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Real-time atomic force microscopy of root dentine during demineralization when subjected to chelating agents

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Abstract

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Aim To explore the potential of atomic force microscopy (AFM) for the examination of changes to dentine surfaces during demineralization and evaluate qualitatively the effect of EDTA, EDTAC and citric acid.

Methodology Nine canine teeth were sectioned transversely at the cemento-enamel junction, and the crowns discarded. Subsequently, each root was embedded in an epoxy cylinder and discs approximately 5 mm thick were cut. A standard metallographic procedure was then used to prepare the surfaces for observation. From the central portion of these samples, two specimens were symmetrically prepared per tooth so that a total number of 18 samples was produced. To allow the use of a liquid cell during AFM, the samples were embedded in silicone rubber and were then randomly divided into three groups, as follows: group 1: 17% EDTA (pH 7.7), group 2: 17% EDTAC (pH 7.7) and group 3: 10% citric acid (pH 1.4). Topographical images were acquired during the demineralization process, allowing real-time observation of the dentine surface. Two operators assigned scores to the AFM images using a double-blind method. ANOVA analysis with random effects ($P < 0.05$) was used to compare the results.

Results The average scores were 6.13 ± 0.35 for EDTAC, 7.36 ± 0.23 for EDTA and 14.55 ± 1.21 for citric acid. Citric acid was statistically different from EDTA and EDTAC while EDTA and EDTAC were not statistically different.

Conclusions The most effective demineralizing substance was citric acid. The methodology developed for real-time observation of dentine surfaces is a valuable method to evaluate demineralization.

Keywords: atomic force microscopy, dentine demineralization, endodontic chelators, real-time observation.

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Introduction

Demineralizing agents are used for the removal of the smear layer during root canal treatment. The removal of the smear layer enhances dentine permeability; hence, this mechanism and its results are subject to broad-ranging scientific discussion and research (Pashley *et al.* 1981). The influence of enhanced permeability for disinfecting deeply into dentinal tubules has been reported and several *ex vivo* studies have stressed the importance of the removal of the smear layer in improving the adaptation and adhesion of filling materials to dentine walls (Ørstavik & Haapasalo 1990, Sen *et al.* 1996, De-Deus *et al.* 2004). The enhancement of dentine permeability may also affect negatively the leakage patterns seen with root fillings (Cergneux 1987,

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Wennberg & Ørstavik 1990). Within this context, chelating agents play a leading role in endodontic practice and research.

A variety of demineralizing agents is used in endodontics. They induce different morphological effects and depths of demineralization. The demineralization process results in a reduction in the Ca/P ratio (Garberoglio & Brannstrom 1976). Baumgartner & Mader (1987) reported that the combination of EDTA and NaOCl caused a progressive dissolution of dentine at the expense of peritubular and intertubular areas. The erosive effects of EDTA have also been reported in other studies (Cergneux 1987, Meryon *et al.* 1987). Çalt & Serper (2002) observed that a 10-min application of 17% EDTA, pH 7.4, caused excessive peritubular and intertubular dentine erosion. However, many details of this important process are poorly understood, and methods for its study do not normally allow longitudinal evaluation of the altered dentine surface microstructure (Marshall 1993).

Currently, there is a debate concerning the question of the ideal time-effect of each chelating agent. However, even with the vast amount of research on this topic, no clearly defined irrigation protocol has been established. There are disagreements regarding the ideal chelator, the application time, and the association with hypochlorite. For example, the time these solutions stay in contact with the canal walls has been reported to vary from 30 s to 10 min (Goldman *et al.* 1981, Abbott *et al.* 1991, Garberoglio & Becce 1994, Lloyd *et al.* 1995).

The main factor leading to the lack of conclusions is the qualitative and non-reproducible character of most studies. Scanning electron microscopy (SEM) is still the most common method for obtaining information about dentine surfaces (Crumpton *et al.* 2005, Teixeira *et al.* 2005). However, traditional SEM does not allow the observation of water-containing components of dentine as the sample chamber operates under high vacuum (Silikas *et al.* 1999). Different protocols have been described for the removal of the smear layer. Some studies are only of a descriptive nature while others use predefined scores. The images are quantified by a scoring system which is invariably subjective (Gulabivala *et al.* 2005). From the majority of these publications it is not clear whether the specimens had been coded and the examiner blinded before the SEM investigation, preventing the identification of the preparation instrument or technique in the SEM.

