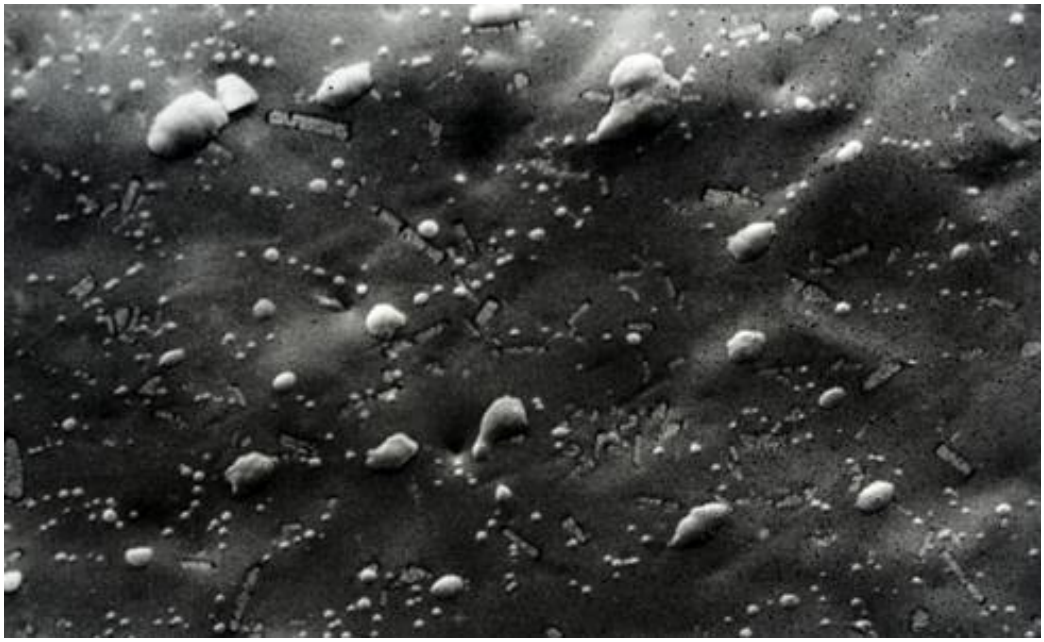
Alma Mater Studiorum – Università di Bologna

Evaluation of fluid transport processes in dental enamel. Methods to assess the relevance of enamel permeability in caries prevention and etching treatments.

Angelica Bertacci



ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

Bologna 2009

Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA
Biotecnologie Mediche

Ciclo XXI

MED/28 MALATTIE ODONTOSTOMATOLOGICHE

Evaluation of fluid transport processes in dental enamel. Methods to assess the relevance of enamel permeability in caries prevention and etching treatments.

Presentata da: ***Angelica Bertacci***

Coordinatore Dottorato

Prof.ssa **Marialuisa Zerbini**

Relatore

Prof. **Carlo Prati**

Esame finale anno 2009

CONTENTS

Chapter 1

Enamel Structure

Dental enamel is a composite of mineral, water, protein and lipid by volume and is the most mineralized tissue of the human body consisting of approximately 97 wt% mineral and 3% organic material and water (Simmmer and Hu, 2001; Klocke *et al.*, 2006). Mature enamel can contain less than 1% organic material (Bartlett and Simmer, 1999).

Enamel is composed of mineral rods sized ~30μm in length and 5 μm in diameter that are orientred roughly perpendicular to the dentino-enamel junction (DEJ). In turn, the rods are built up of crystallites sized ~1μm in length and 40 nm in diameter and aligned with their crystallographic *c*-axis along the rod length (Jongebloed *et al.*, 1975; Klocke *et al.*, 2006).
Crystallites are highly organized, tightly packed and comprise 87% of enamel volume (Simmer and Hu, 2001). In enamel the crystallites are arranged in the enamel prisms and interprismatic substance.

The orientation of the crystallites in enamel is of considerable interest since e.g. the crystallite dissolution in the caries process proceeds faster in the radial direction than parallel to crystallite's c-axis (Arends and Jongebloed, 1978).
Scanning electron microscopic examination showed that demineralization, initiated at core (prism or rod)/wall (prism sheath) interfaces, developed anisotropically along the *c*-axes (Wang *et al.*, 2006).

The inorganic content of enamel is a crystalline calcium phosphate hydroxyapatite having an hexagonal symmetry and a general formula [$Ca_{10}(PO_4)_6(OH)_2$], that contains some impurities, such as carbonates substituring for phosphate in the crystal lattice (Simmer and Hu, 2001; Klocke *et al.*, 2006). Various ions (eg. strontium, magnesium, and fluoride) may be also incorporated into or adsorbed by the hydroxyapatite crystals (Ten Cate, 1998). Dental apatite contains a substantial amount of carbonate groups, which substitute for the OH^- groups (A-type CO_3^{2-}) or for phosphate tetraedal (B-type) (Klocke *et al.*, 2006). The carbonate content represents 2-4 wt% with a reported 90% of type B and 10% of type A (Penel *et al.*, 1998). The carbonate concentration of human enamel has been found to increase on going from the outside to the inside of enamel layer (Wentrup-Byrne *et al.*, 1997). The structure of HAP can be considered as built up of corner-sharing PO_4 and CaO_6 polyhedra forming channels along the crystallographic *c*-axis, in which the hydroxyl-groups are placed (Klocke *et al.*, 2006).

The bulk of the organic material consists of tyrosine-rich amelogenin polypeptide (TRAP) peptide sequence tightly bound to the hydroxyapatite crystals, as well as nonamlelogenin proteins (Ten Cate, 1998).

Dental enamel is extremely hard and brittle. The underlying layer of dentin, more resilient, is necessary to maintain its integrity. Enamel is translucent, and it also varies in thickness, from a maximum of approximately 2.5 mm over working surface to a featheredge at the cervical line (Ten Cate, 1998).

Enamel Rods

Enamel is composed primarily of elongated structure called rods. The rod is shaped like a cylinder and is made up of crystals whose long axes run, for the most part, parallel to the longitudinal axis of the rod. Crystals more distant from the central axis, however, flare laterally to an increasing degree as they approach the rod periphery (Ten Cate, 1998).

Enamel crystals are extremely long relative to their thickness and are higly oriented. They generally extend from the underlying dentin toward the surface of the tooth and are organized into bundles, called prisms (Simmer and Hu, 2001).

The interrod region is an area surrounding each rod in which crystals are differently oriented. The boundary where crystals of the rod meet those of the interrod region at sharp angles is known as the rod sheath. The basic unit of enamel described as a cylindrical rod has a specific spatial relation to the interrod region directly cervical to it (Ten Cate, 1998).

The forming surface of enamel consists of pits, each surrounded by a wall made up of newly formed interrod enamel. During active secretion, each of these walled pits is occupied by a Tomes' process. The interrod region is formed slightly earlier than the rod enamel, which thus constitutes the walls of the pits. These walls are formed by secretion from proximal sites that completely encircle each Tomes' process near its base, where adjacent processes are joined by the distal junctional complexes. Thus each wall (interrod region) is formed as a cooperative effort by adjacent secretory ameloblats.
Each ameloblast is responsible for the formation of one rod (by its distal secretory site) and a portion of the surrounding interrod region (by its cooperative proximal sites).

Enamel rods have an average width of 5 μm, but they vary somewhat in size and morphology troughout the thickness of enamel. In the first 5 μm, next to the dentine, there is no rod structure. As they traverse the enamel the rods gradually increase slightly in diameter. At the enamel surface the rod structure is irregular or absent. Rodless enamel occurs in the outermost 30 μm or so of all primary teeth and in the gingival third of the enamel of permanent teeth. Crystals in these regions are perpendicular to the surface of the enamel.

The rods are aligned in horizontal rows. The rods in each row run in a direction generally perpendicular to the surface of the dentin, with a slight inclination toward the cusp as they pass outward. Near the cusp tip the rows have small radius, and the rods run more vertically. In the cervical enamel the rods run mainly horizontally; only a fey rods are titled apically.

