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Dentine demineralization when subjected to EDTA with or without various wetting agents: a co-site digital optical microscopy study

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Abstract

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Aim To analyse quantitatively the chelating ability of ethylenediaminetetraacetic acid (EDTA) and three common EDTA-based associations with wetting agents.

Methodology Twelve maxillary human molars were selected, from which 3 mm thick discs were obtained from the cervical third of the root. Following the creation of standardized smear layer co-site microscopy image sequences of the dentine surface submitted to EDTA, EDTA plus 0.1% cetavlon® (Sigma Chemical Co., St Louis, MO, USA), EDTA plus 1.25% sodium lauryl ether sulphate and SmearClear™ (Sybron Endo, Orange, CA, USA) were obtained after several cumulative demineralization times. Sixteen images were obtained of each dentine sample for each experimental time, at 1000× magnification. An image processing and analysis sequence was used to measure the area of open tubules for each experimental time. Thus, it was possible to follow the demineralization process and quantitatively analyse the effect of the various substances. The Student's *t*-test was used to assess differences between experimental groups.

Results EDTA solution had the strongest effect at all experimental times whilst the association of EDTA with wetting agents showed a weaker chelating effect and this difference was statistically significant ($P < 0.05$).

Conclusions (i) The EDTA solution had the strongest effect at all experimental times ($P < 0.05$); (ii) the association of EDTA with wetting agents did not improve the chelating power of the solution; (iii) co-site optical microscopy represents a powerful approach to compare directly, longitudinally and quantitatively the ability of the chelating solutions.

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

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Introduction

As a consequence of the instrumentation of dentine surfaces during canal preparation, a smear layer is formed (McComb & Smith 1975). The smear layer is currently thought to be a thin layer that occludes dentinal tubules and covers the intertubular dentine of prepared canals (McComb & Smith 1975, Hülsmann *et al.* 2003, Gulabivala *et al.* 2005). Whether it is beneficial or detrimental to the outcome of root canal treatment is still controversial (Gulabivala *et al.* 2005). However, the smear layer may be considered deleterious because it prevents the penetration of irrigants, medicaments and filling materials into the dentinal tubules and impede their contact with the canal wall (Cergneux *et al.* 1987, De-Deus *et al.* 2004).

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Nontoxic chelating solutions are advocated for smear layer removal and ethylenediaminetetraacetic acid (EDTA) is the most frequently used chelator in endodontics (Hülsmann *et al.* 2003). The fact that the EDTA solution acts only through direct contact with the substrate led both Hill (1959) and later Nygaard-Östby (1962) to add a cationic detergent (Cetavlon®) to improve the action of EDTA, reducing the surface tension of the liquid and increasing its antiseptic capacity. This combined substance is known as EDTAC and acts on the dentine walls to produce a clean surface, as well as open dentinal tubules (Goldberg & Spielberg 1982). The aqueous EDTA solution has a surface tension of 69.23 d cm^{-2}. The addition of Cetavlon® reduces this figure to 33.92 d cm^{-2} (≈50%) (Hill 1959). Moreover, the association of EDTA with a wetting agent enhances its bactericidal effectiveness (Guerisoli *et al.* 2002) and may lead to improved clinical performance. Following this premise, a new irrigating solution has recently been developed; SmearClear™ is a solution consisting of 17% EDTA combined with cetrimide and additional proprietary surfactants.

Little information about SmearClear™ appears in the literature. To date, a search of Google Scholar electronic database with 'SmearClear' as the keyword revealed only two published studies: the first one compared the efficacy of root canal irrigants against *E. faecalis* biofilms (Dunavant *et al.* 2006) and the other compared the surface tension of four endodontic irrigants (Giardino *et al.* 2006). Thus, an evaluation regarding the chelating ability of SmearClear™ is appropriate.