Recently, Gulabivala *et al.* (2005) described some of the main methodological problems found in traditional smear layer studies. The authors mentioned that the magnifications used in the SEM differ widely, in some studies such data are not presented at all, or different magnifications were used during the investigation. A certain observer bias may occur in the SEM when working with higher magnifications, as only a small area of the root canal wall can be observed. This area may be adjusted on the screen by chance or be selected by the SEM operator. It is a common finding that most SEM operators tend to select clean canal areas with open dentinal tubules rather than areas with large bulk of debris (Hülsmann *et al.* 2005).

With the aim of developing a method for longitudinal observation of dentine during demineralization, atomic force microscopy (AFM) was the technique of choice. AFM is a member of the scanning probe microscopy family of instruments that includes the scanning tunnelling microscope (Jahanmir *et al.* 1992, Radmacher *et al.* 1992, Marshall *et al.* 1993). The main application of AFM in Dental Sciences has been in dental research, dealing mainly with dentine changes such as areas affected by caries (Marshall *et al.* 2001), hybrid layer analysis (Eliades *et al.* 1999), and dentine roughness (Silikas *et al.* 1999). Silikas *et al.* (1999) described the advantages of the use of AFM for dentine mapping such as the ease of observation of the dentine surface with minimal sample preparation. Moreover, the authors highlighted the fact that the technique is non-destructive, the possibility of longitudinal studies, and of manipulation at near atomic level.

Atomic force microscopy offers the opportunity to image the three-dimensional surface topography of biological specimens with high spatial resolution under a wide variety of conditions. These include exposure to air, water and other storage solutions at elevated or reduced temperatures. These features extend the environmental control options and the scope of applications in life sciences (Marshall *et al.* 1993, Eliades *et al.* 1999). Moreover, sensitive, non-conducting samples that are difficult to examine using traditional SEM due to the high vacuum requirement, can be studied by AFM.

Recent studies have demonstrated that AFM offers a powerful tool for directly observing demineralization, drying, bonding processes and mechanical properties of calcified tissues. Marshall *et al.* (1993) pioneered the investigation of nitric acid etching of dentine surfaces with AFM. Cassinelli & Morra (1994) also used AFM to investigate the interaction of a dentine adhesive with hard tooth tissue. Other AFM investigations have focused on the effects of conditioning agents on dentine surfaces (Marshall *et al.* 1995), on dentinal tubules

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(Linden *et al.* 1995), and dentine hardness (Kinney *et al.* 1995).

The purpose of the present work was to explore the powerful potential of AFM for the examination of dentine surface microstructure changes during the demineralization process. The use of a liquid cell containing the chelator agent allowed real-time observation of the process. Image sequences were acquired to evaluate qualitatively the effect of EDTA, EDTAC and citric acid on root dentine. The development of this methodology employing real-time microscopy is the main goal of the present research.

Materials and methods

Specimen selection and preparation

This study was reviewed and approved by the Ethics Committee, Nucleus of Collective Health Studies (Rio de Janeiro, Brazil). Nine maxillary human canines were selected from the tooth bank of Rio de Janeiro State University. The teeth were stored in 10% neutral formalin. Subsequently, each sample was embedded in an epoxy resin cylinder (Arazyn 1.0; Ara Quìmica, SP, Brazil) to ease manipulation and improve the metallographic preparation.

Discs approximately 5 mm thick were cut using a low-speed saw (Isomet; Buhler, Ltd, Lake Bluff, NY, USA) with a diamond disc (Ø 125 mm × 0.35 mm × 12.7 mm; 330C), with continuous water irrigation in order to prevent overheating. Interfaces prepared for AFM require an adequate cross-sectioning technique and surface treatment to avoid contamination and smearing effects that would obscure detailed surface topography. A standard metallographic procedure was employed in the pulpal surfaces of the sections, involving grinding and polishing, to prepare the surfaces for the experimental process and to produce a standardized smear layer. From the central portion of these dentine samples, two specimens (5 × 5 × 5 mm) were symmetrically prepared per tooth so a total number of 18 dentine samples was produced. All samples were inspected with an optical microscope and found free of any defects, such as cracks or pores and completely covered with smear layer.

To allow the use of the liquid cell during the AFM observations, which required appropriate sealing to avoid leaks, the samples were embedded in silicone rubber impression, leaving a margin of approximately 1.3 cm around the samples, providing a good contact surface for the O-ring of the liquid cell.

