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Co-site digital optical microscopy and image analysis: an approach to evaluate the process of dentine demineralization

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Abstract

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Aim To introduce and explore the potential of digital optical co-site microscopy and image analysis for the observation of changes in dentine surfaces during demineralization. The effect of ethylenediamine tetra-acetic acid (EDTA) was evaluated quantitatively and longitudinally.

Methodology Three maxillary human molars were sectioned transversely at the cemento-enamel junction, and the crowns discarded. Subsequently, discs approximately 3 mm thick were cut in the cervical third of the root and a standardized smear layer produced. Co-site image sequences of the dentine surface subjected to 17% EDTA were obtained over the experimental period (15, 30, 60, 180 and 300 s). Sixteen images were obtained in each dentine sample for each experimental time, thus, a total of 48 image fields were obtained. For each field, an image analysis routine automatically discriminated open dentine tubules and measured their number, area fraction and minimum diameter, thus allowing the quantification of the demineralization process. The Student *t*-test was used to analyse the data.

Results The number of open tubules remained essentially constant during the demineralization process. The area fraction increased from 9% to 32%. Tubule minimum diameter increased from 1.5 to 3.0 μm. The changes over time for the area fraction and minimum diameter were significant for comparison between all experimental times ($P < 0.05$).

Conclusions The methodology developed for longitudinal observation of dentinal surfaces was fast, robust and reproducible. It could be easily extended to other chelating substances, thus contributing to the understanding of the demineralization process and in establishing an optimal time-effect relationship in the clinical application.

Keywords: co-site optical microscopy, dentine demineralization, digital image analysis, endodontic chelators, longitudinal observation.

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Introduction

Microscopy techniques have been employed in endodontics for several decades. For example, the use of scanning electron microscopy (SEM) allowed the visualization of the smear layer, leading to a discussion of its role in endodontic therapy, and stimulating the development of methods for its removal (McComb & Smith 1975). Since then, non-toxic calcium chelating solutions and their effects in dentine morphology, as well as the relevance of the smear layer on treatment outcomes have played a leading role in endodontic research.

Currently, there is a debate over the ideal time-effect of each chelating agent. However, even with the vast

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amount of research on this topic, no clearly defined irrigation protocol has been established. Ethylenediamine tetraacetic acid (EDTA) is probably the most frequently used chelator in endodontics (Hülsmann *et al.* 2003). However, there remain disagreements regarding the most effective chelator, application times and the interaction with sodium hypochlorite. For example, the times necessary for these solutions stay in contact with the canal walls, has been reported to be from 30 s to 10 min (Goldman *et al.* 1981, Garberoglio & Becce 1994).

The main factor leading to the lack of consensus is the qualitative and nonreproducible character of most research studies. Indeed, Hülsmann *et al.* (2003) pointed out that even after several investigations the real clinical relevance of the tests for evaluating the efficiency of the chelating solutions remains undefined. Effectively, the experimental conditions of laboratory tests differ substantially from the clinical situation. SEM is still the most common method for obtaining information about dentine surfaces (Crumpton *et al.* 2005, Teixeira *et al.* 2005). An English language literature search of the MEDLINE electronic database from 1990 to 2006, having 'smear layer' and 'SEM' as the keywords, revealed a total of 200 published articles. 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). Some studies are only of a descriptive nature whilst others use pre-defined scores. The images are quantified by a scoring system which is invariably subjective. 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 the technique in the SEM (Gulabivala *et al.* 2005).

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).

During the past decade, advances in information technology have increased computer speed by a factor of 100, and an increase by a factor of 1000 is expected in the next decade (Dunning *et al.* 2002). These accelerating advances in information technology are having a parallel impact on the techniques used to conduct dental research and consequently on the methods and materials used to provide oral health care to patients. The impact on dental research has helped increase our knowledge of dental caries, oral candidiasis, periodontal disease and other oral health diseases in addition to helping to map the human genome (Venter *et al.* 2001).

In this context, a new set of methods, described as digital microscopy, has attracted interest. This technique consists of the association between a motorized/computer controlled microscope, digital image acquisition and image analysis software to automate a complete experimental sequence (Paciornik & Mauricio 2004).

In one particular application of these techniques, here referred to as co-site microscopy (CsM), a set of images is obtained from a large number of *x*-*y* positions of a sample at different experimental times. Between each acquisition time, the sample can be removed from the microscope to undergo some kind of modification such as, for instance, chemical etching. Thus, the changes in the sample can be followed over time for the same *x*-*y* positions, providing a longitudinal character to the experiment. 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). These aspects represent an evolution over the traditional microscopy studies of the dentine surface, most of them based on a qualitative SEM analysis.

The aim of the present work is to present and explore the powerful potential of the association of CsM with image processing and analysis for the longitudinal evaluation of changes to dentine morphology during the demineralization process. Image sequences were acquired with a motorized optical microscope and an image processing and analysis routine was developed to evaluate quantitatively and longitudinally the effect of EDTA on dentine surface. The advantages and disadvantages of the methodology are presented and discussed.