The present work aimed to assess, both longitudinally and quantitatively, the efficacy of EDTAC, EDTAT and SmearClear™ in reducing the smear layer on standardized human root dentine specimens. EDTA was used as a reference solution. For this, the recently introduced co-site optical microscopy (CSOM) technique was employed, followed by an image analysis routine developed to quantify the effect of chelator solutions on dentine surface. The null hypothesis tested was that there is no difference between the chelating abilities of either EDTAC or EDTAT or SmearClear™ or EDTA.

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, Rio de Janeiro, Brazil. Twelve 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 mounted in epoxy resin and dentine discs approximately 3 mm thick were cut at the root cervical using a low-speed saw (Isomet, Buhler, Ltd, Lake Bluff, NY, USA) with a diamond disc (∅ 125 mm × 0.35 mm × 12.7 mm at 330C), with continuous water irrigation in order to prevent overheating. A standard metallographic procedure [griding with SiC paper (200, 300, 400, 600) grits and polishing with 3 μm diamond paste] was employed on the surfaces of the teeth, to prepare them for the experimental process and to produce a standardized smear layer (De-Deus *et al.* 2006a, 2007). At this point the samples were randomly divided into four groups according to the chelating agent used, as follows (n = 3 per group):

- G1: 17% EDTA (pH 7.7)
- G2: 17% EDTAC [EDTA plus 0.1% Cetavlon® (Sigma Chemical Co., St Louis, MO, USA), pH 7.7]
- G3: 17% EDTAT [EDTA plus 1.25% sodium lauryl ether sulphate (pH 7.7)]
- G4: SmearClear™

All EDTA-based used solutions were freshly prepared by the manufacturer Formula & Ação Ltda (São Paulo, SP, Brazil). SmearClear™ is produced by Sybron Endo (Orange, CA, USA).

Experimental procedure (co-site microscopy)

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

In the co-site microscopy experiment, a group of image fields of a given sample was acquired in specific x–y positions for several cumulative demineralization times (15, 30, 60, 180 and 300 s). A special holder allowed application of the chelating solutions without removing the dentine sample from the microscope. The motorized specimen stage was then used to automatically acquire 15 images of each sample for each demineralization time. Thus, it was possible to follow the same fields with high reproducibility of the x–y positions and auto focus, allowing the observation of the effect of demineralization in the same regions. The details of the procedure are described earlier by De-Deus *et al.* (2007). The complete image acquisition sequence was controlled by a special

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routine implemented under the AxioVision 4.5 software (Carl Zeiss Vision).

Image analysis

A previously developed image analysis routine (De-Deus *et al.* 2007) was used to enhance image contrast, discriminate and measure open dentine tubules in each acquired image. The ratio between the total area of open tubules and the image field area, the tubular area fraction (AF), was then measured. The time evolution of AF was used to quantify the demineralization process. The routine was applied without operator influence to the vast majority of the images acquired for different samples at different times. All steps were implemented as a macro routine under the KS400 3.0 software (Carl Zeiss Vision). The initial images with the standardized smear layer were not analysed and served as controls.

Data presentation and analysis

Data were presented as tubular AF in % of the whole dentine area. The parametric Student's *t*-test was used to compare mean values between groups at respective times. The level of significance was set at $P < 0.05$.

Results

The image montages in Figs 1–4 show the evolution over time of the demineralization process for each chelator. The montages show a given field of view for different experimental times and in each figure, after ≈ 15/30 s of etching, the enlargement of the dentinal tubules can be observed, as expected in the typical progression of demineralization.

At 15 s, EDTAT and SmearClear™ showed similar results whilst the effect of EDTAC was significantly weaker. From 15 to 60 s, EDTAT and SmearClear™ showed similar effects ($P = 0.229$ for 15 s; $P = 0.224$ for 30 s and $P = 0.221$ for 60 s). However, from 180 to 300 s, EDTAT revealed a stronger chelating effect than EDTAC and SmearClear™. At 180 s, EDTAC still had a weaker chelating effect than SmearClear™, although at 300 s, both solutions had a similar effect.

