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Biofilm on the apical region of roots in primary teeth with vital and necrotic pulps with or without radiographically evident apical pathosis

C. T. Rocha[1], M. A. Rossi[2], M. R. Leonardo[1], L. B. Rocha[2], P. Nelson-Filho[1] & L. A. B. Silva[1]

[1]Department of Paediatric, Preventive and Community Dentistry, School of Dentistry of Ribeirão Preto; and [2]Department of Pathology, Medical School of Ribeirão Preto, University of São Paulo, Ribeirão Preto, São Paulo, Brazil

Abstract

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Aim To evaluate, by scanning electron microscopy (SEM), the presence of biofilms on the external surfaces of the apical third of roots of human primary teeth with vital or necrotic pulps with and without radiographically evident periradicular pathosis.

Methodology Eighteen teeth were selected: group I – normal pulp (n = 5), group II – pulp necrosis without radiographic evidence of periapical pathosis (n = 7) and group III – pulp necrosis with well-defined radiographic periapical pathosis (n = 6). After extraction, the teeth were washed with saline and immersed in 0.03 g mL^{-1} trypsin solution for 20 min. The teeth were then washed in sodium cacodilate buffer and stored in receptacles containing modified Karnovsky solution. The teeth were sectioned, dehydrated in an ethanol series, critical-point dried with CO_2, sputter coated with gold and the external root surface in the apical third examined by SEM.

Results In the teeth of groups I and II, the apical root surfaces were covered by collagen fibres, with no evidence of bacteria (100%). In the teeth of group III, the root apices had no collagen fibres but revealed resorptive areas containing microorganisms (cocci, bacilli, filaments and spirochetes) in all cases (100%).

Conclusion Microorganisms organized as biofilms on the external root surface (extraradicular infection) were detected in primary teeth with pulp necrosis and radiographically visible periapical pathosis.

Keywords: apical biofilm, extraradicular infection, microorganism, periapical pathosis, primary teeth, scanning electron microscopy.

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Introduction

The major role of microorganisms in the pathogenesis of pulpal and periapical alterations is well known (Kakehashi *et al.* 1965, Möller *et al.* 2004).

Following pulp exposure as a result of caries, the microorganisms that initially occupy the pulp chamber and root canal lumen, invade the entire root canal system, i.e. the dentinal tubules, lateral canals, accessory canals, secondary canals, apical delta ramifications, apical foramen and apical root cementum surface (Sen *et al.* 1995, Leonardo *et al.* 2002) as well as invade the periapical tissues (Nair *et al.* 1990). This infection leads to the development of apical periodontitis. Extraradicular infection is inaccessible to biomechanical root canal preparation and allows the persistence and multiplication of microorganisms (Tronstad *et al.* 1987, 1990, Ferreira *et al.* 2004). This explains the persistence of post-treatment disease following root canal treatment of these teeth (Nair *et al.* 1990, Sjögren *et al.* 1997, Sundqvist *et al.* 1998).

Correspondence: Profa Dra Léa Assed Bezerra da Silva, Departamento de Clinica Infantil, Odontologia Preventiva e Social, Faculdade de Odontologia de Ribeirão Preto, Universidade de São Paulo, Avenida do Café S/N, CEP: 14040-904 Ribeirão Preto, SP, Brazil (Tel.: +55 16 3602 3995; fax: +55 16 3633 0999; e-mail: lea@forp.usp.br).

Another key factor accounting for the persistence of periapical infections (Tronstad *et al.* 1987, Nair *et al.* 1990) is the presence of a gelatinous matrix or extracellular matrix that protects the microorganisms and aids their organization as a biofilm (Lomçali *et al.* 1996, Leonardo *et al.* 2002). The apical biofilm constitutes a mechanical barrier against the action of antimicrobial substances and the host's defence mechanisms (Costerton *et al.* 1999).

Several scanning microscopic studies have evaluated the presence of biofilm on the root apexes of nonroot-filled permanent teeth (Tronstad *et al.* 1990, Lomçali *et al.* 1996, Siqueira & Lopes 2001, Leonardo *et al.* 2002). Nevertheless, the literature is scarce on studies investigating infections associated with the root canal system of primary teeth (Silva *et al.* 2006, Ruviére *et al.* 2007), especially in terms of extraradicular infection. Therefore, the purpose of this study was to evaluate, by scanning electronic microscopy (SEM), the presence of microorganisms organized as biofilms on the external root surface of the apical third of human primary teeth with vital pulp and those with pulp necrosis (with and without radiographically visible periapical pathosis).