Materials and methods

Specimen selection and preparation

This study was revised and approved by the Ethics Committee, Nucleus of Collective Health Studies, Rio de

Janeiro State University, Brazil. Three maxillary human molars 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 Química, SP, Brazil) to facilitate manipulation and improve the metallographic preparation.

Dentine discs approximately 3 mm thick were cut from the cervical third of the root using a low-speed saw (Isomet, Buhler, Ltd; Lake Bluff, NY, USA) with a diamond disc (∅ 125 mm × 0.35 mm × 12.7 mm – 330C), with continuous water irrigation to prevent overheating. 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 (De-Deus *et al.* 2006a). The endodontic chelator used was 17% EDTA with pH 7.7 buffered with sodium hydroxide (Formula & Ação Ltda.; São Paulo, SP, Brazil).

Experimental procedure (co-site microscopy)

The experiments were developed in an Axioplan 2 Imaging motorized microscope (Carl Zeiss Vision, Hallbergmoos, Germany). An Epiplan 100× HD objective (Carl Zeiss Vision) was used coupled to a 1300 × 1030 pixels digital camera (Axiocam HR, Carl Zeiss Vision), leading to a total magnification of approximately 1000×, and a resolution of 0.1 μm/pixel.

A special holder was built to allow the application of the chelating solutions without removing the dentine sample from the microscope. The holder was attached to a motorized *x*-*y*-*z* stage that allowed the following sequence of steps.

1 A reference dentine sample ($t = 0$ s) covered with smear layer was placed on the holder and brought into focus. An interactive automation procedure allowed the user to select an area of interest on the sample and the system automatically captured a collection of 16 field images at equally spaced *x*-*y* positions, covering the whole region (Fig. 1).

2 The motorized *z*-axis was used to lower the sample to a safe position, away from the objective lens, to minimize the risk of damage to microscope components.

3 One millilitre of 17% EDTA was applied with a pipette and left in contact with the sample for a certain amount of time, after which the chelating process was interrupted with 5 mL of distilled water. The sample was air-dried and automatically returned to focus.

4 The same fields captured in step 1 were captured again, with high reproducibility of the *x*-*y* positions and autofocus, allowing the observation of the effect of demineralization across the whole region of analysis. See Figs 3–5.

5 For each field, an image analysis routine automatically discriminated open dentine tubules and measured several size and shape parameters, thus allowing the

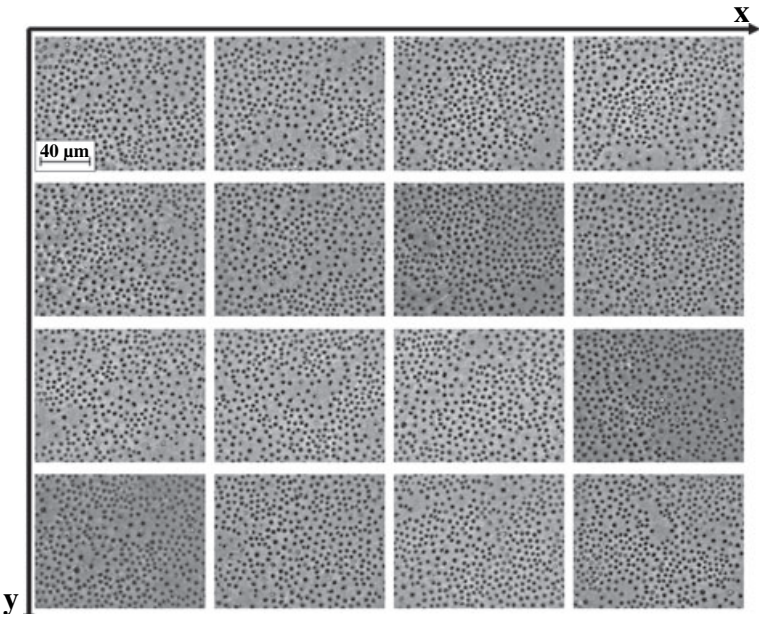

Figure 1 Set of 16 field images automatically obtained from different *x*-*y* coordinates of the sample.