Figure 5 shows the time evolution of AF of open tubules for each group. Each point in the graph corresponds to the average of 45 image fields, for three different samples (16 fields per sample) for each solution. The statistical comparison between groups and experimental times for this parameter is shown in Table 1.

Discussion

Results

The current study showed that EDTA was a more powerful agent in removing the smear layer than the other tested solutions. Consequently, the null hypothesis was rejected. Generally Fig. 5 showed that the EDTA solution had the strongest effect at all experimental times whilst the association of EDTA with wetting agents (EDTAT, EDTAC and SmearClear™) revealed a weaker chelating effect.

The results of this study are in line with the results of a few *ex vivo* studies in which a reduced chelating power was detected when EDTA was associated with a wetting agent (De-Deus *et al.* 2006a,b). Zehnder *et al.* (2005) suggested that the association of an endodontic chelator solution with a wetting agent did not improve the effectiveness of Ca ion removal. De-Deus *et al.* (2006a), in a microhardness dentine analysis, reported that EDTAC was not more effective than EDTA. Another study (Scelza *et al.* 2004), in which demineralized dentine scanning electron microscopy (SEM) images were digitally analysed, did not reveal any advantage in the use of an endodontic chelator solution associated with a wetting agent (EDTAT solution). More recently, Lui *et al.* (2007) concluded that the addition of surfactants to EDTA in SmearClear™ did not result in better smear layer removal compared with EDTA alone. These findings lead to the assumption that the quaternary ammonium compounds associated with the EDTA solution have a negative effect on its chelating ability when asserting direct action upon the dentine substrate.

Methodology

Several previous papers have analysed EDTA and its association with wetting agents. An English language literature search of the MEDLINE electronic database from 1990 to 2006, having 'EDTA' and 'smear layer' as the keywords, revealed a total of 194 published articles. However, when 'EDTAC' was included as a keyword this number decreased to five articles. It may be reasonable to conclude that it is rare to find a direct comparison between EDTA, EDTAC and EDTAT. The efficiency of each chelating solution is still inadequately understood.

Methodological problems appear in several papers and Gulabivala *et al.* (2005), Hülsmann *et al.* (2005) and De-Deus *et al.* (2006b, 2007) have pointed out