Striae of Retius and bands of Hunter-Schreger

The striae of Retzius are incremental growth lines. They are formed as the results of a temporary constriction of Tomes' process associated with a corresponding increase in the secretory face forming interrod enamel. As a result enamel structure is altered along the lines. Electron micrographs reveal a possible decrease in the number of crystals in the striae, suggesting that enemel rods bend as they cross an incremental line (Ten Cate, 1998).

The striae of Retius often extend from the dentino-enamel junction to the outer surface of enamel, where they end in shallow furrows known as perikymata. Peikymata run in circumferentially horizontal lines across the face of the crown. In addition, lamellae or craks in the enamel apprear as jagged lines in various regions of the tooth surface. In unerupted teeth the enamel surface consits of a structureless layer some 0.5 to 1.5 μm thick. Immediately below it is a layer of small, losely packed crystallites, some 5 nm thick, with undemineralized material between them. Interspersed among, in and on these fine crystallites are randomly distributed large, platelike crystals. The fine crystallite layer merges into the subsurface enamel where crystals are closely packed and approximately 50 nm in size. In erupted teeth the structureless surface layer and the surface layer of small crystallites are rapidly lost by abrasion, attrition and ersosion (Ten Cate, 1998).

Human enamel is known to form at a rate of approximately 4 μm per day. Situated between the rods at approximately 4 μm intervals are interrod regions. Cross striation probably indicate a daily (or circadian) variation in the secretory activity of the ameloblasts and the striae of Retius represent a weekly rhythm of the same cells (Ten Cate, 1998).

The bands of Hunter and Schreger are an optical phenomenon produced solely by changes in rod direction. They are seen most clearly in longitudinal ground section viewed by reflect light and are found in the inner four fifths of the enamel. They appear as dark and light alternating zone that can be reversed by altering the direction of incident illumination (Ten Cate, 1998).

Enamel tufts and lamellae

Enamel tufts project from the dentino-enamel junction for a short distance into the enamel; they appear to be branched and contain greater concentrations of enamel protein than the rest of the enamel. Lamellae extend for varying dephts from the surface of enamel and consist of linear, longitudinally oriented defects filled with enamel protein or organic debris from the oral cavity. The protein of tufts is a high-molecular-weight variety similar to enamelin. Tufts are believed to occur developmentally baecause of abrupt changes in the direction of groups of rods that arise from different regions of the scalloped dentino-enamel junction. A different ratio of interrod and rod enamel in these groups creates less mineralized and weakened planes. Faulting of blocks of enamel relieves internal strains produced by dimensional changes as the tissue matures. When a fault occurs, it blocks the normal exit for enamel protein, causing the higher organic content of tufts and lamellae (Ten Cate, 1998).

Dentino-enamel junction

Dentin, enamel and cementum formation involves a remarkable mechanism of complex molecular and cellular events that conclude in specific structured tissue joined at distinctive interfaces.

The dentino-enamel junction (DEJ) is the natural junction that unites functionally dentin and enamel (Habelitz *et al*, 2001; Marshall *et al.*, 2001; Schulze *et al.*, 2004).

The junction between enamel and dentin is established as these two hard tissue begin to form and it appear as a series of ridges that increase the surface area and probably enhance the adhesion between enamel and dentin. The DEJ has a unique structure with at least three levels of microstructure (Schulze *et al.*, 2004).

The DEJ is the junction of coronal dentin and enamel and is formed by the secretion of dentine on one side and of enamel on the other side.

Before enamel forms, some newly forming odontoblast processes push between adjoining ameloblasts and when enamel formation begins, become trapped to form enamel spindles that do not follw the direction of enamel rods (Ten Cate, 1998).

The scallops house microscallops that contain finer nanoscale structures (Marshall *et al.*, 2001; Schulze *et al.*, 2004). The scallops and the presence of a smooth gradient of mechanical properties at the junction are believed to contribute to reduce stress concentra-

tion. This gradation of properties is initiated by biomineralization starting from the DEJ in both directions (Ten Cate, 1998).

It has been demonstrated that DEJ is resistant to acid attacks as well as to mechanical forces such as cracks propagation (Tramini *et al.*, 2000; Marshall *et al.*, 2001; Schulze *et al.*, 2004).

Enamel formation

Mineralization involves the crystallization of ions from supersaturated solutions (Simmer and Fincham, 1995).

Particularly mineralization process ivolves the net movement of ions out of solutions, where their charges are dissipated by interaction with water molecules, and into a solid structure stabilized by covalent interactions between oppositely charged ions (Simmer and Hu, 2001).

The stages of enamel formation include (Reith, 1970):

- secretion of an organic matrix;
- crystal nucleation;
- crystal eleongation;
- removal of the organic matrix, and
- crystal maturation.

Dentine and enamel formation take place simultaneously starting along the DEJ (dentino-enamel junction). On enamel side of the DEJ, crystal nuclei elongate into long thin ribbons. These ribbons are evenly spaced, oriented parallel to each other, and extend from the DEJ to the mineralization front just outside the membrane of ameloblasts (the cells lining the extracellular compartment on enamel side) (Simmer and Hu, 2001).

The shape and growth of the earliest crystallites appeared at the DEJ can be interpreted as evidence for a precursor phase of octocalcium phosphate (OCP). An OCP crystal displays on its face a surface that may act as a template for hydroxyapatite (OHAp) precipitation. Octocalcium phosphate is less stable than hydroxypapatite and can hydrolyze to OHAp. During this process one unit cell of octocalcium phosphate is converted into two unit cells of hydroxyapaptite (Simmer and Fincham, 1999).

As ameloblasts secrete enamel proteins, the crystallites continue to growth on length but grow very little in width and thickness. The final length of enamel crystals is determined

by how long the ameloblasts continue to add enamel proteins, which also determines the final thickness of the enamel layer as a whole (Simmer and Hu, 2001).

At a certain point, which is decided by the genetic program, ameloblasts undergo a transition that greatly reduced their secretion of enamel proteins. Instead of structural proteins, proteinases are secreted and the organic matrix is degraded and suddenly disappears from the extracellular compartment. These changes terminate the growth of enamel crystallites in length and vastly accelerate their growth in width and thickness. Crystal elongation is arrested by curbing the secretion of enamel matrix constituents such as amelogenin, ameloblastin, and enamelin. Mineral deposition on the sides of the crystallites accelerates, in part because of the degradation and removal of growth-inhibhiting enamel protein, cleavage products (Simmer and Hu, 2001).

In humans, the maturation stage, during which the crystallites growth in width and thickness, takes about three to four years. This process is necessary to harden the enamel layer and is directed by maturation stage of ameloblasts as they cycle trought smooth and ruffle-ended phases. Fluoride is incorporated into crystal structure during the maturation stage. Disturbance during the maturation stage of amelogenesis results in pathologically soft (hypomaturation) enamel of normal thickness (Simmer and Hu, 2001).

Dental enamel formation is highly specialized and, the proteins most directly involved in enamel biomineralization are specific for it. As a consequence, defect in the gene encoding enamel proteins generally cause enamel malformations without affecting other parts of the body. There are, however, numerous genetic syndromes associated with dental defect of all types (Simmer and Hu 2001).

References

Arends J, Jongebloed WL. Crystallites dimension of enamel. J Biol Buccale 1978;6:161-71.

Bartlett JD, Simmer JP. Proteinase in developing dental enamel. Crtit Rev Oral Biol Med 1999;10:425-441.

Habelitz S, Marshall SJ, Marshall GW, Balooch M. The functional width of the dentino-enamel junction determined by AFM-based nanoscratching. J Struct Biol 2001;135:294-301.

Jongebloed WL, Molenaar I, Arends J. Morphology and size-distribution of sound and acid-treated enamel crystallites. Calcif Tissue Res. 1975 Dec 18;19:109-23.