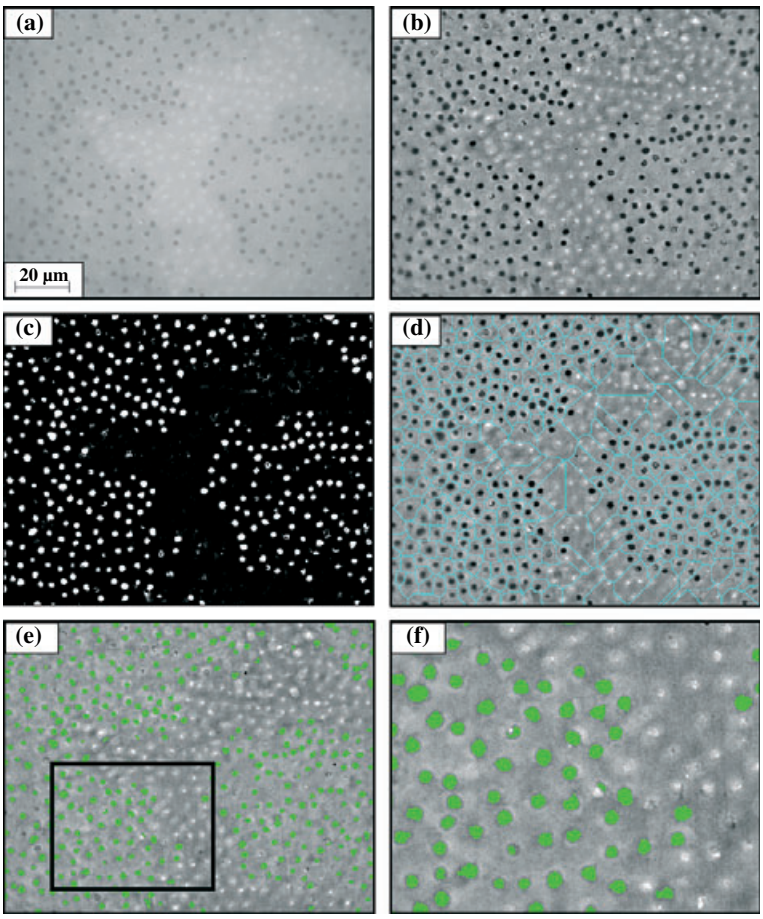

Figure 2 Image processing sequence. (a) Original image. (b) After background subtraction and contrast expansion. (c) After segmentation. Tubules appear white on the dark background. (d) After spurious objects elimination and watershed operation. The blue lines represent the detected boundaries between tubules. (e) Detected tubules, in green, superimposed on original image. (f) Magnification of the framed region in Fig. 2e.

quantification of the demineralization process. More details of the image analysis are given in the following section.

6 Steps 2–5 were repeated for several cumulative demineralization times (15, 30, 60, 180 and 300 s) revealing the complete time evolution of the effect of 17% EDTA on the dentine surface.

The complete image acquisition sequence was controlled by a special routine implemented under the AxioVision software (Version 4.5, Carl Zeiss Vision).

Image analysis

The initial images with the standardized smear layer were not analysed and served as controls. The typical sequence of image processing and analysis involves the steps of pre-processing, segmentation, post-processing and feature extraction (Paciornik *et al.* 2003); it is summarized in Fig. 2. All steps were implemented as a macro routine under the KS400 software (version 3.0, Carl Zeiss Vision).

The *pre-processing* step involves operations that aim at correcting basic defects of the acquired image such as uneven illumination, insufficient contrast, noise, etc. In the present experiments, it was necessary to apply a well-known background correction procedure based on a high-pass filter (Russ 1992) to correct uneven illumination, followed by a standard contrast expansion (Paciornik & Mauricio 2004). See Fig. 2a,b.

The *segmentation* step aims at discriminating the desired objects from the background. Several methods of segmentation are described in the literature (Cocquerez & Philipp 1995), but there is no general rule to choose a best method for a given set of images. In the present case, as the contrast between tubules and dentine was good (except for the images for $t = 0$, with standardized smear layer), the segmentation was accomplished with the automatic Otsu method (Otsu

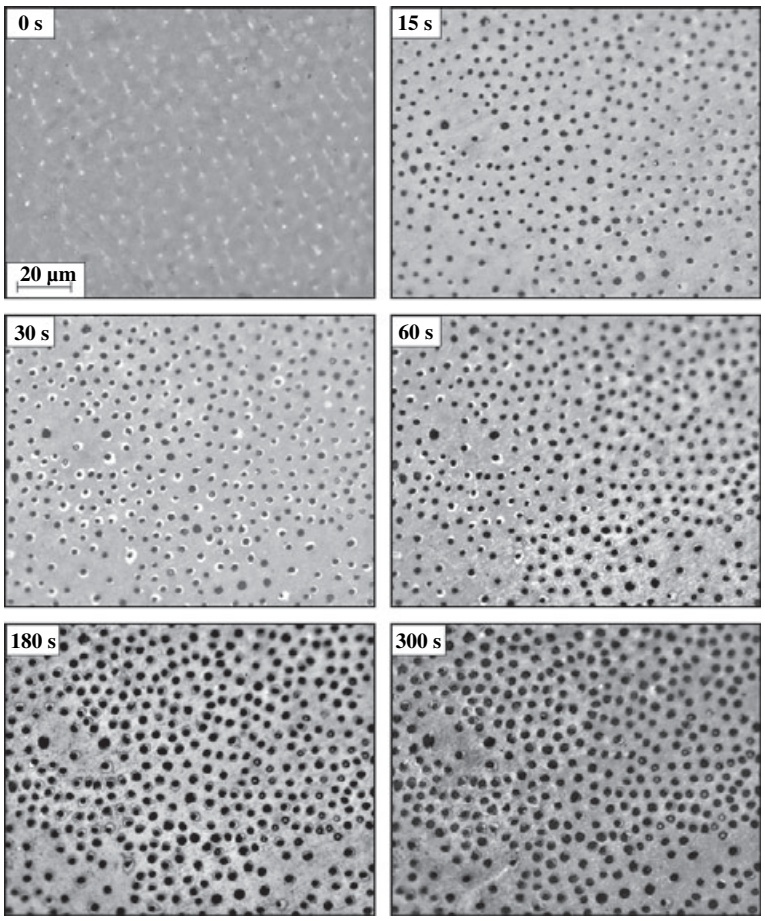

Figure 3 Time evolution of a given region of sample 1, during demineralization with ethylenediamine tetraacetic acid.