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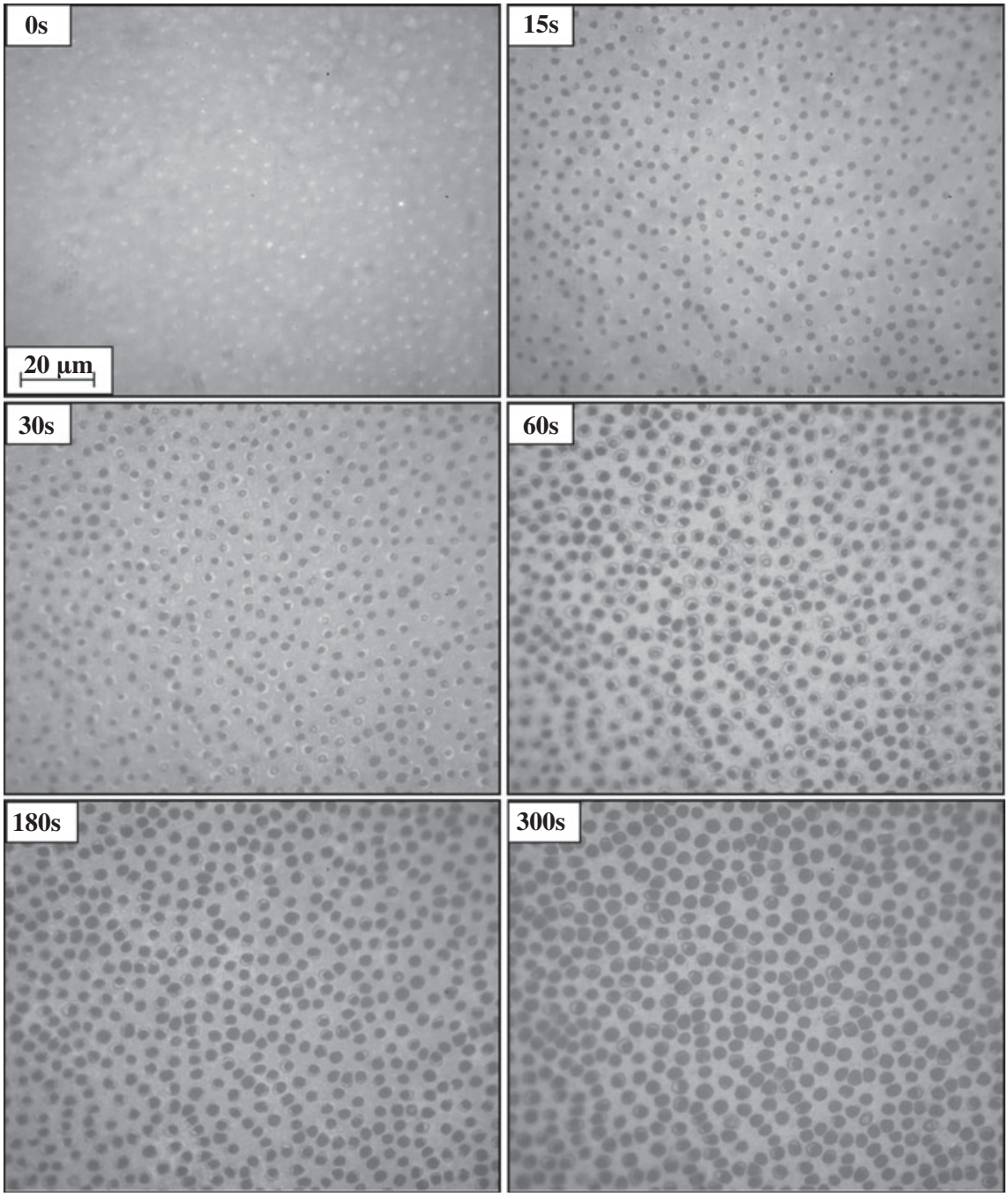

Figure 1 Time evolution of a given dentine region during demineralization with EDTA.

that the main factor leading to the lack of conclusions regarding this issue is the qualitative and nonreproducible character of most studies. In addition, the magnifications used in the traditional SEM studies differ widely – in some studies such data are not even presented. A certain observer bias may occur in 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