Klocke A, Mihailova B, Zhang S, Gasharova B, Stosch R, Guttler B, Kahl-Nieke B, Henriot P, Ritschel B, Bismayer U. CO2 laser-induced zonation in dental enamel: a raman and IR microspectroscopic study. J Biomed Mater Res Part B: 2007 Appl Biomater 81 B:499-507.

Marshall GW, Balooch M, Gallagher RP, Gansky SA, Marshall SJ. Mechanical properties of the dentino-enamel junction: AFM studies of nanohardness, elastic modulus, and fracture. J Biomed Mater Res 2001;54:87-95.

Reith EJ. The stages of amelogenesis as observed in molar teeth of young rats. J Ultrastruct Res 1970;30:111-151.

Schulze KA, Balooch M, Balooch G, Marshall GW, Marshall SJ. Micro-Raman spectroscopic investigation of dental calcified tissues. J Biomed Mater Res A. 2004;69:286-93.

Simmer JP, Fincham AG. Molecular mechanisms of dental enamel formation. Crtit Rev Oral Biol Med 1995;6:84-108.

Simmer JP, Hu JC-C. Dental enamel formation and its impact on clinical dentistry. J Dent Ed 2001;65:896-905.

Ten Cate AR. Oral histology: development, structure and function, 5th ed. St. Louis, MO: Mosby; 1998. p218-235.

Tramini P, Pelissier B, Valcarcel J, Bonnet B, Maury L. A Raman spectroscopy investigation of dentin and enamel structures modified by lactic acid. Caries Res 2000;34:233-240.

Wang LJ, Tang R, Bonstein T, Bush P, Nancollas GH. Enamel demineralization in primary and permanent teeth. J Dent Res 2006;85:359-363.

Wentrup-Byrne E, Armstrong CA, Armstrong RS, Collins BM. Fourier Transform Raman Microscopic Mapping of the molecular components in a human tooth. J Raman Spectr 1997, 28:151-158.

Chapter 2

Fluid transport processes

Enamel permeability

Enamel does not behave as an inert tissue because water and organic material occur between the prisms (Bartlestone, 1951; Lindén 1968; Pashley 1996; Shellis 1996; Ten Bosch 2000).

Bergman and Lindén showed that small quantities of a fluid passing through the enamel *in vivo* (Bergman and Lindéen 1965; Lindén 1968).

Enamel fluid flowing is related to permeability but is not well documented *in vivo* (Bergman and Lindén 1965; Bakhos *et al.*, 1977).

The transport of water across dental enamel *in vitro* is not a simple diffusion process in which enamel behaves as an inert porous medium as enamel behaves as an osmotic membrane (Burke and Moreno, 1975). The diffusion of molecules and ions in the dental enamel plays an important role in the development of caries (Dibdin 1993; Kuhar *et al.*, 1999).

Many studies in the literature investigated enamel permeability. In particular most of all related this property with caries trying to explain the tissues changes in the phases of demineralization and remineralization (Fearnhead *et al.*, 1982; Ten Cate 2001) even if the importance of enamel permeability in caries, restorative materials and pulp-dentine-enamel interaction is still not fully understood (Byers *et al.*, 2003).

From the beginning of the last century several authors investigated the penetration of enamel with dyes (Beust 1912, Berggren 1943, Tarbet and Forsick, 1971), the diffusion

of organic components (Poole *et al.*, 1963), the permeability to inorganic ions (Sognnaes and Shaws 1952), radioactive elements (Braden, 1971; Sognnaes and Shows 1952; Joystone-Bechal *et al.*, 1971), and water (Poole *et al.*, 1963; Lindén 1968; Shellis and Dibdin, 2000). The main studies in the literature evaluated enamel permeability and fluid flowing *in vitro* and *in vivo* through scanning (Whittaker, 1982) and transmission microscopy (Poole *et al.*, 1981), measurement of diffusion coefficients (Lindén 1968; Borggreven *et al.*, 1977) and through physical parameters such as electrical resistance (Hoppenbrouwers et al, 1986; Wang *et al.*, 2005) conductance (Ie *et al.*, 1995; Ten Bosch *et al.*, 2000), impedance (Scholberg *et al.*, 1984). These results demonstrated the bidirectional permeability of enamel.

Enamel fluid transport processes

Zahradnik and Moreno showed that dental enamel has a bimodal pore distribution (Zahradnik and Moreno, 1975) and that the transport processes related to mineralization and demineralization are significantly affected by the amount of water avaible in the tissue as well as by its porous structure (Moreno and Zahradnik, 1973).

Diffusion in the aqueous phase which fills enamel pores is the main transport of the ions in the early stages of caries progression, in remineralization, and in fluoride treatment (Dibdin, 1993).
The organic matter of the enamel is probably the route of diffusion although the role of organic material needs further investigation to be clearified (Shellis, 2000). Microscopic examinations of enamel section showed that the main channels of diffusion were the interprismatic substance (Whittaker, 1982). Microscopical observations show that the prism junction provide the main pathways (Tarbet and Forsick, 1971) although, in inner enamel some transport was observed within the prism (Lindén, 1968).

Because enamel mineral exists as a very small crystals organised in an elaborate structure, the internal pores are small and variable in form, orientation and distribution. The largest pores in enamel are associated with the prism junctions, but these constitute only a small fraction of the total porosity, most of which is associated with the prism bodies and tails (Shellis and Dibdin, 2000).

Structure, porosity and enamel solubility are linked. Because the prism-junction material is more soluble than the interprismatic material, it is possible for prism junctions to be opened up under conditions where the enamel fluid is still supersaturated with respect to the intraprismatic mineral (Shellis, 1996).

Enamel permeability is variable depending to age and demineralization (Kotsanos and Darling, 1991) is greatest in teeth with immature enamel, and it appears to require a partnership with dentine (Byers *et al.*, 2003). Epidemiologic studies with animals have suggested that caries susceptibility decreases with age: a process commonly referred as "posteruptive maturation" of enamel may be responsible for this phenomenon reducing the permeability of enamel (Fearnhead *et al.*, 1982; Kotsanos and Darling, 1991).

In extracted young human teeth, cervical enamel has more dye flow than the rest of the crown (Lindén 1968) and appears to be the preferred pathway for fluid flowing (Poole *et al.*, 1981; Byers *et al.*, 2003). Moreover in young teeth the enamel interprismatic region is proportionally greater than in old teeth and the dentin is much thinner and odontoblast processes reach closer to the dentino-enamel junction (Byers and Sugaya, 1995).

Newly-erupted teeth acquire F more readily than older teeth; younger permanent enamel takes up more F, exhibits a higer-water sorption capacity and imbibes more iodide than older permanent enamel (Lindén *et al.*, 1986).
Enamel fluoride concentration of permanent enamel is always higher than that for primary enamel (Issa *et al.*, 2003).

Enamel of deciduous teeth contains more organic matter, more water, less mineral and is more porous in agreement with clinical studies that have shown caries formation and progression to be faster in primary than in permanent teeth (Sønju Clasen *et al.*, 1997; Issa *et al.*, 2003).

Caries and enamel fluid movement

The development of carious lesions in enamel involves transport of acids into and dissolution of minerals from the tooth surface. Accordingly the rate of diffusion of cariogenic

and cariostatic substances (ions and molecules) plays a crucial role in the dynamic process of caries (Van Dijk *et al.*, 1983; Featherstone, 1983; Lindèn *et al.*, 1986).

The fluoride content of the mid-coronal buccal surface enamel in increasing age was found to decrease posteruptively with age, therefore not accounting for the decreasing caries susceptibility (Kostanos an Darling, 1991). Recently it has been demonstrated that recently erupted teeth are more sensitive to dental caries than teeth that have remained free from caries lesions for a few years after eruption (Ten Bosch *et al.*, 2000).