1979), which does not require any user defined parameter. Figure 2c shows a typical result of the segmentation step, with the discrimination of the tubules. In this *binary* image the tubules appear as white images on a black background.

Post-processing of these binary images was necessary to treat some typical artefacts of the segmentation step. Initially, small white regions that did not correspond to real tubules were discarded with an area-based scrap operator. A minimum size threshold of 30 pixels was used. This corresponds to ≈1/10 the size of a typical tubule. The next problem addressed was the joining of neighbouring tubules, an effect that increased as the demineralization evolved. This could lead to an incorrect measurement of tubule count, size and shape. The correction required the use of the well-known morphological watershed operator (Beucher 1992) that locates boundaries between touching objects. This method required previous steps of closing and filling of irregular tubules, to avoid the creation of false boundaries (Russ 1992). The detected boundaries between tubules are shown in Fig. 2d.

The final result is illustrated in Fig. 2e, where the detected tubules are shown in colour, superimposed on the greyscale image. The outlined frame is shown at higher magnification in Fig. 2f. The employed sequence was robust and reliable and was applied without changes to the vast majority of images acquired for different samples.

Once the images were correctly segmented and post-processed to discriminate the tubules with their true numbers, size and shape, several microstructural parameters were measured. *Field parameters* refer to each image as a whole. The two main parameters are the *number* and the *area fraction of tubules* within the field.

Region parameters refer to individual tubules in each image. The region parameters obtained were the *area* and *minimum diameter* for each tubule. The area is a basic characterization of the tubule size and is easily measured digitally, counting the number of pixels in

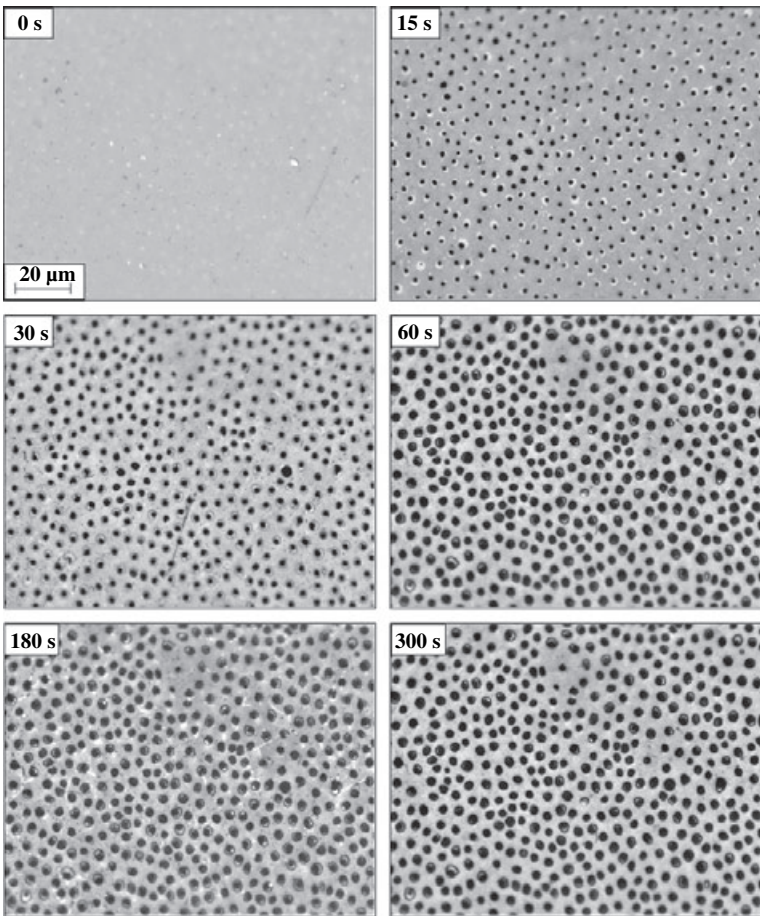

Figure 4 Time evolution of a given region of sample 2, during demineralization with ethylenediamine tetraacetic acid.

each object. However, as this is a projected area of the tubule on the dentine plane, it depends on the inclination of the tubule in relation to the dentine surface. The minimum diameter can be used as a reasonable estimate for the tubule diameter, as it is not affected by tubule inclination.

Statistics

After image analysis and processing, data were analysed by Student's *t*-test in Origin 6.0 (Microcal Software, Inc.; Northampton, MA, USA) at a significance level of $P < 0.05$.