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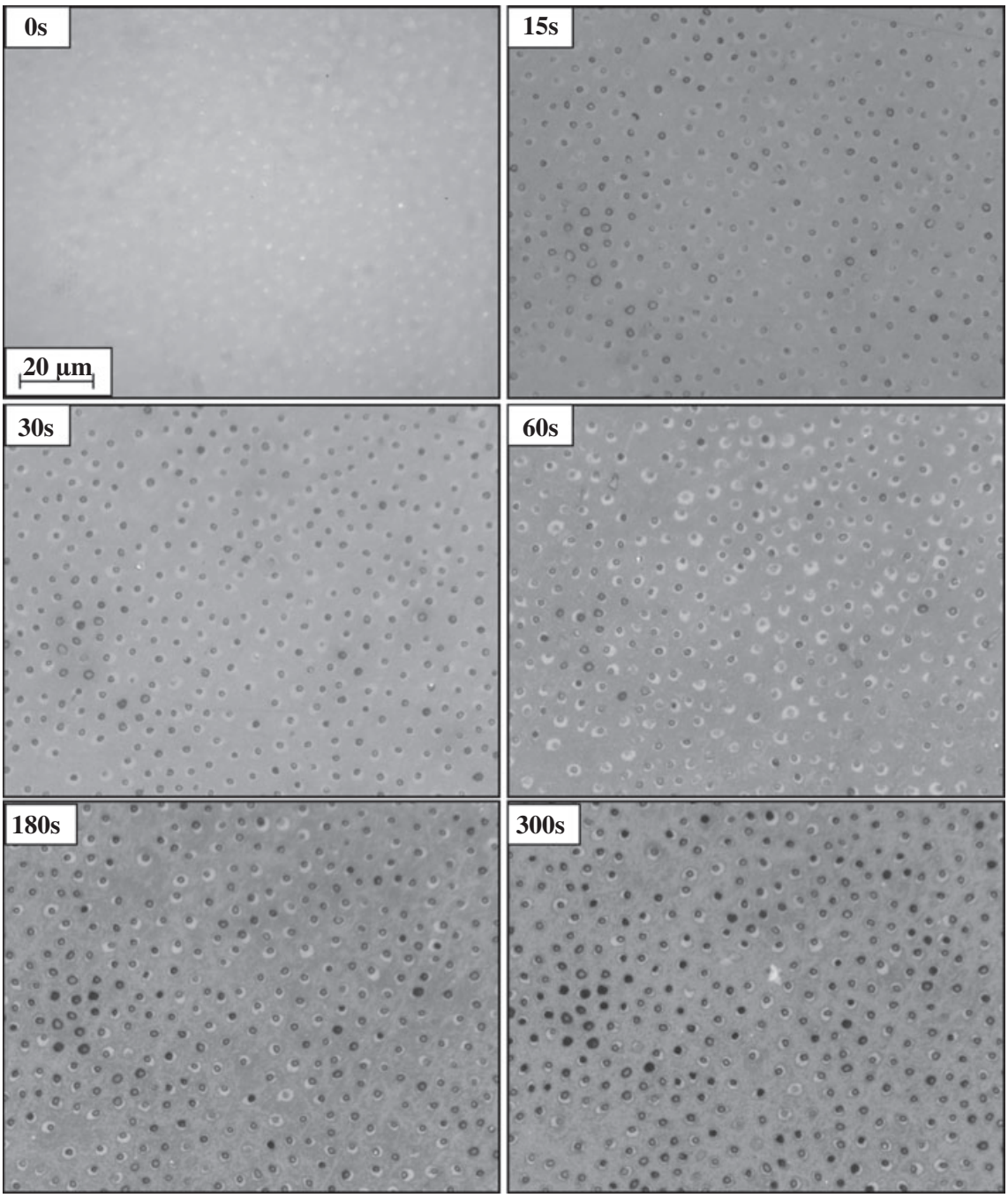

Figure 2 Time evolution of a given dentine region during demineralization with EDTA plus 0.1% cetavlon® (EDTAC).

a common finding that most SEM operators tend to select clean canal areas with open dentinal tubules rather than areas with large amounts of debris (Gulabivala *et al.* 2005, Hülsmann *et al.* 2005).

This project is a part of a larger study comparing the demineralization power of the chelating agents available in Endodontic practice. The accuracy and reproducibility of the method used in this study has been verified previously and it proved to be fast, robust and