This was confirmed with experiments in which artificial caries lesions were produced in extracted teeth of different post-eruptive ages (Kostanos an Darling, 1991; Ten Bosch *et al.*, 2000). It has been hypothesized that these differences could be ascribed to differences in enamel porosity consequent to intra-oral maturation presumably due to incorporation of calcium-phosphate into the enamel (Ten Bosch *et al.,* 2000).

Studies on enamel physical properties showed that the resistivity of enamel layers increased from the DEJ to the outer surface, the permeability increases from the outer surface towards the EDJ (Lindén 1968, Hoppenbrouwers *et al.*, 1986) and that the resistivity in erupted teeth was considerable higher than in unerupted teeth confirming the effects of post eruptive mineralization (Hoppenbrouwers *et al.*, 1986).

The formation of caries lesions is strongly influenced by the pathways for diffusion and by electrochemical effects arising from the charge on the pore walls (Shellis and Dibdin, 2000).
Mineral loss during caries progress results in an increase in porosity: the resulting changes in porosity could affect the flow of an electrical current through the enamel (Wang *et al.*, 2005).

References

Bakhos Y, Brudevold F, Aasenden R. In vivo estimation of the permeability of surface human enamel. Arch Oral Biol 1977;22: 599–603.
Berggren H. Penetration of dyes in living human enamel. J Dent Res 1943;22:1-6.

Bergman G, Lindén L. Techniques for microscopic study of enamel fluid in vivo. J Dent Res 1965; 44: 1409.

Borggreven JMPM, Van Dijk JWE, Driessens FCM. A quantitative radiochemical study of ionic and molecular transport in bovine dental enamel. Arch Oral Biol 1977; 22:467–472.

Braden M, Duckworth R, Joyston-Bechal S. The uptake of ^{24}Na by human dental enamel. Arch Oral Biol 1971; 16: 367–374.

Buest T von. A contribution to the study of immunity to dental caries. Dent Cosmos 1912;54:659-663.

Burke EJ, Moreno EC. Diffusion fluxes of tritiated water across human enamel membranes. Arch Oral Biol 1975; 20:327-332.

Byers MR, Sugaya A. Odontoblast processes in dentin revealed by fluorescent Di-I. J Histochem Cytochem 1995;43:159-68.

Byers MR, Yoon Lin KJ. Patterns of fluoro-gold entry into rat molar enamel, dentine, and pulp. J Dent Res 2003; 82: 312–317.

Dibdin GH. The water in human dental enamel and its diffusional exchange measured by clearance of tritiated water from enamel slabs of varying thickness. Caries Res 1993; 27: 81–86.

Fearnhead RW, Kawasaki K, Inoue K. Comments on the porosity of human tooth enamel. J Dent Res 1982; 61: 1524–1530.

Featherstone JDB. Diffusion phenomena and enamel caries development. In: Guggenheim, B, ed. Cariology Today. Basel: Karger, 1983; 259–268.

Hoppenbrouwers PMM, Scholberg HPF, Borgreven JMPM. Measurement of the permeability of dental enamel and its variation with depth using an electrochemical method. J Dent Res 1986; 65: 154–157.

Ie YL, Verdonschot EH, Schaeken MJ, Vant'Hof MA. Electrical conductance of fissure enamel in recently erupted molar teeth as related to caries status. Caries Res 1995; 29: 94–99.

Issa AI, Preston AJ, Preston AJ, Toumba KJ, Duggal MS. A study investigating the formation of artificial sub-surface enamel caries-like lesion in deciduous and permanent teeth in the presence and absence of fluoride. Arch Oral Biol 2003;48:567-571.

Joyston-Bechal S, Duckworth R, Braden M. Diffusion of radioactive ions into human dental enamel. Arch Oral Biol 1971; 16: 375–384.

Kotsanos N, Darling AI. Influence of posteruptive age of enamel on its susceptibility to artificial caries. Caries Res 1991;25: 241–250.

Lindén LA, Björkman S, Hattab F. The diffusion in vitro of fluoride and chlorhexidine in the enamel of human deciduous and permanent teeth. Arch Oral Biol 1986; 31: 33–37.

Lindèn LA. Microscopic observations of fluid flowing through enamel in vitro. Odontol Rev 1968; 19: 349–365.

Moreno EC, Zahradnik RT. The pore structure of human dental enamel. Arch Oral Biol 1973; 18: 1063–1068.

Pashley DH. Dynamics of the pulpo-dentine complex. Crit Rev Oral Biol Med 1996; 7: 104–133.

Poole DF, Tailby PW, Berry DC. The movement of water and other molecules through human enamel. Arch Oral Biol 1963; 38: 771–772.

Poole DFG, Newman HN, Dibdin GH. Structure and porosity of human cervical enamel studied by polarizing microscopy and transmission electron microscopy. Arch Oral Biol 1981; 26:977–982.

Scholberg HPF, Borggreven JMPM, Driessens FCM. A phenomenological interpretation of the frequency-dependent impedance behaviour of bovine dental enamel. Archs Oral Biol 1984;12:965-970.

Shellis RP, Dibdin GH. Enamel microporosity and its functional implications. In: Teaford MF, Ferguson MJ, Smith MM, eds. Teeth: development, evolution and function. Cambridge:Cambridge University Press, 2000; 242–251.

Shellis RP. A scanning electron-microscopic study of solubility variation in human enamel and dentine. Arch Oral Biol 1996; 41: 473–484.

Shellis RP. Transport processes in enamel and dentine. In: Addy M, Embery G, Edgar WM, Orchardson R, eds. Tooth wear and sensivity. London: Martin Duniz, 2000; 19–24.

Sognnaes RF, Shaw JH. Salivary and pulpal contribuitions to the radiophosphorous uptake in enamel and dentin. J Am Dent Assoc 1952;44:489-505.

Sønju Clasen AB, Øgaard B, Duschner H, Ruben J, Arends J, Sønju T. Caries development in fluoridated and non fluoridated deciduous and permanent enamel *in situ* examined by microradiography and confocal laser scanning microscopy. *Adv Dent Res* 1997; 11: 442-7.

Tarbet WJ, Forsick LS. Permeability of human dental esame to acriflavine and potassium fluoride. Arch Oral Biol 1971; 16:951–961.

Ten Bosch JJ, Fennis IEY, Verdonschot EH. Time-dependent decrease and seasonal variation of the porosity of recently erupted sound dental enamel in vivo. J Dent Res 2000; 79: 1556–1559.

Ten Cate M. Remineralization of caries lesions extending into dentine. J Dent Res 2001; 80: 1407–1411.

Van Dijk JW, Borggreven JM, Driessens FC. Diffusion in mammalian tooth enamel in relation to the caries process. Arch Oral Biol 1983; 28: 591–597.

Wang J, Someya Y, Inaba D, Longbottom C, Miyazaki H. Relationship between electrical resistance measurement and microradiographic variables during remineralization of softened enamel lesions. Caries Res 2005; 39: 60–64.

Whittaker DK. Structural variations in the surface zone of human tooth enamel observed by scanning electron microscopy. Arch Oral Biol 1982; 27: 383–392.

Zahradnik RT, Moreno EC. Structural features of human dental enamel as revealed by isothermal water vapour sorption. Arch Oral Biol 1975;20:317-25.

Chapter 3

Methods to assess enamel permeability and structure

Replica Technique

The replica technique performed in these studies has been previously described in the literature (Barnes, 1977; Ittahgarun and Tay, 2000; Chersoni *et al.*, 2005). This technique allows the evaluation of fluid outflow from enamel surface and is able to detect the presence of small quantities of fluid. The specific characteristics of replica technique, that is not invasive and risk-free for the patient, make possible to perform *in vivo* studies on fluid outflow, that represents enamel permeability, in different clinical conditions.

Hydrophobic polyvinyl siloxane impression material was applied on observational area and after 4 minutes, the polymerised impression material was removed and degassed for at least 48 hours and finally later cast in polyether impression material.
The absence of any chemical reaction between the two impression materials makes this tecniqua effective in showing water exudation.