The samples were then randomly divided into three groups ($n = 6$ teeth *per* subgroup) according to the employed chelator agent, as follows:

- Group 1: 17% EDTA (pH 7.7)
- Group 2: 17% EDTAC (pH 7.7)
- Group 3: 10% citric acid (pH 1.4).

All solutions were freshly prepared by the manufacturer (Formula & Ação Ltd, São Paulo, SP, Brazil).

Experimental procedure

A NanoscopeIIIa atomic force microscope (Veeco Instruments Inc., Santa Barbara, CA, USA) equipped with a fluid cell was used to perform the analysis of the dentine samples during chemical etching. The measurements were performed with the use of NP-type, V-shaped, gold-coated Si_3N_4 cantilevers with length, width and thickness of 190 ± 3, 21 ± 2 and 0.62 ± 0.02 μm, respectively. The cantilevers' normal bending constant, measured by the supplier, was 0.06 N m^{-1}. Prior to any measurement and tip exchange, the microscope glass fluid cell, the silicon o-ring, the fittings and tubing used to inject the solution into the cell were cleaned with distilled water and blown dry using a high-velocity stream of high-purity compressed nitrogen.

The microscope tip was then brought into contact with the sample using the smallest force possible to minimize any undesired surface modification. The approach was performed with the fluid cell empty and an optical microscope (×450) that allowed a top view of the tip and sample surface during the approach was used. A first image of the dentine surface was then obtained in order to choose a suitable defect-free surface area for imaging. The tip was then retracted and 5 mL of the chemical solution were inserted in the microscope liquid cell. A new adjustment of the laser beam in the microscope detector was then performed in order to correct for changes in the microscope optical path and the tip approach, now with a filled cell.

Topographical images were acquired continuously while the demineralization process evolved, allowing real-time observation of the dentine surface. The images were levelled with a first-order plane fit in order to correct any tilt between the microscope tip and the sample surface.

As image formation in an AFM is a sequential line scanning process, any given image takes a certain time to be fully formed. During image formation, etching is proceeding and the final acquired image contains information of different etching times. Thus, it is

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important to speed up image formation to allow a more accurate description of the phenomenon at any given time. The image formation time depends on the line scan speed, on the number of scan lines, and on the line length. Scan speed depends on the imaging mode, either *tapping mode* or *contact mode*.

Atomic force microscopic tapping mode, in which the probe periodically touches the sample surface, can produce higher quality images with fewer artefacts (Eliades *et al.* 1999). However, contact mode, in which the probe is in permanent contact with the dentine surface, is much faster. Thus, the AFM was operated in contact mode, with a resolution of 256 lines, with 256 image pixels per line. Even with these parameters, the typical image formation time was approximately 25 s, covering a square of 50×50 μm^2. The total image sequence acquisition procedure took between 400 and 550 s. Figure 1 shows a typical sequence obtained when EDTA was used as chelator substance.

Statistical analysis

In order to evaluate the demineralization process, two operators observed the AFM images using a double-blind method according to the scoring scale of Ahmad *et al.* (1987). The amount of smear was graded