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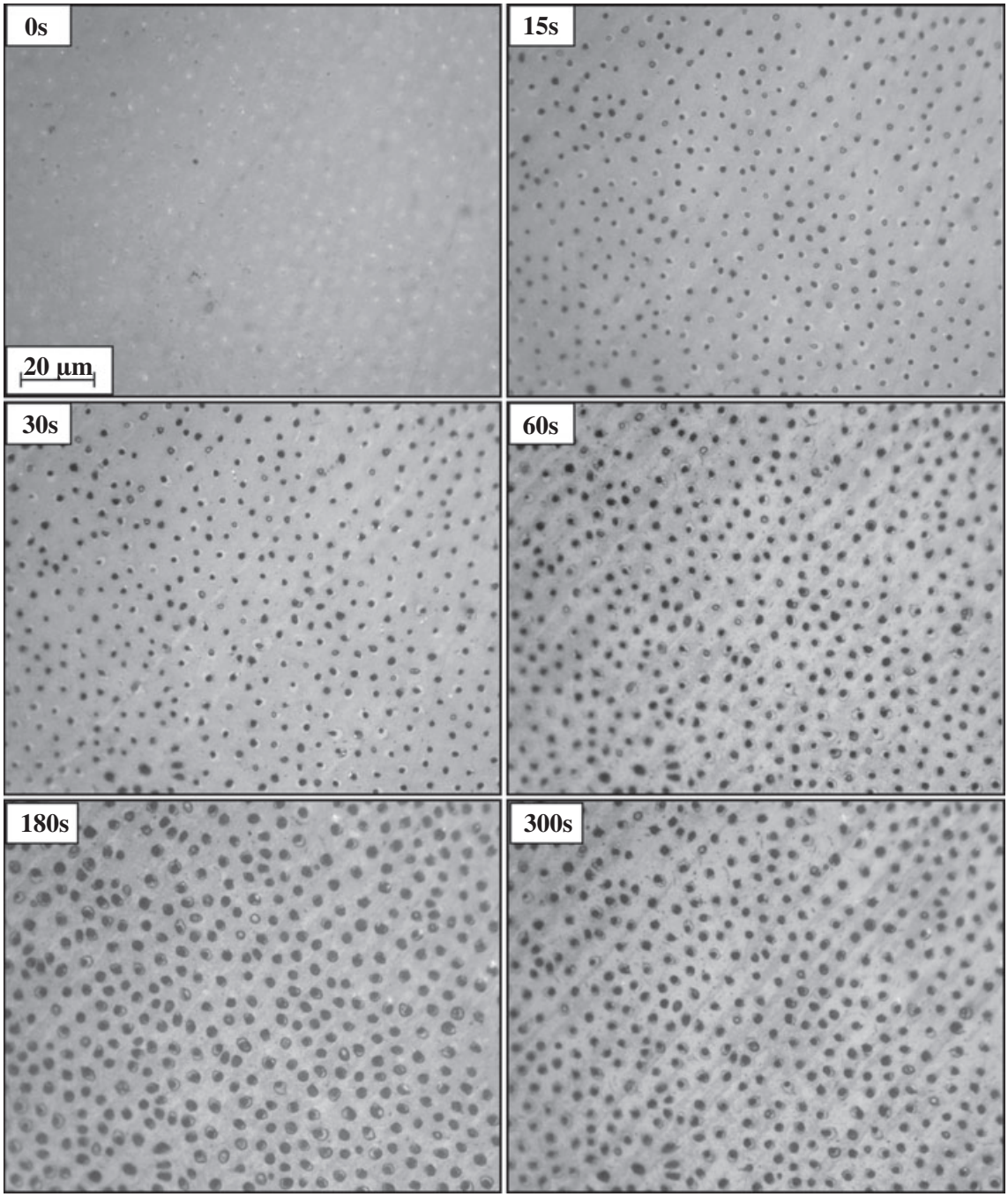

Figure 3 Time evolution of a given dentine region during demineralization with EDTA plus 1.25% sodium lauryl sulphate (EDTAT).

reproducible (De-Deus *et al.* 2007). Moreover, the method provides quantitative data linked to the longitudinal observation of the dentinal substrate changes.