The *in vivo* application of this technique yielded qualitative and quantitative findings on outward fluid flow on enamel surfaces by means of scanning electron microscopy inspection of polyether replicas. This technique uses droplet formation to display the discharge of liquid from enamel during the setting time of the polyvinyl siloxane impression material that is able to recall water by osmotic gradient. Moreover water droplets are not incorporated into the polyvinyl siloxane and remain at the observation interface level.

All the replicas obtained, morphological expression of fluid outflow, were gold sputtered and observed by a Scannig Electron Microscope.

Raman and IR Spectroscopy

Raman spectroscopy is an advanced, fast analytic technique to determine the structure and the chemical composition of materials. It provides chemical information based on molecular vibrations of the molecules in the samples.
The Raman spectroscopy technique allows obtaining vibrational spectra of minerals by analysing scattered light caused by monochromatic laser excitation (Tsuda and Arends, 1997). Raman spectra analysis and infrared absorption (IR) spectroscopy are complementary techniques. While IR measures the light absorption by specific molecules using a broadband light source the Raman technique measures the characteristics Raman emission induced from muolecules under monochromatic laser irradiation (Tsuda and Arends, 1997).
Raman signals are emitted in the form of light scattering and can be observed from all directions, unlike the co-linear optical arrangement of IR. The axes of excitation light and detection can be chosen independently, resulting in a considerable instrumental flexibility of Raman. An optical microscope (micro) can be incorporated into a Raman specrtroscopy system (Tsuda and Arends, 1997).
Despite the advantages often Raman technique has the problem of fluorescence exhibited by most biological materials when irradiated by laser light. In normal Raman spectroscopy, fluorescence spectra due to organic materials often dominate the much weakr Raman signals. Therefore Raman spectroscopy studies have been limited to enamel which contains only few organic matter (Tsuda and Arends, 1997).

Raman spectroscopy, as a versatile and non-destructive technique, allows for simultaneous characterization of the inorganic and organic phases of the tooth. Furthermore, Raman spectra exhibit little interference with water, making Raman spectroscopy advantageous for the study of many biological specimens (Carden and Morris, 2000).

Raman microspectrometry produces the capability to characterize the spatial distributions of organic and inorganic compounds with spatial resolution of about 1μm. The intensity values of the spectra obtained from the inorganic component reveals the chemi-

cal composition; shift analysis of the peaks in the spectra allows the chemical contents of the tissues to be differentiate (Schulze *et al.*, 2004).
The micro-Raman technique has considerable potential for studies of crystallite orientation in enamel. Spectral variations were taken as a function of rotation angle for transverse or longitudinal arrangements. Similarly spectra were also obtained with the enamel samples at various orientation angles (Tsuda and Arends 1994).

Applications of Raman spectroscopy in dental research have included studies of enamel powder, artificial apatite, synthetic, carbonated apatite, synthetic fluorapatite (De Mul *et al.*, 1986; Bertoluzza *et al.*, 1996; Penel *et al.*, 1998 Liu and Hsu, 2007). The depeosition of CaF_2-like crystals after fluoride treatment and the relative orientation of single crystals in dental enamel were also investigated through Raman spectroscopy (Tsuda and Arends, 1993; Tsuda and Arends 1994).

The spectra from human dental hard tissue were analyzed in two specific wave number locations, the phosphate stretching band and the C-H stretching mode.
The enamel Raman spectrum is dominated by bands that can be attributing to the mineral apatite at 591, 961, and 1071 cm^{-1}.
The phosphate/C-H ratio clearly showed that enamel had a different average composition than the adjacent hard tissues. The cementum had the lowest (2.8) and the enamel has the highest ratio (94.2). The phosphate/C-H intensity ratio for dentine was approximatively 10% that of enamel, and varied from 7.1 for dentine to 19.6 for enamel (Schulze *et al.*, 2004).

The apatite crystallites in enamel are preferentially oriented with their crystallographic *c*-axis perpendicular to enamel-dentine junction, which results in different Raman scattering intensity depending on the experimental geometry (Klocke *et al.*, 2006).

Sound tooth enamel exhibited strong Raman polarization anisotropy, whereas early caries consistently showed a lower degree of Raman polarization anisotropy in particular for sound enamel the Raman peak arising from the symmetric ν1 vibration of PO^{3-}_4 from hydroxyapatite at 959 cm^{-1} is strongly polarized (Sowa *et al.*, 2007).

FT-IR (Fourier Transform InfraRed) spectroscopy can result in a positive identification (qualitative analysis) of every different kind of material.
An infrared spectrum represents a fingerprint of a sample with absorption peaks which correspond to the frequencies of vibrations between the bonds of the atoms making up the material.

Because each different material is a unique combination of atoms, no two compounds produce the exact same infrared spectrum. In addition, the size of the peaks in the spectrum is a direct indication of the amount of material present. With modern software algorithms, infrared is an excellent tool for quantitative analysis.

Fourier transform infrared (FTIR) spectroscopy and Raman spectroscopy are chemical analytical methods that have been used to collect information about mineral tissues (Wentrup-Byrne *et al.*, 1997; Klocke et la., 2006). The outputs from these methods are FTIR and Raman spectra that contain signals from the organic functional groups in the sample. Since the bands in FTIR spectra are due to polar functional groups while the bands in Raman spectra are due to nonpolar functional groups, FTIR and Raman spectroscopy are complementary techniques.

SEM-EDX

Energy Dispersive X-ray (EDX) analysis is a valuable tool for qualitative and quantitative element analysis. This method allows a fast and non-destructive chemical analysis with a spatial resolution in the micrometer regime. It is based on the spectral analysis of the characteristic X-ray radiation emitted from the sample atoms upon irradiation by the focussed electron beam of a SEM.
The incident beam electrons excite electrons in a lower energy states, prompting their ejection and resulting in the formation of electron holes within the atom's electronic structure. Electrons from an outer, higher-energy shell then fill the holes, and the excess energy of those electrons is released in the form of X-ray photons. The release of these X-rays creates spectral lines that are highly specific to individual elements. In this way the X-ray emission data can be analyzed to characterize the sample.

The data generated by EDX analysis consist of spectra showing peaks corresponding to the elements making up the true composition of the specimen being analysed.
The technique can be qualitative, semi-quantitative, quantitative and also provide spatial distribution of elements through mapping.
The EDX technique is non-destructive and if required specimens of interest can be examined in situ with little or no sample preparation. The EDX systems also have Image Analysis packages that can be applied to any images generated by the SEM / EDX technique allows for the identification of the critical characteristics of particles. It offers the ability to gather information about finer particles than by optical microscopes and can readily distinguish between clusters and agglomerates of particles in addition to the chemical analysis available by EDX. The strength of this analysis technique is its ability to gather statistically significant data on the size, morphology and composition of the particles in a time efficient manner, beyond the capabilities of conventional optical microscopy.

Severals studies used EDX investigating the effect of different treatment (such as fluoride, peroxide etc) on the chemical composition of enamel surface (Takagi *et al.*, 2000; Barbour and Rees, 2004; Schougall Vilchis *et al.*, 2008).

References

Barbour ME, Rees JS. The laboratory assessment of enamel erosion: a review. J Dent 2004;32:591-602.
Barnes IE. The adaptation of composite resins to tooth structure. Part 1. Study 1: introduction and the adaptation of composite resins to the unetched enamel cavity wall. Br Dent J 1977; 142: 122–129.
Bertoluzza A, Bottura G, Taddei P, Tinti A. Vibrational spectrs of controlled-structure hydroxyapatite coatings obtained by the polymeric route. J Raman Spectroscopy 1996;27:759-764.
Carden A, Morris MD. Appplication of vibrational spectroscopy to the study of mineralized tissue (review). J Biomed Opt 2002;5:259-68.
Chersoni S, Suppa P, Breschi L, Ferrari M, Tay FR, Pashley DH, Prati C. Water movement in the hybrid layer after different dentin treatments. Dent Mater 2004; 20: 796–803.