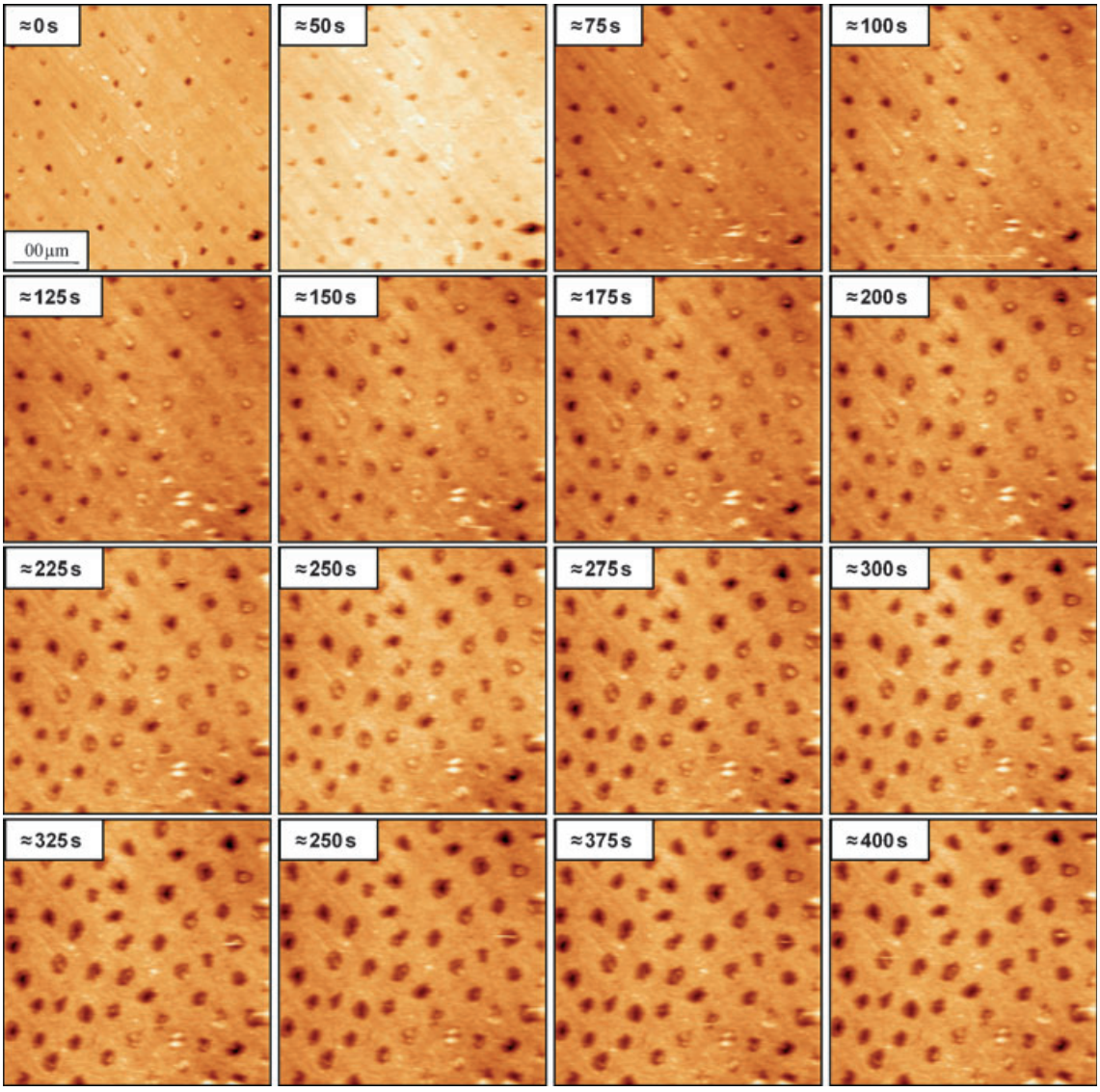

Figure 1 A representative image sequence for the EDTA group.

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between 0 and 3 as follows: Smear scores: 0 = no smear on the dentine wall, all tubules opened; 1 = light smear, more than 50% tubules opened; 2 = moderate smear with fewer than 50% tubules opened; 3 = heavy smear with outlines of tubules obliterated. The images were re-evaluated 3 weeks later to assess the intraoperator reliability and the reproducibility of the results.

A linear regression model (SPSS/PC+ Statistics 4.0 software; SPDD International BV, Gorinchem, The Netherlands) was used and the scores were statistically evaluated using ANOVA with random effects. A global analysis of the groups was conducted. Least-square means (LSM) of smear layer were calculated for each group. LSM were adjusted by observer, magnification, number of images and repetition of the observation. Statistical significance was set at $P < 0.05$.

Results

Figure 1 shows a typical image sequence for the EDTA group. Figure 2 shows the topographical representation of some images from the sequence. The opening of dentinal tubules promoted by EDTA is clearly shown in these figures. Figure 2(a) shows the initial condition of the sample, with the standardized smear layer, before any etching. Figure 2(b) was obtained after approximately 50 s of etching. The demineralization becomes visible at this stage of the process. In Fig. 2(c), after approximately 200 s of etching, the increase in open tubule fraction can be observed, as expected in the typical evolution of demineralization. Figure 2(d–f) shows the continuation of the process up to the final time of 400 s. There is a clear saturation of the effect at approximately 200 s, confirming the well known self limiting characteristic of EDTA.

Equivalent groups of topographical images are shown in Fig. 3 for EDTAC and Figs 4 and 5 for citric acid. Overall, the images for EDTAC show a similar behaviour to EDTA, although demineralization was less intense. In contrast, citric acid had a much stronger effect, with extensive demineralization occurring after approximately 50 s.