Material and methods

Specimen selection

The research proposal was reviewed by the Ethics in Research Committee of the School of Dentistry of Ribeirão Preto, University of São Paulo (Process #2006.1.1311.58.9) and the study design was approved. The apical root third of recently extracted human primary incisor and molar teeth without previous dental treatment were evaluated. Group I: teeth with normal pulps (sound) ($n = 5$), group II: teeth with pulp necrosis without radiographically visible periapical pathosis ($n = 7$) and group III: teeth with pulp necrosis and well-defined radiographically visible periapical pathosis ($n = 6$).

Eligible participants were selected from patients of both sexes aged 4–8 years who had been referred for dental treatment at the Paediatric Dentistry Clinic of the School of Dentistry of Ribeirão Preto, University of São Paulo, Brazil. Children who had used antibiotics within the previous 3 months were excluded from the trial. The study purposes were fully explained to the parents/guardians, who signed a written informed consent form authorizing the enrolment of the children in the study. The patients were submitted to a clinical interview, review of dental/medical history and clinical/radiographic examination for tooth selection. Periapical radiographs were taken according to the parallel radiographic technique using a paediatric film holder to minimize image distortions. Teeth with pulp vitality confirmed by pulp sensibility tests had prolonged retention, intact crowns, no fistula, less than 2/3 of physiological root resorption, periodontal probing depths <3 mm and normal periodontal ligament space. The teeth with pulp necrosis (with and without well-defined radiographically visible periapical pathosis) had pulps exposed through carious lesions, presence/absence of fistula, less than 2/3 of root resorption and periodontal probing depth <3 mm. They were scheduled for extraction because of extensive crown destruction not amenable to restoration.

Specimen preparation

After tooth extraction, the roots were gently rinsed with sterile saline. Care was taken not to damage the apical root surface. In teeth with pulp necrosis and apical periodontitis, the chronic periapical lesion was removed using a scalpel without damage to the apical root surface.

Thereafter, the teeth were placed for 20 min in sterile glass receptacles containing 0.15 g trypsin (Sigma Chemical Co., St Louis, MO, USA) weighed in a precision analytical balance (model FA2104N, Bioprecisa, Curitiba, PR, Brazil) and diluted in 5 mL distilled water to reach a concentration of 0.03 g mL^{-1}. Next, the teeth were washed in 0.1 mol L^{-1} sodium cacodilate buffer and placed in individual receptacles containing modified Karnovsky solution (8% glutaraldehyde, 12% paraformaldehyde, in 0.2 mol L^{-1} sodium cacodylate, pH 7.2–7.4) for a minimum of 5 days.

After this period, using a carborundum disk mounted in a low-speed handpiece, a groove was made perpendicular to the long axis of the root at a distance of 3 mm from the root apex. This groove served as a guide for the placement of a surgical chisel that was used together with surgical hammer to sever the apical portion from the tooth. In molars, the root that was least affected by physiological resorption was used. All procedures were performed in a laminar flow chamber with care not to damage the apical portion of the root.

Specimen processing for SEM

The root apexes (apical 3 mm) were dehydrated in an increasing ethanol series (70, 95 and 100%), each solution being changed at 15-min intervals for 1 h *per*

concentration. The specimens were critical-point dried with CO_2 (Bal-Tec CPD 030, Fürstentum, Liechtenstein), sputter-coated with gold to obtain an approximately 200-μm-thick layer (Emitech K650 Sputter Coater, London, UK) and examined with a scanning electron microscope (DSM 940A; Zeiss, Oberkochen, Germany), operating at 15 kV.

Specimen analysis

For analysis of the specimens, ×50 magnification was used initially to locate the apical root third to obtain an overview of the surface morphology. The areas corresponding to the main apical foramen were examined under ×100, 200 and 500 magnification, using the apical region as a landmark. Greater magnifications (×1000, 2000, 3000 and 5000) were used to identify the presence of apical biofilm, microorganisms on root cementum and areas of root resorption (cementum and dentine). The microorganisms were characterized morphologically as cocci, bacilli, filaments and spirochetes. The incidence of microorganisms in the apical root biofilm in the three experimental groups was recorded as percentages.

Results

Microorganisms were absent in all the teeth with normal pulps (group I) and those with pulp necrosis without radiographically visible periapical pathosis (group II). In the teeth of these groups, the apical root surface was covered with a large amount of adherent periodontal ligament remnants, which hindered direct visualization of the apical foramen. Collagen fibres arranged in different directions extended from the apical surface in either thick or thin bundles interwoven within each other (Fig. 1). Physiological root resorption was observed in one tooth of group I (20%) and three teeth of group II (43%).