De Mul FF, Hottenhuis MH, Bouter P, Greve J, Arends J, ten Bosch JJ. Micro- Raman line broadening in synthetic carbonated hydroxyapaptite. J Dent Res 1986;65:437-40.

Itthagarun A, Tay FR. Self-contamination of deep dentin by dentin fluid. Am J Dent 2000; 13: 195–200.

Klocke A, Mihailova B, Zhang S, Gasharova B, Stosch R, Güttler B, Kahl-Nieke B, Henriot P, Ritschel B, Bismayer U. CO_2 laser-induced zonation in dental enamel: a Raman and IR microspectrometry study. J Biomed Mat Res Part B: Appl Biomater; 2007: 81B:499-507.

Liu Y, Hsu C-YS. Laser-induced compositional changes on enamel: A Ft-Raman study. J Dent 2007;35:226-30.

Penel G, Leroy G, Rey C, Bres E. Micro-Raman spectral study of the PO_4 and CO_3 vibrational modes in synthetic and biological apatites. Calcif Tissue Int 1998;63:475-481.

Schulze KA, Balooch M, Balooch G, Marshall GW, Marshall SJ. Micro-Raman spectroscopic investigation of dental calcified tissues. J Biomed Mater Res A. 2004;69:286-93.

Sowa MG, Popescu DP, Werner J, Hewko M, Ko A C-T, Payette J, Dong CCS, Cleghorn B, Choo-Smith L-P. Precision of Raman depolarization and optical attenuation measurements of sound tooth enamel. Anal Bioanal Chem 2007;387:1613-1619.

Tsuda H, Arends J. Detection and quantification of calcium fluoride using micro-Raman spectroscopy. Caries Res 1993;27:249-57.

Tsuda H, Arends J. Orientational micro-Raman spectroscopy on hydroxyapatite single crystals and human enamel crystallites. J Dent Res 1994;73:1703-1710.

Tsuda H, Arends J. Raman spectroscopy in dental research: a short review of recent studies. Adv Dent Res 1997;11:539-547.

Wentrup-Byrne E., Armstrong CA, Armstrong RS, Bradley MC. Fourier transform Raman microscopic mapping of the molecular components in a human tooth. J Raman Spectroscopy 1997;28:151-158.

Takagi S, Liao H, Chow LC. Effect of tooth-bound fluoride on enamel demineralization/ remineralization in vitro. Caries Res 2000; 34: 281-8.

Schougall Vilchis RJ, Hotta Y, Yamamoto K. Examination of Six Orthodontic Adhesives with Electron Microscopy, Hardness Tester and Energy Dispersive X-ray Microanalyzer. Angle Orthodontist 2008; 78: 655-61.

Chapter 4

In vivo enamel fluid movement

The aim of this study was to visualize fluid movement through dental enamel *in vivo*. Fifty permanent upper central incisors, from subjects aged 10–70 yr, and 5 permanent central just-erupted incisors, from subjects aged 6–7 yr, were included in the study. An impression was obtained by vinyl polyxiloxane, and replicas were then obtained by polyether impression material. The hydrophobic vinyl polyxiloxane material yielded a morphological image in situ of outward fluid flow through tooth enamel. The study confirmed *in vivo* that enamel is a permeable substrate, as shown by the presence of droplets on its surface, and demonstrated that age and enamel permeability are closely related. Samples from subjects of different ages showed a decreasing number and size of droplets with increasing age: freshly erupted permanent teeth showed many droplets covering the entire enamel surface. Droplets in permanent teeth were prominent along enamel perikymata.

Introduction

Enamel is not a completely dense inorganic material as its prismatic structure also contains water and organic material (Lindén, 1968; Pashley, 1996; Schellis, 1996; Ten Bosch *et al.*, 2000). Many studies on enamel have focused on caries research to explain the morphology of demineralization and remineralization (Fearnhead *et al.*, 1982; Ten Cate, 2001). Despite what is known about enamel permeability in caries, the efficacy of restorative materials and pulp–dentine–enamel interactions remain unresolved (Byers and Yoon Lin, 2003).

Throughout the last century, enamel permeability was investigated in different ways, including dye penetration (Tarbet and Forsick, 1971), diffusion of organic components (Borggrevven *et al.*, 1977), inorganic ions (Byers and Yoon Lin, 2003) or radioactive tracers (Braden *et al.*, 1971; Joyston-Bechal *et al.*, 1971), and water (Lindén *et al.*, 1968; Poole *et al.*, 1963; Shellis, 2000). Studies have applied *in vitro* and/or *in vivo* monitoring techniques, ranging from scanning electron microscopy (SEM) (Whittaker, 1982) and transmission microscopy (Poole *et al.*, 1981), to the measurement of diffusion coefficients (Lindén, 1968; Borggrevven *et al.*, 1980), electrical resistance (Hoppenbrouwers *et al.*, 1986; Wang *et al.*, 2005) or conductance (Ten Bosch *et al.*, 2000; Ie *et al.*, 1995).

The diffusion rate of cariogenic and cariostatic substances, ions and molecules through the aqueous phase in the enamel and pores plays a crucial role in the dynamics of the caries process (Van Dijk *et al.*, 1983; Featherstone, 1983; Lindén *et al.*, 1986) and fluoride treatment (Dibdin, 1993). These transport processes are significantly affected by enamel porosity and the amount of water available in the tissue (Moreno and Zaharadnik, 1973).

Fluid flowing through enamel is related to permeability: it is important to correlate enamel permeability to age and the extent of enamel demineralization, as caries susceptibility decreases with age (Kostanos and Darling, 1991). In addition, posteruptive (continuing) maturation (Fearnhead *et al.*, 1982; Kostanos and Darling, 1991) could reduce the permeability of enamel, making it clinically important to determine enamel permeability *in situ*, despite the dearth of information currently available (Lindén, 1968, Bergman and Lindén 1965; Bakhos *et al.*, 1977).

The aim of this study was to visualize fluid flow through tooth enamel *in vivo* in permanent immature and mature teeth using a replica technique and SEM observations to test the effect of enamel posteruptive maturation.

The test null hypothesis was that patient age did not affect enamel permeability.

Materials and Methods

Fifty permanent upper central incisors, with no visual signs of caries, cracks, erosion or restorations, in subjects aged 10–70 yr, and 5 permanent central just-erupted incisors, in subjects aged 6–7 yr, were selected for this study.
Four permanent teeth (premolars), extracted for orthodontic reasons from young patients (range age 20–40 yr), were used as controls. The extractions were carried out with great care to prevent any type of alteration to the enamel surface. All subjects enrolled in the study (parents for subjects aged 6–17 yr) gave their informed consent to the procedure, which was non-invasive and risk-free.

Enamel surface replica

Each tooth was brushed with a prophylactic paste for 10 s, gently washed and finally air dried for 10 s with a dental chair air syringe (Castellini, Castel Maggiore, Bologna,Italy). The method used to investigate the morphology of enamel, by detecting the presence of droplets, has been described previously (Itthagarun and Tay, 2000; Chersoni *et al.*, 2004). Immediately after enamel preparation, as previously described, an impression of the surface was made using polyvinylsiloxane impression material (Affinis ligth body; Coltene, Alstatten, Switzerland). After 4 min, the material was removed from the enamel surface, degassed for at least 48 h and later cast in polyether impression material (Permadyne Garant; 3M ESPE, St Paul, MN, USA). Samples were gold-sputtered and inspected by a scanning electron microscope (Model 5400; JEOL, Tokyo, Japan).

Evaluation and statistical analysis

High, moderate, and low numbers of droplets were evaluated at X200 magnification by two operators, randomly examining, in a double-blind manner, three different points representative of the enamel in the cervical, medium and incisal thirds of each sample.