The graph in Fig. 6 shows the results from the score analysis for the three substances. The average scores were 6.13 ± 0.35 for EDTAC, 7.36 ± 0.23 for EDTA and 14.55 ± 1.21 for citric acid. Citric acid was statistically different from EDTA and EDTAC

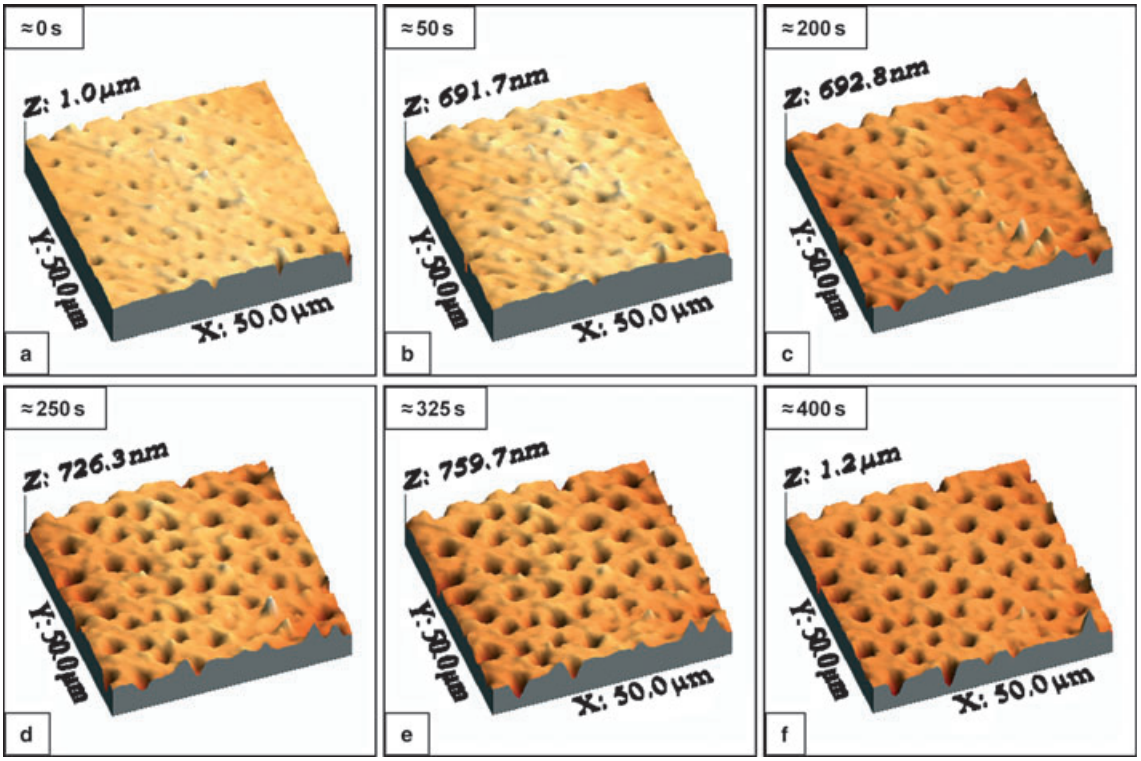

Figure 2 Topographical representation of some images from the sequence shown in Fig. 1.