Results

The image montages in Figs 3–5 show the time evolution of the demineralization process for three different samples. Each montage shows a given field of view for different experimental times. The opening of dentinal tubules promoted by EDTA was clearly revealed in these figures. Moreover, the claim of high reproducibility of *x*-*y* positions was confirmed by these figures as almost the exact same dentine features were visible for all times. An estimate of *x*-*y* displacement between fields was obtained by measuring the position of the centre of gravity of specific tubules, at different times. The largest measured displacement occurred in the *x*-direction and reached a maximum value of 40 pixels within a 1300 pixels wide image (≈3%).

The box plot in Fig. 6 presents the evolution of the number of tubules per field against time for the 48 fields of the three samples. The number of tubules varied from 363 to 1047 per field, with a mean of 588. Figure 7 shows the time evolution of area fraction of open tubules for each sample and the average for the three samples. The statistical comparison between experimental times for the two field parameters is shown in Table 1.

Figure 8 shows the time evolution of minimum tubule diameter for each sample. For each experimental time, the plotted result is the average of the measurements

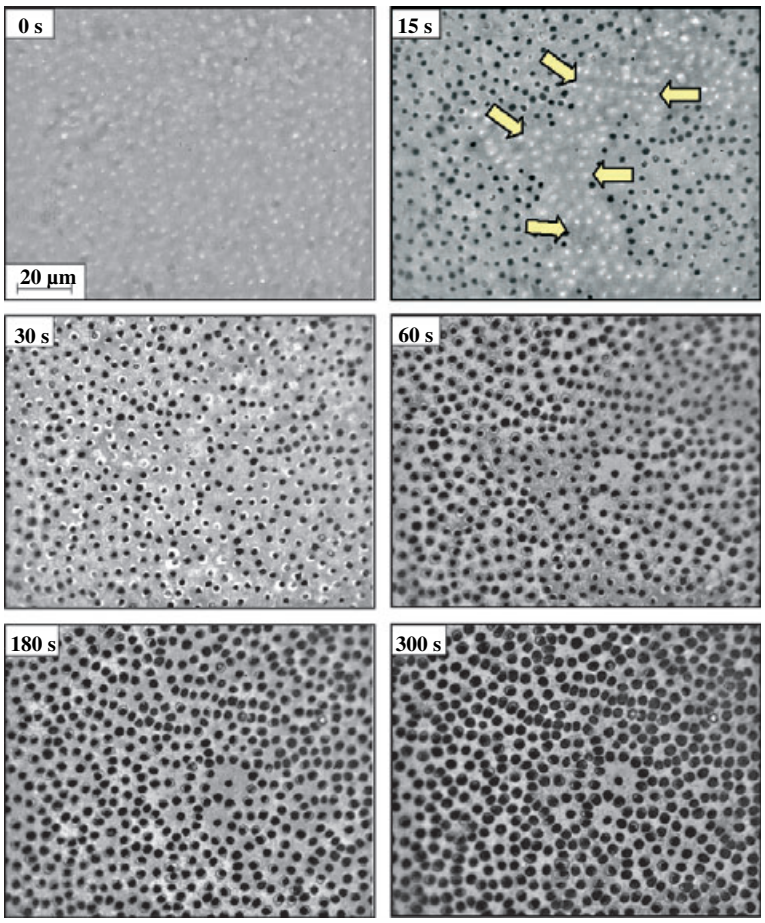

Figure 5 Time evolution of a given region of sample 3, during demineralization with ethylenediamine tetraacetic acid. For 15 s, the arrows point to a region were tubules are still obliterated. See text for details.

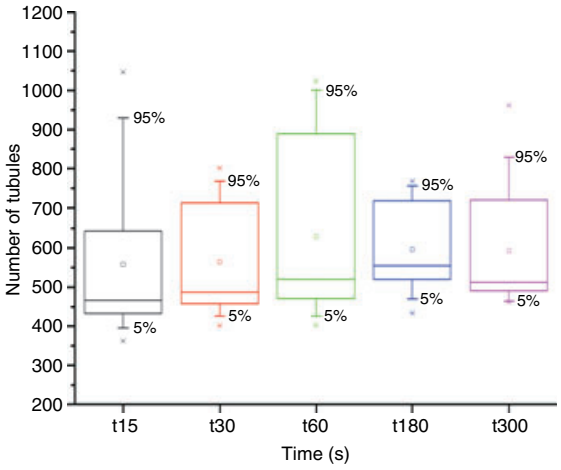

Figure 6 Time evolution of number of tubules for all samples.

of ≈6500 tubules, corresponding to 16 fields for each sample. The average for the three samples is also shown in the figure. The statistical comparison between times for the minimum tubule diameter is shown in Table 2.

To illustrate the intrinsic dispersion of measurements within fields in each sample histograms for each parameter for the 16 fields were plotted for each experimental time. Figures 9 and 10 show the histograms for tubule area fraction and minimum tubule diameter, respectively.