The following visual scale was employed:

- high: more than 75% of the entire enamel surface was covered with droplets;
- moderate: less than 75% but more than 5% of the entire enamel surface was covered with droplets;
- low: less than 5% of entire enamel surface was covered with droplets.

Statistical analysis was performed by the chi-square test.

Results

Figure 1 summarizes the statistical analysis and shows the results related to healthy teeth. The percentage distribution revealed a strong relationship ($P < 0.01$) with age: data showed that all the samples from subjects aged 6–20 yr presented more than 75% of the enamel surface covered with droplets. Samples from older subjects showed a decreasing percentage: samples from the 30–50 yr age group predominantly presented a moderate (5–75%) percentage, whereas in the 50–60 yr age group the number of samples with a low (< 5%) percentage of enamel area covered with droplets increased up to the last group (age > 60 yr), where all the samples showed less than 5% of the enamel surface covered with droplets (Fig. 2A–D and 3A–D). Figure 4A–D shows details of an enamel pore, an enamel crack, and white spot lesions, respectively. The number of droplets disclosed by SEM observation confirmed that enamel is a permeable substrate. Our results demonstrated that permeability was related to age: freshly erupted permanent teeth showed more droplets covering the entire enamel surface. Samples from subjects of different ages showed a decreasing number and size of droplets.

Permanent mature teeth showed many droplets mainly localized along the perikymata, and only a few droplets were detected away from these.
In vitro testing on extracted teeth showed a similar morphology. Droplets were still present along the perikymata.

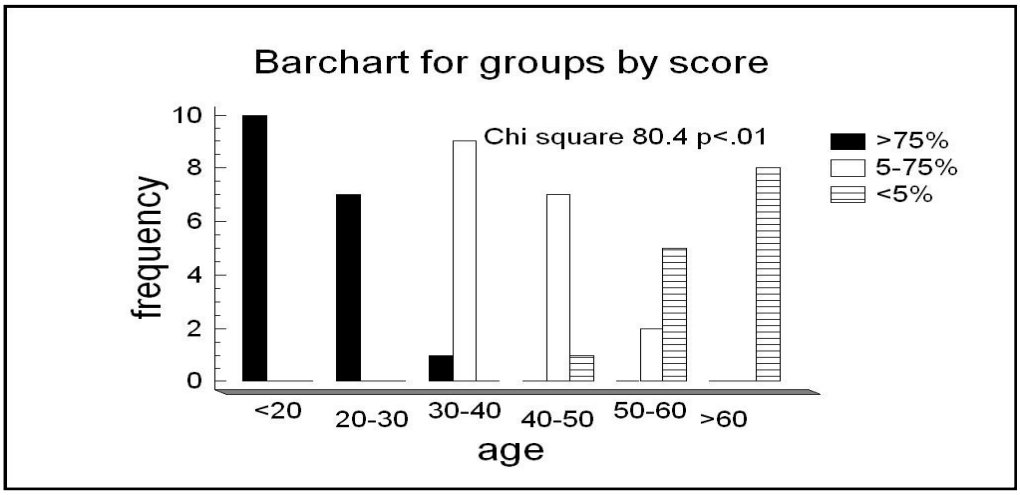


Fig. 1 Percentage distribution of enamel area covered with droplets related to age. Barchart for groups by score.

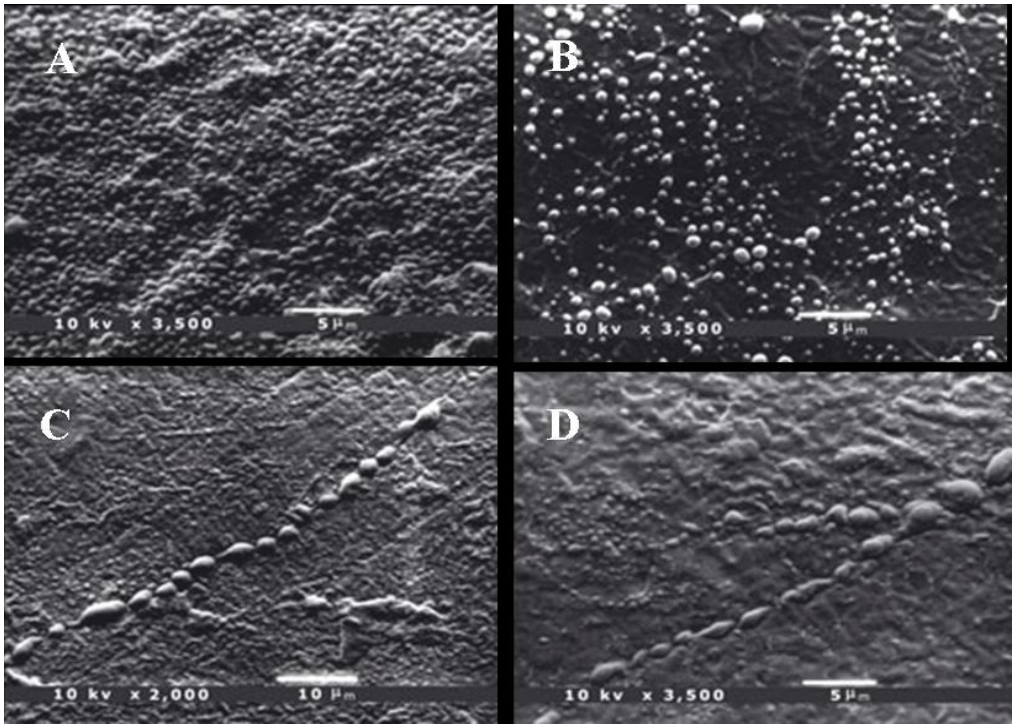


Fig. 2 The arrangement of droplets in samples according to increasing age of the subject. Scanning electron microscopy (SEM) photomicrographs of enamel from 6-yr-old (A) and 17-yr-old (B) patients, showing many more droplets on the enamel surface, covering the whole surface in several areas. Permanent teeth showed many droplets, mainly localized along the perikymata. SEM photomicrograph of 28-yr-old (C) and 30-yr-old (D) patients, showing typical droplet distribution along the perikymata. These droplets measured approximately 1 lm or less in diameter.

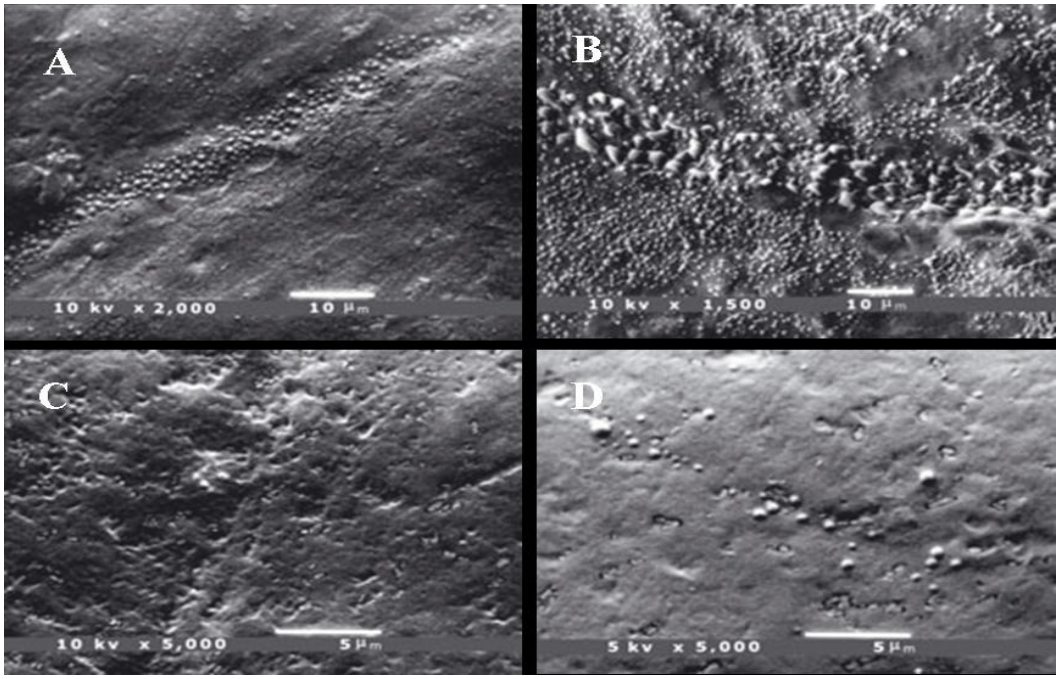


Fig. 3 Scanning electron microscopy (SEM) photomicrograph of 33-yr-old (A) and 39-yr-old (B) patients, showing the perikymata covered with droplets that appeared to be much larger than those of the adjacent enamel. SEM photomicrograph of 67-yr-old (C) and 70- yr-old (D) patients, showing only a few small droplets, probably as a result of the reduced enamel water content.

