 2 Presentation of the relative MMP activity of the coronal, medial and apical dentin slices in µU/mg dentin. Mean values (+ SD) for each group of 10 teeth (n = 10) during the 2hr incubation at t = 0 min, 30 min, 60 min and 120 min. (**a**) Gp1: endodontically treated teeth, (**b**) Gp2: non-endodontically treated teeth, (**c**) Gp3: unerupted teeth.

0 min, 30 min, 60 min and 120 min. A characteristic distribution pattern of the enzyme activity values over the 3 root areas is obvious. Mean enzyme activity values for the total measurement period were 4.4×10^{-1} µU/mg dentin coronally, 4.7×10^{-1} µU/mg dentin medially and 5.4×10^{-1} µU/mg dentin apically. The differences between all 3 areas were statistically significant (coronal vs. medial: p = 0.008, coronal vs. apical: p = 0.006, medial vs. apical p = 0.04; Fig. 3a).

Gp2 (non-endodontically treated teeth)

Gp2 had an average weight per sample of 35.2 mg (± 5.1 mg). Throughout the measurement period the distribution pattern of the enzyme activity values for the 3 root areas was comparable to Gp1 (Fig. 2b). In each case, the highest values were apically and the lowest were measured coronally. Mean enzyme activity values were 4.2×10^{-1} µU/mg dentin coronally, 4.7×10^{-1} µU/mg dentin medially and 5.1×10^{-1} µU/mg dentin apically. The differences between two of the areas were statistically significant (coronal vs. medial: p = 0.002 and coronal vs. apical: p = 0.0002). There was no significant difference for medial vs. apical with p = 0.07 (Fig. 3a).

Gp3 (unerupted teeth)

Gp3 had an average weight per sample of 34.4 mg (± 7.9 mg). Figure 2c again illustrates the characteristic pattern of the enzyme activity values increasing over the 3 root areas from coronally to apically that was also seen in both Gp1 and Gp2. Mean enzyme activity values were 3.8×10^{-1} µU/mg dentin coronally, 4.5×10^{-1} µU/mg dentin medially and 6.0×10^{-1} µU/mg dentin apically. The differences between all 3 areas at every time interval were statistically significant (p = 0.001 to p = 0.0001; Fig. 3a).

Comparison of enzyme activity values between Gp1, Gp2 and Gp3

The mean enzyme activity values over the 2 hours for the coronal and medial areas of Gp1 (4.8×10^{-1} µU/mg), Gp2 (4.7×10^{-1} µU/mg) and Gp3 (4.8×10^{-1} µU/mg) did not differ with statistical significance (p = 0,05 to 0.83). Only the comparison between the apical areas of Gp2 and Gp3 showed a statistically significant difference (p < 0.02, Fig. 3a).

There were no statistically significant differences in the total root dentin enzymatic activities between any of the groups (p = 0.53 to p = 0.79, Fig. 3b).

Discussion

In this study the activity of MMPs was detected for the first time on root dentin slices by using the gelatinase/collagenase assay method. In contrast, previous studies by Pashley et al. [24], von Hebling et al. [12] and Nishitani [23] used dentin milled into a fine powder by using a vibrating mill. By this method it was not possible to measure the distribution of the MMPs in the root dentin. Changes of the collagen structure and the MMPs could also not be completely ruled out.

In contrast, this study used solid dentin in the form of dentin slices to

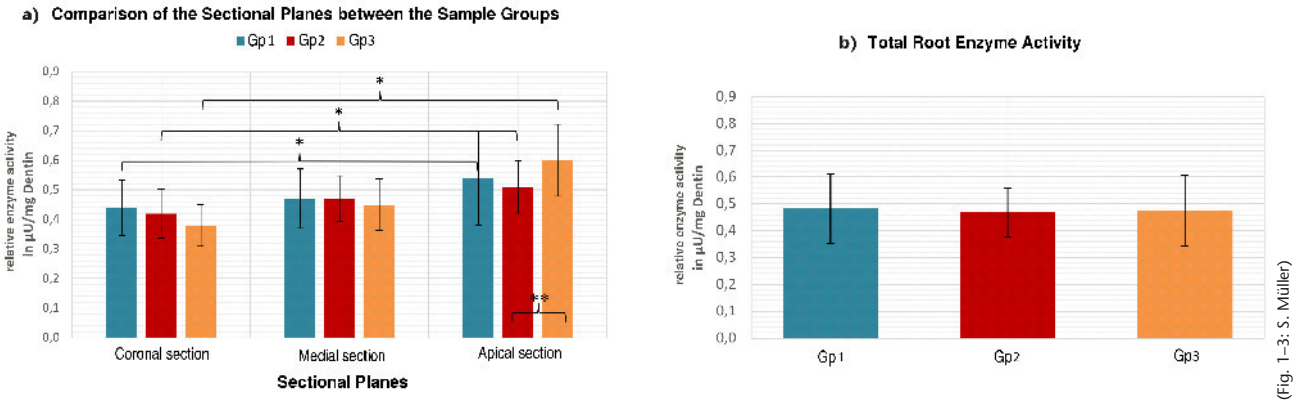

Figure 3 Comparison of MMP activity in µU/mg dentin of groups (Gp1: endodontically treated teeth, Gp2: non-endodontically treated teeth, Gp3: unerupted teeth). Mean values (+ SD) from the measurements at t = 0 min to t = 120 min. (**a**) coronal, medial and apical dentin slices of Gp1 to Gp3, statistical differences within the groups: *; between the groups: **; $p < 0.05$ (**b**) mean MMP activity of the entire roots of Gp1, Gp2 and Gp3. No statistically significant differences.

investigate MMP enzyme activity. In addition, the distribution of enzyme activity within the roots could be visualized over the 3 anatomical root areas, (coronal, medial and apical). The method used, however, is also an in vitro method for which the results cannot be fully transferred to the clinical situation. For the fluorescence measurement, a method established in the literature was used [12, 23, 24, 29] which, in contrast to the Western blot method [6, 7, 19, 27, 30] and zymography [13, 32], represents a rather time-consuming and costly procedure. According to Lynch and Matrisian [17] this method is appropriate for MMP-1, MMP-3, MMP-8, as well as for MMP-2 and MMP-9.