The images in Figs 1–4 show the time evolution of the demineralization process in the same region of a sample thus highlighting the longitudinal character of the study. The possibility of observing microscopic

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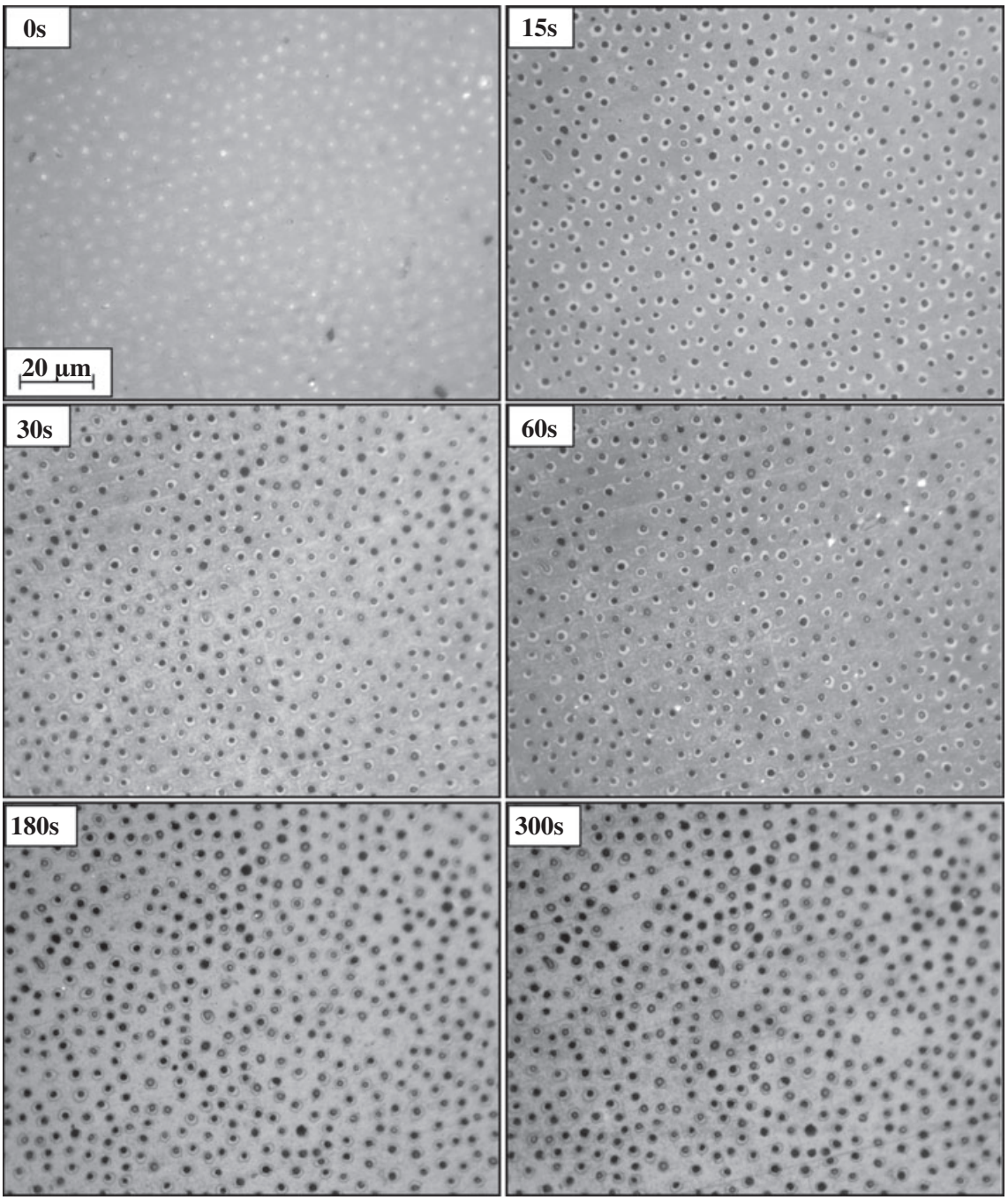

Figure 4 Time evolution of a given dentine region during demineralization with SmearClear™.

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 surface.