Discussion

Results

The area fraction of open tubules and the minimum tubule diameter were readily measured by the digital analysis procedure developed and these parameters represent important microstructural features for dentine analysis. Nevertheless these parameters are seldom mentioned in the literature. Probably one of the causes of this lack of data is the difficulty and uncertainty of the measurements by a human operator directly from

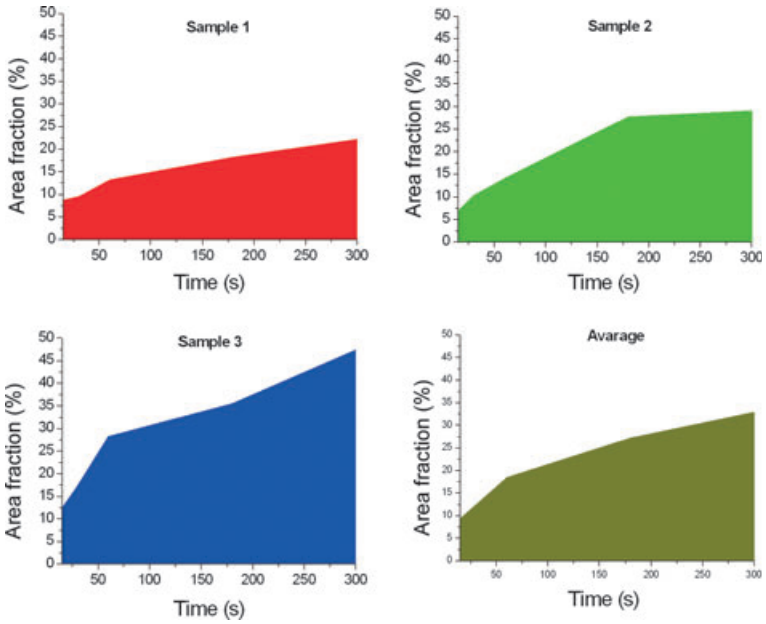

Figure 7 Time evolution of open tubule area fraction for each sample and their average.

Table 1 Statistical comparison between experimental times for field parameters (*t*-test, $P < 0.05$)

Parameter	Exp. times (s)	15	30	60	180	300
Number of tubules	15		No	No	No	No
	30			No	No	No
	60				No	No
	180					No
	300					
Area fraction	15		0.0078	0.0031	0.0048	0.00071
	30			0.0024	0.0019	0.00986
	60				0.0041	0.0054
	180					0.0121
	300					

'No' means no statistical difference. A *P*-value is shown when there is a significant difference.

micrographs. These restrictions lead to the prevalence of qualitative scores evaluation.

The processing and analysis sequence was totally automatic and allowed the measurement of all fields without operator influence. The macro routine automatically opened a sequence of images and applied all steps of processing and analysis, in the exact same fashion, to each image. The typical processing time for each image was approximately 3 s in a typical computer. Thus, the full characterization of one dentine sample, with 16 fields, took <40 s. For each experimental time, a total number of ≈6500 tubules was measured, thus providing excellent interfield statistics.

The area is a basic characterization of the tubule size and was easily measured digitally, counting the number of pixels in each object. However, as this is a projected area of the tubule on the dentine plane, it depends on the inclination of the tubule in relation to the dentine surface. The major and minor diameter axes provide linear measurements of tubule size. The minor axis can be used as a reasonable estimate for the tubule diameter, as it is not affected by tubule inclination.

Tables 1 and 2 indicate that the changes over time for the tubule area fraction and minimum diameter were significant for comparison between all experimental times. This evolution is clearly displayed in the graphs of Figs 7 and 8. Thus, both parameters are candidates for characterizing and comparing the effect of different chelators. This comparison is ongoing and will be presented elsewhere.

In comparison, the number of tubules does not seem to be a relevant parameter for characterizing the time evolution of demineralization. As shown in Table 1, there were no significant differences

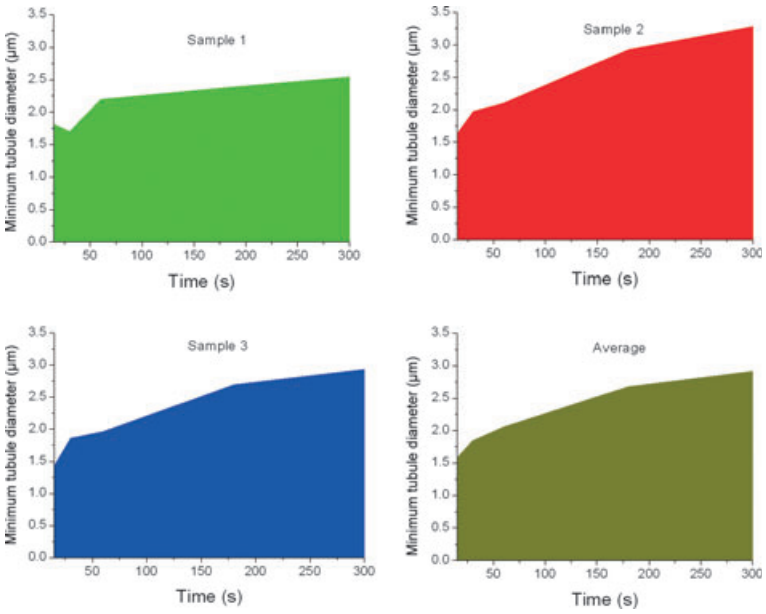

Figure 8 Time evolution of minimum tubule diameter for each sample and their average.