The results of this study proved that similar enzyme activity is present in endodontically treated teeth (Gp1), those without endodontic interference (Gp2), but also in unerupted teeth that had been surgically removed (Gp3). This study verified MMP activity from different regions of the roots and demonstrated that the enzyme activity increased from the coronal to the apical zones. This result could be expected, but interestingly, until now hasn't been presented in the literature. The basic condition for inclusion in the study was caries-free root dentin. The used endodontically treated teeth had extensive coronal restorations, the non endodontically treated teeth and the teeth not exposed in the oral cavity had intact crowns. Since the influence of the restoration of the teeth crown on the quality of the root dentin is not known, no statement can be made about the influence of coronal restorations on the activity of MMPs in the root dentin area. The teeth of groups Gp1 and Gp2 could have had their roots exposed to the oral cavity because of periodontal bone loss or other non-caries lesions. The effect that any such exposure to the oral environment may have had on MMP activity cannot be estimated [5, 30]. This can only be excluded for teeth of the Gp3. In our study, however, the possible influencing factors for a decrease in enzyme activity described above had no effect. MMP activity was detectable in all the teeth of Gp1 and Gp2.

The MMPs inside the dentin matrix structure remain inactivated as long as the dentin matrix structure is mineralized [23, 28]. The literature reports that limited demineralization of dentin, e.g. when using etch-and-rinse or self-etching bonding techniques, results in MMP activation (MMP-2 and MMP-9) in the dentin [20]. Adhesive dentistry procedures demineralize the dentin for 15 sec with 30 to 37 % phosphoric acid. Longer acid exposure times result in a deeper demineralization, which then results in deeper resin impregnation and consequently in a thicker hybrid layer. However, a thicker hybrid layer does not necessarily improve bond [31]. Here, a possible decrease in the stability of the composite dentin interaction is suspected due to a loss of the integrity of the dentin matrix [9]. After acid etching, incomplete wetting with primers may result in imperfect impregnation of the collagen matrix [37]. It has been shown that MMP's enclosed in the dentin matrix can attack and degrade exposed collagen fibers in the hybrid layer [20, 23, 24, 37].

While the exposure of the dentin collagen network by acid etching with etch-and-rinse adhesives and thus an activation of dentin-trapped MMP's is verified, the present study situation regarding an activation of the MMP's by self-etch adhesives is not conclusive [20, 23, 24]. It should be considered that thin adhesive layers can have the effect of a semi-permeable membrane. Due to its water permeability it offers activated MMP's the possibility to perform their hydrolytic function against collagen fibrils and thus destroy the collagen network [37]. From a clinical perspective, it would therefore be advantageous to prevent the activation of MMPs [24]. Therefore, the use of MMP inhibitors such as EDTA [19,

24] and chlorhexidine [12, 37] seems to be recommendable at present. Under physiological conditions, the activities of MMP's are regulated at the level of transcription, at the level of activation of zymogenic precursors of so-called Pro-MMP's, but also at the level of inhibition by endogenous inhibitors such as tissue inhibitors, metalloproteinases themselves and TIMP's [14]. The MMP's also have a high variability to act in complex processes under pathophysiological conditions. The most interesting implications for dentistry are where MMPs are involved in the physiological and pathophysiological processes of the oral cavity and the teeth. For example, it is discussed that dentin-bound MMPs may play a role in regenerative processes and in the regulation of complex dentin-pulpa defense reactions in carious lesions by solving dentin-bound growth factors [30, 27].

MMPs which at the time of their synthesis and secretion, were incorporated into mineralized dentin during dentinogenesis, possess a remarkable capability to reactivate; but, these enzymes also have the impressive property of being able to remain inert and stable for long periods, even in endodontically treated teeth [15].

Summary and conclusion

In the present study, root dentin slices were tested for non-specific enzymatic activity of MMPs. In each of the 3 studied groups, Gp1 (endodontically treated teeth), Gp2 (non-endodontically treated teeth) and Gp3 (unerupted teeth), enzyme activity in the dentin could be detected. In addition, for the first time information on the distribution of MMPs from coronal to apical within the root dentin could be obtained.

Follow-up studies are necessary to classify the non-specific MMP activity to specific MMPs. Also, the distribution of MMP's within the root from the root canal to the dentin root cement junction has not yet been clarified.

Clinically, the detection MMP activity in endodontically treated teeth is relevant. The therapeutic use of MMP inhibitors, e.g. Chlorhexidine, is recommended during endodontic procedures. The use of an MMP inhibitor is also important for adhesive dentistry (etch-and-rinse techniques) to ensure durable bonding to dentin.

Conflicts of interest:

The authors declare that there is no conflict of interest within the meaning of the guidelines of the International Committee of Medical Journal Editors.

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