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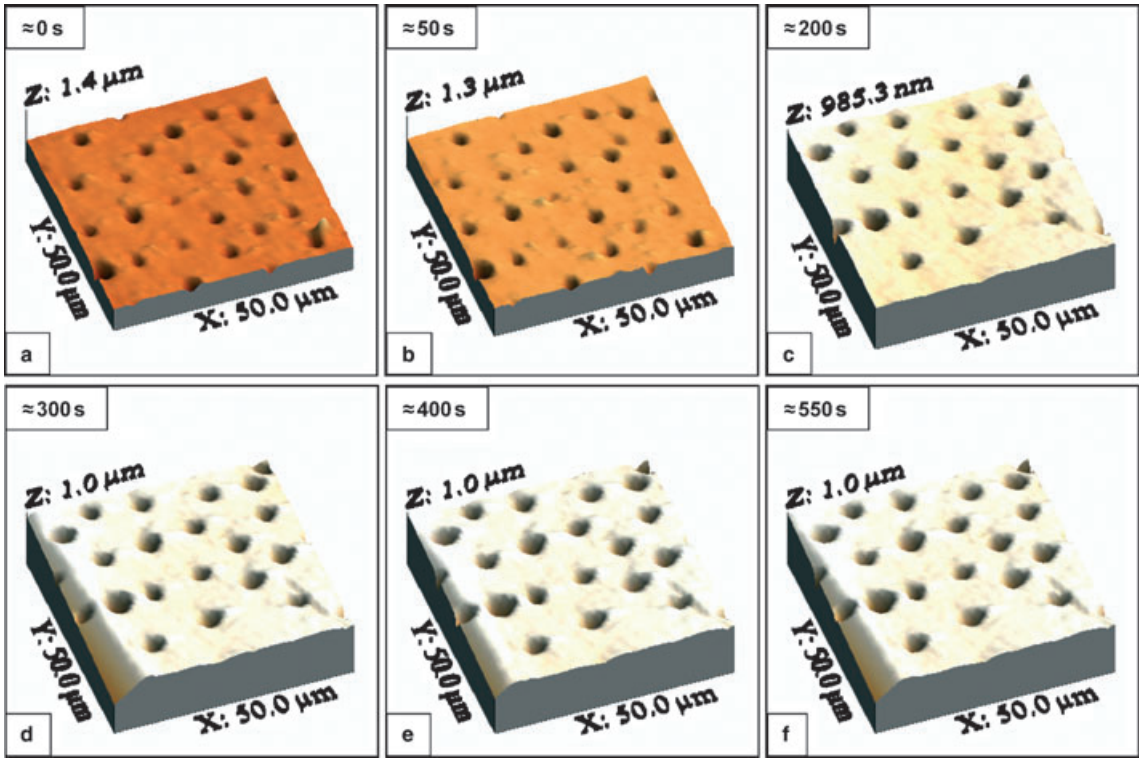

Figure 3 A representative topographical image sequence for the EDTAC group.

($P < 0.05$) while EDTA and EDTAC were not significantly different.

Discussion

Methodology

Longitudinal experiments, in which the sample is in permanent contact with endodontic chelator solutions, and the demineralization process is observed in real-time, can be achieved using AFM and a liquid cell. The images in Figs 1–5 clearly show the time evolution of the demineralization process in the same region of the sample. This represents an evolution over the traditional SEM method for the characterization of dentine surface. Moreover, the image sequence is acquired in digital form and can be processed by Image Analysis software to provide accurate quantification of its features (Paciornik & Mauricio 2004).

Even though there are several references to the employment of AFM in Dental Science, few technical details are discussed. One of the few papers that refers to *in situ* analysis was published by Eliades *et al.* (1999) but no detail of the use of the liquid cell is provided. The expression *in situ*, in this case, refers to experiments in which any changes in the sample are observed, in the microscope, while they are occurring. However, to avoid confusion with the more common use of the expression, *in situ* referring to the oral cavity, the expression *real time* is used in the present paper.

The main problems in the development of the methodology for the present work were related to sample surface height variations and to speed of image acquisition. Figure 5 illustrates the first kind of problem. A strong variation in brightness and contrast is visible across the field. Remembering that image intensity in the AFM is proportional to surface height, this variation indicates that sample surface was not flat. When the surface presents large height variations this can lead to saturation in the image formation system of the AFM. These images cannot be accurately analysed leading, many times, to the loss of a full experimental sequence. This condition is particularly critical for observing the demineralization process for samples in which the smear layer is nonuniform. Thus, it is necessary to prepare the sample through grinding and polishing, to render a flat surface, before the experiment can be reliably performed. Evidently, this kind of