Table 2 Statistical comparison between experimental times (*t*-test, $P < 0.05$)

Parameter	Exp. times (s)	15	30	60	180	300
Minimum tubule diameter	15		0.0023	0.0013	0.0019	0.0005
	30			≈0	≈0	≈0
	60				≈0	≈0
	180					≈0
	300					

A *P*-value is shown when there is a significant difference.

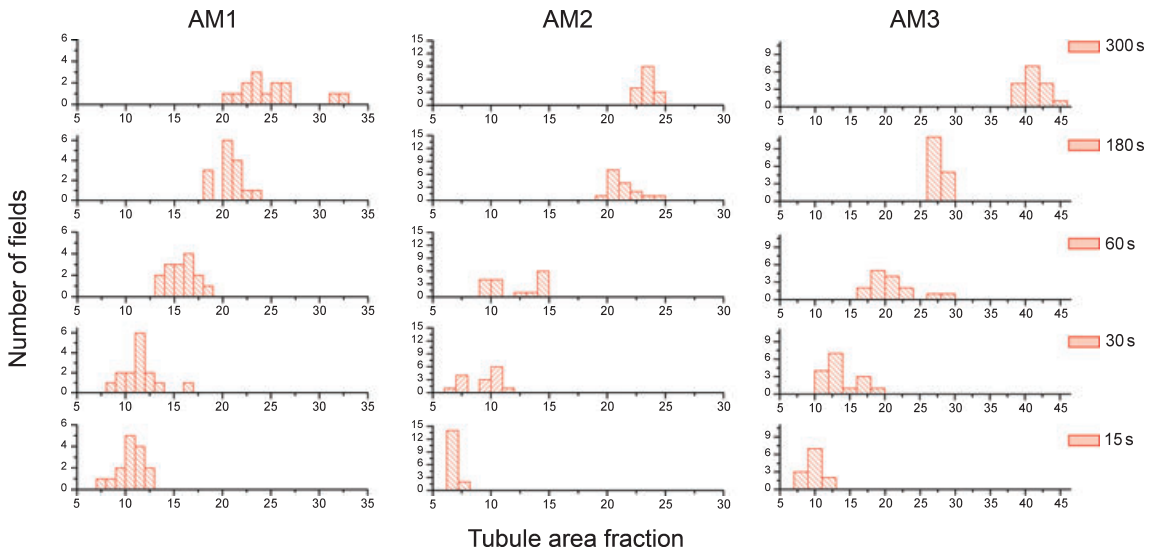

Figure 9 Tubule area fraction histograms of 16 fields for each sample against time.

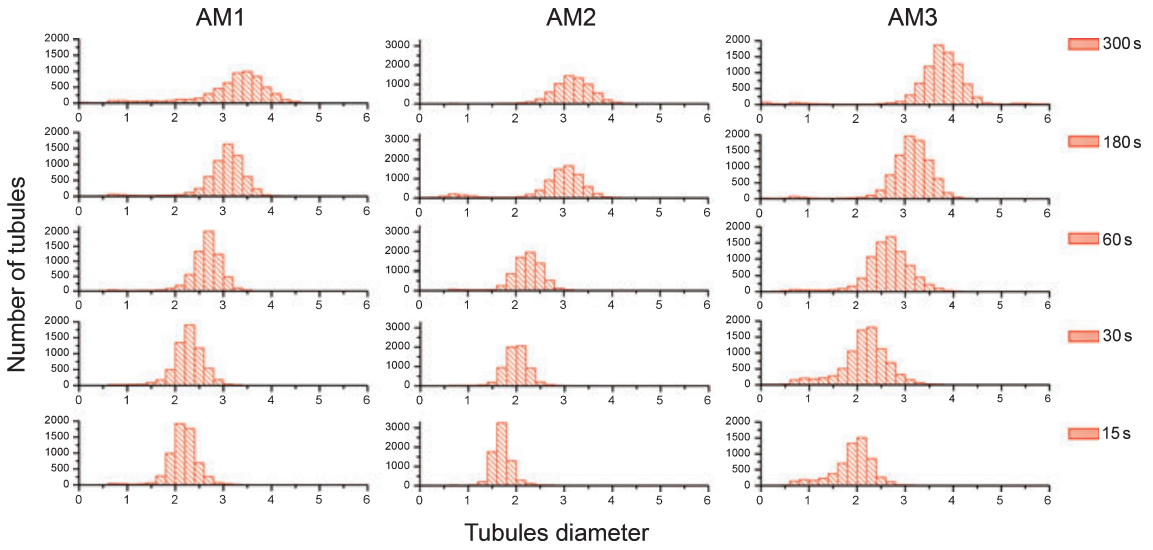

Figure 10 Minimum tubule diameter histograms of 16 fields for each sample against time.

between experimental times for the number of tubules. The dispersion of this parameter, illustrated in Fig. 6, was much more related to intrinsic variations of the dentinal tissue within a given sample and between samples.