All teeth in group III (100%) had microorganisms organized as biofilms. There were resorptive areas on the apical root surface of these teeth, which exhibited a large number of microorganisms under greater magnifications (Fig. 2). In this group, the bacterial morphotypes consisted primarily of cocci and bacilli, although spirochetes and filaments were found as well (Fig. 3). Fungal cells were not observed.

Microbial aggregates were detected around the main apical foramen (Fig. 4). Mononuclear cells were found in the surrounding areas or 'within' this biofilm (Fig. 5).

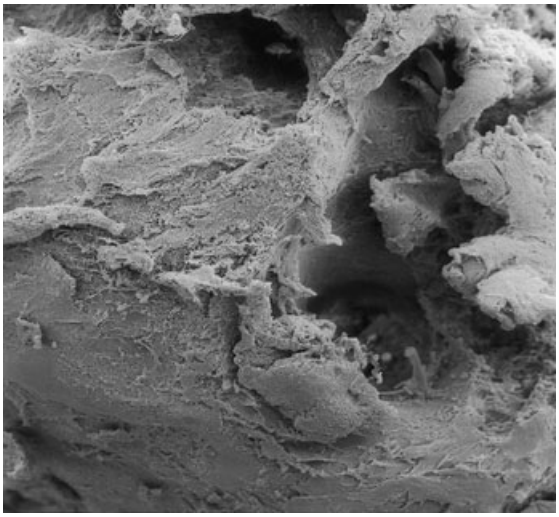

Figure 1 Collagen fibres arranged in different directions extending from the apical surface.

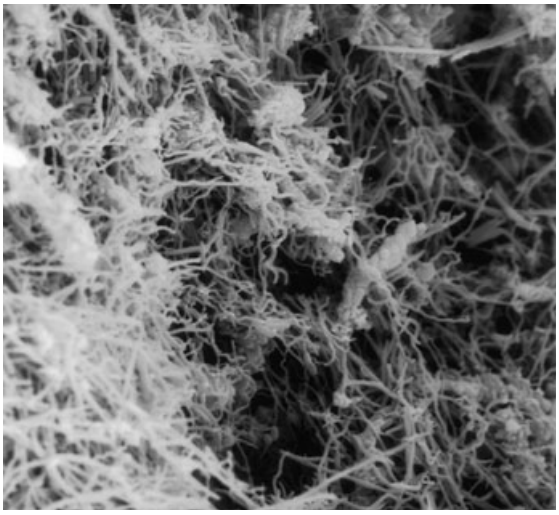

Figure 2 Resorptive areas on the apical root surface with an intense number of microorganisms under greater magnification.

Discussion

Several studies (Kakehashi *et al.* 1965, Möller *et al.* 2004) have demonstrated that microorganisms play an important role in the aetiology of pulpal and periapical pathosis in permanent teeth.

However, few authors have investigated the microbiota in the root canal system of primary teeth. Studies using conventional microbial culture (Silva *et al.* 2006)

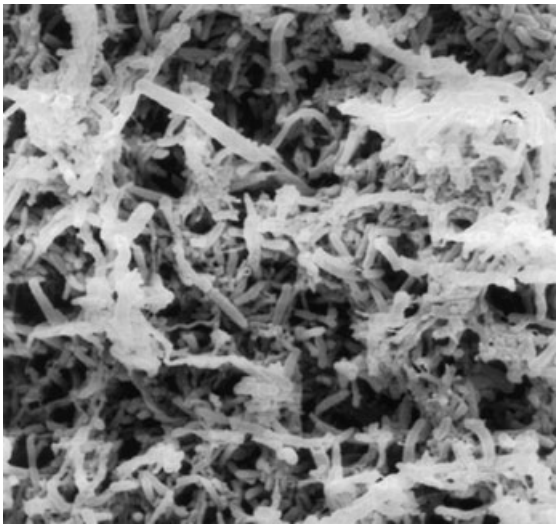

Figure 3 Bacterial morphotypes consisting primarily of cocci and bacilli.

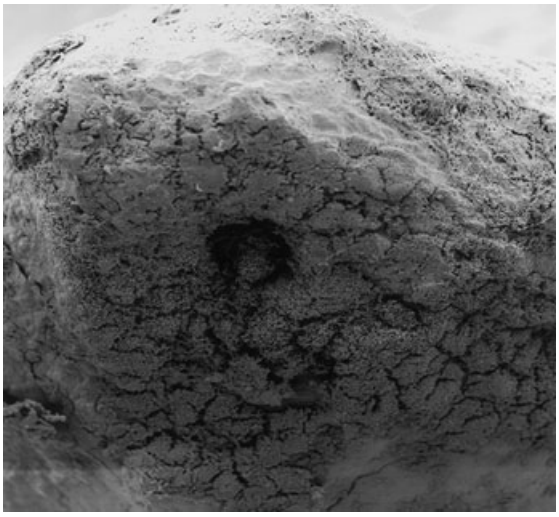

Figure 4 Exuberant microbial aggregates around the main apical foramen.