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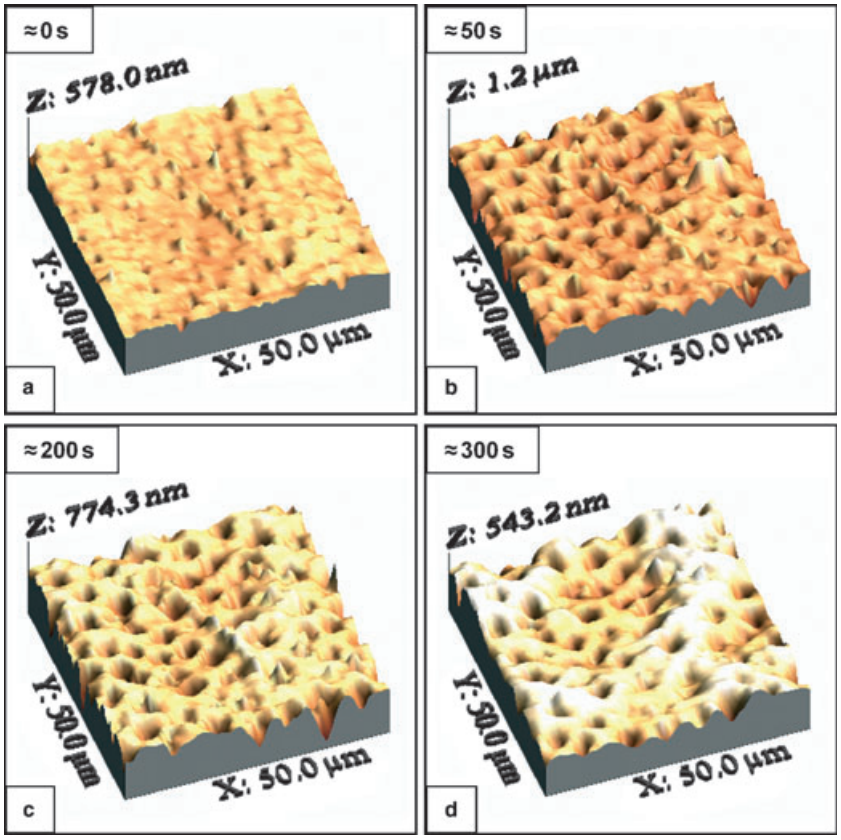

Figure 4 A representative topographical image sequence for the citric acid group.

specimen preparation does not reproduce the real smear layer obtained in clinical conditions.

Speed of image acquisition is the second critical parameter for the observation of the phenomenon. As discussed above, even with the use of contact mode, each image is built by line scanning over a period of 25 s. Any effect occurring at shorter time scales is lost. This problem is compounded by the need to realign the AFM laser beam once the liquid is inserted in the cell. Even an experienced operator needs several seconds (approximately 50 s) to realign the beam leading, in certain cases, to a delay of 60 s before the first image of the etching sequence is fully formed. Thus, the initial stages of the phenomenon cannot be imaged. For faster demineralization processes, this can lead to the loss of the most important period of the experiment.

Results

Several previous papers have analysed endodontic chelator solutions. However, it is rare to find a direct comparison between EDTA, EDTAC and citric acid. It has been reported that both 10 and 19% citric acid can remove Ca^{2+} from the dentine matrix (Yamaguchi *et al.* 1996). While EDTA is usually employed with 17% concentration (O'Connell *et al.* 2000), several different concentrations of citric acid are recommended (Takeda *et al.* 1999, Scelza *et al.* 2000). In the present study 10% citric acid showed a strong demineralization effect, significantly different from EDTA and EDTAC. Machado-Silveiro *et al.* (2004) also found stronger results for 10% citric acid against 17% EDTA. Yamada *et al.* (1983) contradict the results reported herein stating that 25% citric acid can remove smear layer, but less effectively than 17% EDTA or 8.5% REDTA.

Citric acid has been studied in several concentrations and pH values with very distinct results. Tidmarsh (1978) reported that 50% citric acid was effective for the removal of smear layer. Haznedaroglu (2003) studied the effect of pH variation on the chelating effectiveness of citric acid. Solutions with 5, 10 and 50% concentrations had their original pH values compared with buffered citric acid (pH 6). The author reported that citric acid solutions with pH between 1.1 and 1.9 were the most