Overall, the three samples were smear-free after 30 s of etching. This result is not in line with other results from conventional qualitative SEM analysis performed in the root canal system. According to Hülsmann *et al.* (2003) a certain cleaning effect is achieved after application of a chelator for a few minutes. Goldman & Spielberg (1982) concluded that the cleaning effect is only achieved after 15 min. In a general manner, most of the authors reported good cleaning efficacy of liquid EDTA solution after working times between 1 and 5 min (Yamada *et al.* 1983, Cergneux *et al.* 1987, Çalt & Serper 2002, Hülsmann *et al.* 2003, Scelza *et al.* 2003). In an interesting investigation using single-rooted teeth Çalt & Serper (2002) showed that 1 min exposure of EDTA solution was sufficient to remove the smear layer. In the current study, perhaps the EDTA chelating ability was improved because of the aforementioned experimental conditions. In consequence, the correlation of the present results with the clinical situation is not straightforward.

Methodology

The mechanism of the demineralization process and its results are subject to broad-ranging scientific discussion and research (Pashley *et al.* 1981). However, Gulabivala *et al.* (2005) relates that the vast research efforts on smear layer removal are predominantly laboratory studies, but unfortunately are difficult to compare because of lack of standardization in the methodology. De-Deus *et al.* (2006b) point out that the main factor leading to the lack of conclusions is the qualitative and nonreproducible character of most studies. In that study the authors used atomic force microscopy (AFM) to observe the demineralization process. The method showed relevant advantages such as the observation of the process in near real time as the samples were immersed in the chelating substance during observation. However, limitations because of specific characteristics of AFM precluded obtaining quantitative results. Watari (2005) used AFM to obtain quantitative results regarding the acid etching of dentine and enamel. However, these results refer to relief measurements such as roughness and not to the quantification of the dentine tubules.

The methodology described in the present paper proved to be fast, robust and reproducible. The images in Figs 3–5, show the time evolution of the demineralization process in a given region of samples 1, 2 and 3, respectively, thus highlighting the longitudinal character of the study. 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. This represents an evolution over the traditional qualitative SEM studies for the characterization of dentine surfaces.

This comment is highlighted by some features of the images in Fig. 5. For 15 s, one can clearly distinguish regions with and without smear layer obliterating the tubules. After 30 s most tubules in the image were open and no further distinction between regions was visible. Thus, this sample showed a different behaviour when compared with the other two samples, for which the smear layer was removed after 15 s of EDTA action.

Digital image processing and analysis is a computer-based technique which is being steadily used for semi-automatic or automatic stereological analysis of micrographs. Its main advantages include higher statistical value, as many more fields or objects can be considered for analysis, usually without the influence of a human operator; faster data acquisition than manual counting, especially when combined with microscope automation and digital image acquisition methods; the possibility of evaluating complex parameters that cannot be obtained through visual inspection, such as sophisticated area, texture or shape measurements (Paciornik & Mauricio 2004).

There appear to be no reports in the literature of longitudinal and quantitative analysis in the SEM. Atomic absorption spectroscopy analysis (Serper & Çalt 2002, González-López *et al.* 2006) and microhardness tests (De-Deus *et al.* 2006a) provide quantitative data of the demineralization process but do not offer the possibility of observing the process.

The present paper shows that co-site optical microscopy associated with image analysis provides quantitative data linked to the visualization of the dentine microstructure during the demineralization process. Another relevant point is related to excellent sampling given by automatic image analysis, allowing thousands of tubules to be measured automatically, leading to very reliable data analysis. As the aim of the present paper was to present a new methodology, results are shown for a single chelating agent. Comparisons with other substances will be shown elsewhere.

One of the limitations of the proposed method is due to the restricted depth of focus in optical microscopy, requiring a nearly flat sample surface. 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 specimen preparation does not reproduce the real smear layer obtained in clinical conditions. Moreover, in the experiments described EDTA was applied to a flat horizontal surface, eliminating part of the variability present in the clinical situation, in which the contact between the chelating substance and the dentine is affected by the vertical position of the teeth and the intrinsic anatomical variability of the root canal system.

A variety of chelating agents are used in endodontics and they induce different morphological effects and demineralization depths. Despite the large number of studies, there is a lack of comparable and reproducible results regarding the chelating power. The methodology described in the present paper provides a quantitative, reproducible and statistically sound procedure for comparing different chelator solutions.

Conclusions

Under the conditions of this *ex vivo* evaluation it was concluded that:

1 The demineralization ability of 17% EDTA was confirmed.

2 The obtained results were robust, statistically sound and reproducible.

3 The methodology developed for longitudinal evaluation providing quantitative data linked to the visualization of the dentine microstructure during the demineralization process is the main contribution of the present investigation.

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