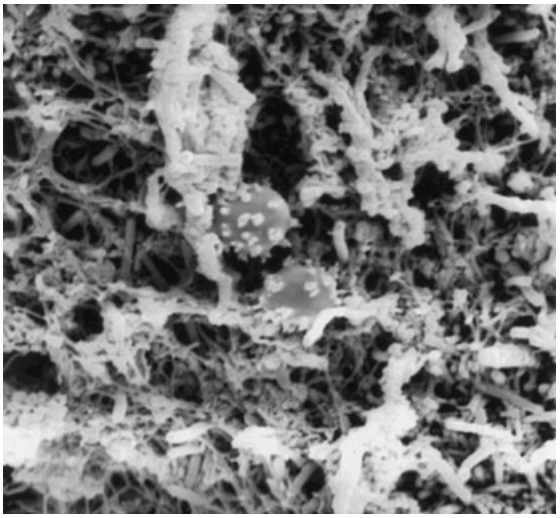

Figure 5 Mononuclear cells in the surroundings or 'within' the biofilm.

and molecular techniques (Ruviére *et al.* 2007) have shown that, in cases of apical periodontitis, the endodontic microbiota is similar in both primary and permanent dentitions, consisting of a polymicrobial infection with predominance of streptococci and anaerobic bacteria. As emphasized by Molven *et al.* (1991) and Sen *et al.* (1995), SEM is an accurate method to evaluate the presence of microbial biofilm and is a routinely employed method in studies investigating extraradicular infection in nonroot filled permanent teeth (Tronstad *et al.* 1990, Lomçali *et al.* 1996, Leonardo *et al.* 2002).

In the present study, the teeth in Group I were clinically sound and thus free from bacterial contamination of the pulp tissue. It has been previously confirmed by scanning electron microscopy (Lomçali *et al.* 1996, Leonardo *et al.* 2002) that no microorganisms exist in such specimens and that a large numbers of collagen fibres on the root apex are present on clinically healthy permanent teeth.

As pointed out by Leonardo (2005), in teeth with pulp necrosis but no radiographically visible periapical pathosis, the microorganisms remain in the lumen of the main root canal. The findings of the present study on primary teeth are in agreement, given that the specimens in group I and group II did not exhibit microorganisms on the outer root surface (extraradicular infection), which indicates that the microbial species were restricted to the main root canal lumen (Leonardo *et al.* 2002). Although some authors have questioned whether the operative procedures, especially those related to tooth extraction, would lead to contamination of the specimens (Sundqvist *et al.* 1998, Siqueira & Lopes 2001), no microorganisms were found in the root apexes of the primary teeth in groups I and II.

Specimens in group III had no collagen fibres but showed microorganisms organized as biofilms in the apical region, including areas of root resorption. These findings do not agree with those of Siqueira & Lopes (2001), who stated that the occurrence of

extraradicular infection is not common in nonroot filled permanent teeth. This difference might be due to the fact that in the present study the teeth were immersed in trypsin after extraction, as recommended by Leonardo *et al.* (2002) and Ferreira *et al.* (2004), which facilitated the identification of the microorganisms.

The bacterial types identified in the teeth of group III consisted predominantly of cocci and bacilli. Filaments and spirochetes were also observed. These findings are consistent with the results of Leonardo *et al.* (2002) with permanent teeth. Although some authors have reported the presence of fungal cells on the root surface of permanent teeth (Tronstad *et al.* 1987, Lomçali *et al.* 1996, Ferreira *et al.* 2004); in the present study these cell types were not found.

The presence of an apical biofilm on primary teeth may lead to the persistence of the inflammatory process, delaying or even precluding periapical healing, which might cause alterations in the developing permanent successor tooth germ (Cordeiro & Rocha 2005), changes in the patient's general health status (Brook 2000) and acceleration of the physiological root resorption process (Haralabakis *et al.* 1994). These factors may cause the premature loss of the primary teeth (Leroy *et al.* 2003), with functional and/or aesthetic sequelae, including loss of space in the dental arch, loss of eruption guide for the permanent successor, phonetic problems and development of deleterious habits (Cuoghi *et al.* 1998).