There appear to be few reports in the literature involving longitudinal and quantitative analysis of the process of dentine demineralization. Atomic absorption

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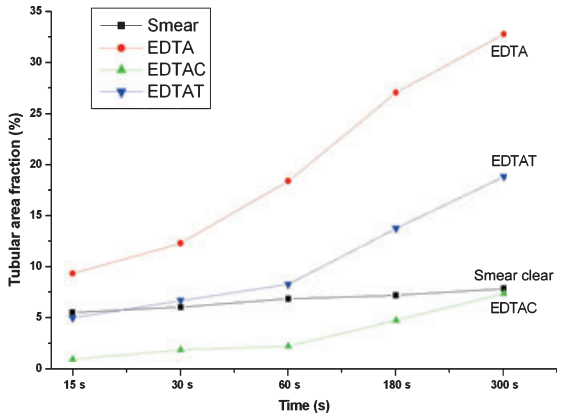

Figure 5 Time evolution of the open tubule area fraction (AF). Data points are the average of 45 measurements from three different samples.

Table 1 Statistical comparison between substances for each experimental time (*t*-test, $P < 0.05$)

Experimental time (s)	Substance	EDTAT	SmearClear™	EDTAC
15	EDTA	$P \approx 0$	$P \approx 0$	$P \approx 0$
	EDTAT		No	$P \approx 0$
	SmearClear™			$P \approx 0$
30	EDTA	$P \approx 0$	$P \approx 0$	$P \approx 0$
	EDTAT		No	$P \approx 0$
	SmearClear™			$P \approx 0$
60	EDTA	$P \approx 0$	$P \approx 0$	$P \approx 0$
	EDTAT		No	$P \approx 0$
	SmearClear™			$P \approx 0$
180	EDTA	$P \approx 0$	$P \approx 0$	$P \approx 0$
	EDTAT		$P \approx 0$	$P \approx 0$
	SmearClear™			$P \approx 0$
300	EDTA	$P \approx 0$	$P \approx 0$	$P \approx 0$
	EDTAT		$P \approx 0$	$P \approx 0$
	SmearClear™			No

No, no statistical difference. A *P*-value is shown when there is significant difference.

spectroscopy analysis (Çalt & Serper 2002, Gonzalez-Lopez *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. De-Deus *et al.* (2006b) 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 due to 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.

Another relevant point of the current method is related to the excellent sampling given by digital microscopy and image analysis, allowing thousands of tubules to be measured automatically, leading to more reliable analysis. The processing and analysis sequence was fully automatic and allowed an unbiased measurement process – in other words without operator influence.

As described by De-Deus *et al.* (2007), the typical processing time for each image was approximately 3 s in a typical computer. Thus, the full characterization of one dentine sample, with 15 fields, took <40 s. In each field, ≈350 tubules were detected, leading to ≈6000 tubules per sample and ≈18 000 tubules for each chelator at each experimental time. As published previously, the tubular AF of open dentinal tubules is readily measured by the digital analysis procedure developed and this parameter represents an important microstructural feature for dentine analysis (Schilke *et al.* 2000, Coutinho *et al.* 2003, De-Deus *et al.* 2007).

As the goal of the present work was restricted to a direct longitudinal and quantitative comparison of the chelating ability of EDTA with or without wetting agents, the application of these results to the clinical situation is not straightforward. Furthermore, one of the limitations of the current method is that the chelator solution was applied to a flat horizontal dentine surface, different from the clinical situation, in which the contact between the chelating substance and the dentine surface is affected by the vertical position of the teeth and the intrinsic anatomical variability of the root canal system. On the other hand, several experimental parameters are better controlled in the current method such as: the relationship between the amount of available chelator solution and the canal wall surface area, the contact time, the depth of penetration of the irrigation needle, root canal length as well as the solution age, pH, temperature and concentration.

Conclusions

Under the conditions of the present laboratory evaluation, the following conclusions can be drawn: (i) at all experimental times the EDTA solution had the strongest chelating effect; (ii) the association of EDTA with

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wetting agents had a negative influence on the chelating ability of the solution; (iii) CSOM represents a powerful approach to compare directly, longitudinally and quantitatively the ability of the chelating solutions.

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