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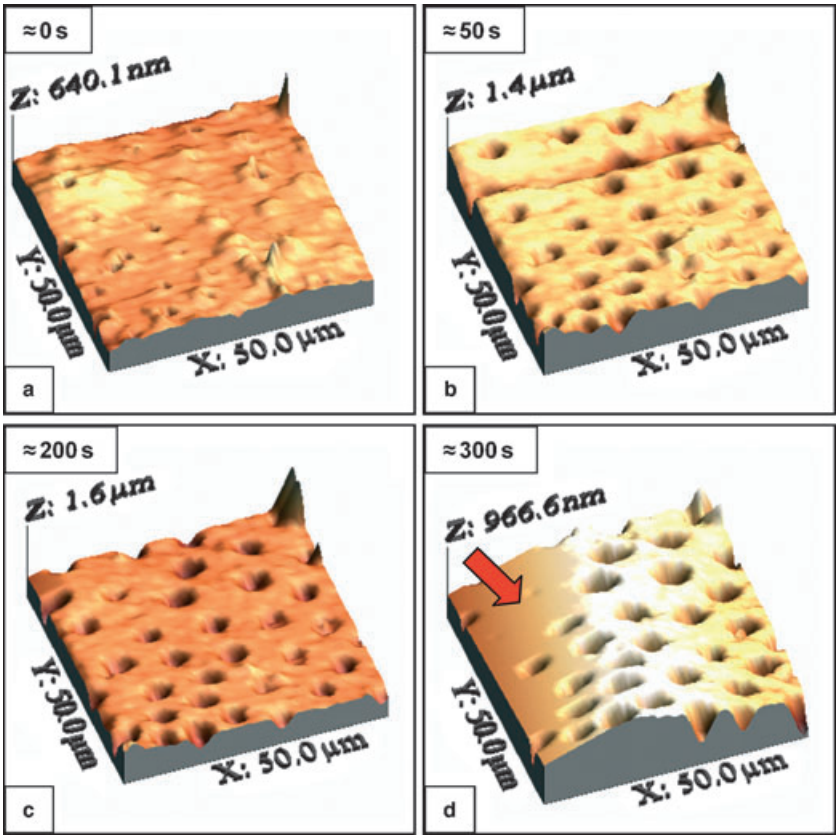

Figure 5 A topographical image sequence for the citric acid group, in which a large variation in height restricted the full observation of the process (arrow, Fig. 5d).

effective. These results are in agreement with Hennequin *et al.* (1994). Haznedaroglu (2003) reports that buffered citric acid solutions at 5 and 10% led to incomplete smear layer removal and concluded that pH was more important than concentration. These conclusions can be indirectly supported by the results of previous studies in which citric acid was found to be less effective than EDTA and EDTAC (De-Deus *et al.* in press).

In the present investigation, EDTA promoted much weaker demineralization when compared with 10% citric acid. Moreover, 17% EDTA and EDTAC caused less peritubular and intertubular dentine erosion.

These results corroborate previous results by Haznedaroglu (2003), in which 50% citric acid with its original pH (1.1) not only removed the smear layer, but also caused extensive demineralization. Dentinal tubules were widened and peritubular dentine was almost completely removed. Furthermore, it has been mentioned that the consequences of this effect on the adaptation of the root canal filling material are unclear (Garberoglio & Becce 1994).

The AFM method described in the present paper allows real-time mapping of dentine morphology allowing the observation of the demineralization kinetics with unprecedented detail. Even though the method is not ideal, given the speed limitations discussed above, the present study revealed results supported by the longitudinal character of the experiments. As a result, it is possible to state that citric acid promoted much faster demineralization that EDTA or EDTAC (see Figs 4 and 5). Among the analysed substances, EDTAC showed the weakest effect. It must be noted that the results might be over estimated as the smear layer produced had to be made thinner and more uniform through polishing, due to the limitations of the AFM, as mentioned before.

Conclusions

The most effective demineralizing substance was 10% citric acid, followed by 17% EDTA and EDTAC. The demineralization kinetics promoted by 10% citric acid

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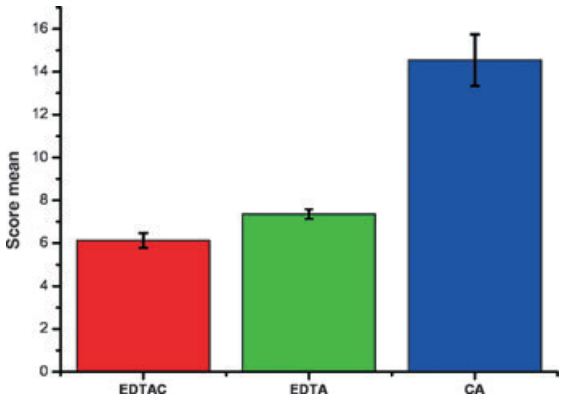

Figure 6 Graphical representation of the score data for the three groups. Bar heights represent the average score and error bars correspond to 1 standard deviation.

was clearly faster than for the other substances. Citric acid also caused strong damage to the dentine matrix while EDTA and EDTAC had little effect.

The methodology developed for real-time observation of the demineralization process in radicular dentine is the main contribution of the present investigation. The possibility of observing microscopic changes in dentine morphology during demineralization is crucial for understanding the phenomenon and may help in establishing an optimal time-effect relationship for the clinical application of chelating substances.

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