Conclusion

Microorganisms organized as biofilms on the external root surface (extraradicular infection) was found only in primary teeth with pulp necrosis and radiographically visible periapical pathosis.

Acknowledgements

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References

Brook I (2000) Anaerobic infections in children. *Advances in Pediatrics* **47**, 395–437.

Cordeiro MM, Rocha MJ (2005) The effects of periradicular inflamation and infection on a primary tooth and permanent successor. *The Journal of Clinical Pediatric Dentistry* **29**, 193–200.

Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. *Science* **284**, 1318–22.

Cuoghi OA, Bertoz FA, de Mendonca MR, Santos EC (1998) Loss of space and dental arch length after the loss of the lower first primary molar: a longitudinal study. *The Journal of Clinical Pediatric Dentistry* **22**, 117–20.

Ferreira FB, Ferreira AL, Gomes BP, Souza-Filho FJ (2004) Resolution of persistent periapical infection by endodontic surgery. *International Endodontic Journal* **37**, 61–9.

Haralabakis NB, Yiagtzis SC, Toutountzakis NM (1994) Premature or delayed exfoliation of deciduous teeth and root resorption and formation. *Angle Orthodontist* **64**, 151–7.

Kakehashi S, Stanley HR, Fitzgerald RJ (1965) The effects of surgical exposures of dental pulps in germ-free and conventional laboratory rats. *Oral Surgery, Oral Medicine, and Oral Pathology* **20**, 340–9.

Leonardo MR. *Endodoncia: Tratamiento de conductos radiculares – Princípios técnicos e biológicos*. São Paulo: Artes Médicas, 2005.

Leonardo MR, Rossi MA, Silva LA, Ito IY, Bonifacio KC (2002) EM evaluation of bacterial biofilm and microorganisms on the apical external root surface of human teeth. *Journal of Endodontics* **28**, 815–8.

Leroy R, Bogaerts K, Lesaffre E, Declerck D (2003) Impact of caries experience in the deciduous molars on the emergence of the successors. *European Journal of Oral Sciences* **111**, 106–10.

Lomçali G, Sen BH, Cankaya H (1996) Scanning electron microscopic observations of apical root surfaces of teeth with apical periodontitis. *Endodontics & Dental Traumatology* **12**, 70–6.

Möller AJ, Fabricius L, Dahlen G, Sundqvist G, Happonen RP (2004) Apical periodontitis development and bacterial response to endodontic treatment. Experimental root canal infections in monkeys with selected bacterial strains. *European Journal of Oral Sciences* **112**, 207–15.

Molven O, Olsen I, Kerekes K (1991) Scanning electron microscopy of bacteria in the apical part of root canals in permanent teeth with periapical lesions. *Endodontics & Dental Traumatology* **7**, 226–9.

Nair PN, Sjogren U, Krey G, Kahnberg KE, Sundqvist G (1990) Intraradicular bacteria and fungi in root-filled, asymptomatic human teeth with therapy-resistant periapical lesions: a long-term light and electron microscopic follow-up study. *Journal of Endodontics* **16**, 580–8.

Ruviére D, Ito I, Leonardo M, Silva L, Nelson-Filho P (2007) Assessment of the microbiota in root canals of human primary teeth by Checkerboard DNA–DNA hybridization. *Journal of Dentistry for Children* **74**, 118–23.

Sen BH, Piskin B, Demirci T (1995) Observation of bacteria and fungi in infected root canals and dentinal tubules by SEM. *Endodontics & Dental Traumatology* **11**, 6–9.

Silva LA, Nelson-Filho P, Faria G, de Souza-Gugelmin MC, Ito IY (2006) Bacterial profile in primary teeth with necrotic pulp and periapical lesions. *Brazilian Dental Journal* **17**, 144–8.

Siqueira JF Jr, Lopes HP (2001) Bacteria on the apical root surfaces of untreated teeth with periradicular lesions: a scanning electron microscopy study. *International Endodontic Journal* **34**, 216–20.

Sjögren U, Figdor D, Persson S, Sundqvist G (1997) Influence of infection at the time of root filling on the outcome of endodontic treatment of teeth with apical periodontitis. *International Endodontic Journal* **30**, 297–306.

Sundqvist G, Figdor D, Persson S, Sjögren U (1998) Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics* **85**, 86–93.

Tronstad L, Barnett F, Riso K, Slots J (1987) Extraradicular endodontic infections. *Endodontics & Dental Traumatology* **3**, 86–90.

Tronstad L, Kreshtool D, Barnett F (1990) Microbiological monitoring and results of treatment of extraradicular endodontic infection. *Endodontics & Dental Traumatology* **6**, 129–36.