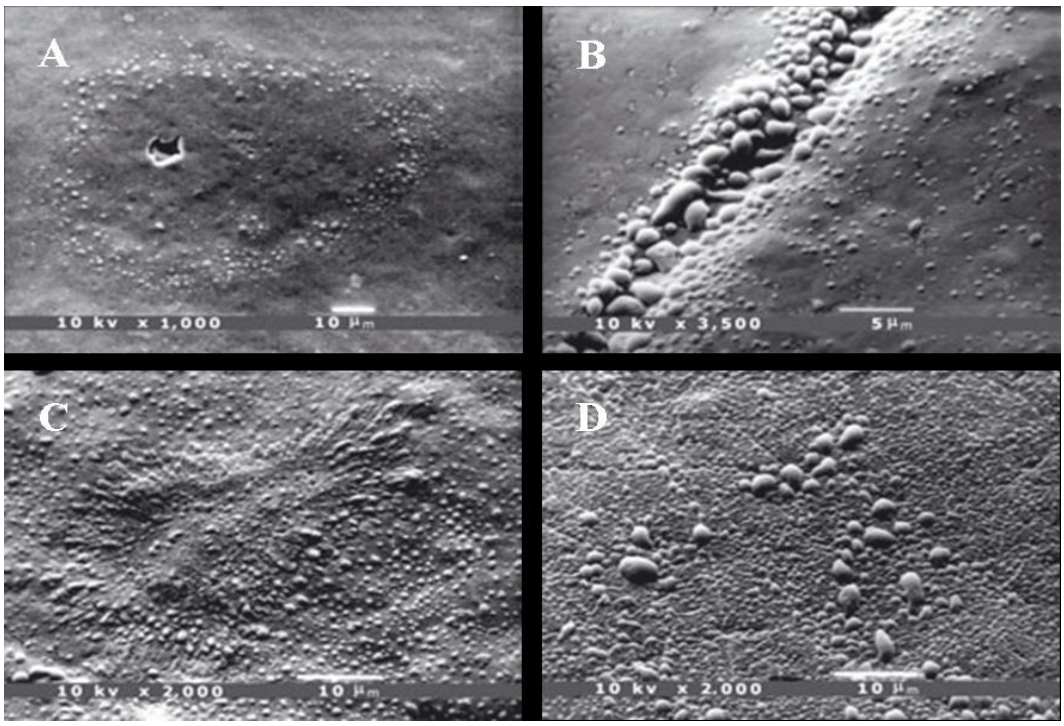


Fig. 4 Details of an enamel pore (A) and an enamel crack (B), and samples of white spot lesions (C,D).

Discussion

Enamel permeability has been demonstrated *in vivo* and *in vitro* (Lindén, 1968; Ten Bosch *et al.*, 2000; Byers and Yoon Lin, 2003). Permeability is more substantial in teeth with immature enamel and appears to require a partnership with dentine (Byers and Yoon Lin, 2003, Shellis and Dibidin, 2000). Permeability is also correlated with enamel pores, which may cause water uptake and release (Shellis, 1996). Most permeability studies recorded electrical variables, such as electrical resistance (Hoppenbrouwers *et al.*, 1986; Wang *et al.*, 2005) or conductance (Ten Bosh *et al.*, 2000; Ie *et al.*, 1995), providing an indirect evaluation of enamel thickness, mineral loss and uptake (Ten Bosch *et al.*, 2000), and enamel porosity (Flaitz *et al.*, 1986; Rock and Kidd, 1988; Huysmans *et al.*, 1995; Ricketts *et al.*, 1996). The present study yielded qualitative and quantitative findings on outward fluid flow on tooth enamel surfaces *in vivo* by means of scanning electron microscopy inspection of polyether replicas. This technique uses droplet formation to display the discharge of liquid from enamel during the setting time of the impression material, as demonstrated *in vitro* by Barnes (Barnes, 1977). The fluid forming these droplets may come from free, unbound water in blind outer enamel porosities and partly in deeper structures, as suggested by the droplet distribution on enamel surface related to age. Presumably, the mechanism of droplet formation is simply diffusion. When a water-free impression material is applied to hydrated enamel, water diffuses out of the enamel down its concentration gradient and accumulates over the pores, without wetting or spreading, on the light-bodied hydrophobic material.

Droplet formation appeared to be typical in its location on the enamel surface of permanent mature teeth, with a strong preference for the perikymata. The enamel surface of recently erupted teeth presents these and other open structures containing proteins produced during tooth development. Indeed, the enamel of freshly erupted permanent teeth showed more discharge of droplets than mature enamel. It is likely that these structures and interprismatic spaces form diffusion pathways, which alter with time in the oral cavity as a result of intermittent pH shift, traumas, and mineral deposition (Shellis and Dibdin, 2000). The results of this study appear to predict that the water content of outer enamel decreases with age.

Moreover, increasing enamel maturation and age involve a progressive localization of outward fluid flow on the enamel surface along perikymata those are anatomically correlated to deep enamel structures.

The results of this *in vivo* study, obtained with a new, non-invasive technique, could be correlated to epidemiological data on caries.

Recently erupted teeth are more prone to dental caries than teeth that have remained free from caries lesions for a few years after eruption (Ten Bosch *et al.*, 2000), as confirmed by experiments in which artificial caries lesions were produced in extracted teeth of different posteruptive ages (Ten Bosh *et al.*, 2000, Kostanos and Darling, 1991).

This may be ascribed to differences in enamel porosity dependent on intra-oral maturation, presumably caused by congestion of the pathways by deposition of calcium-phosphates in the outer layer of the tooth surface (Ten Bosh *et al.*, 2000).

Therefore, enamel surface alterations, interpreted as posteruptive maturation and, consequently, enamel permeability, are of paramount importance for caries pathogenesis. Enamel fluid could also interfere with adhesive procedures. On the other hand, clinical procedures, such as acid etching and reshaping of teeth by grinding off some of the enamel outer surface, will increase the permeability of dental enamel. Partial recovery from such damage takes several months *in vivo*, and in the meantime the tooth is more susceptible to carious decay (Kuhar *et al.*, 1999).

The replica procedure described identified the location of the pathway openings in the outer surface of tooth enamel *in vivo* by demonstrating fluid outflow, namely along the perikymata. Furthermore, the null hypothesis was rejected; the enamel of freshly erupted teeth presented higher outflow than mature enamel. We speculate that this outflow reflects both enamel permeability and, possibly, caries susceptibility. Specific obstruction of these pathways may increase caries resistance.

References

Bakhos Y, Brudevold F, Aasenden R. In vivo estimation of the permeability of surface human enamel. Arch Oral Biol 1977;22: 599–603.

Barnes IE. The adaptation of composite resins to tooth structure. Part 1. Study 1: introduction and the adaptation of composite resins to the unetched enamel cavity wall. Br Dent J 1977; 142: 122–129.

Bergman G, Lindén L. Techniques for microscopic study of enamel fluid in vivo. J Dent Res 1965; 44: 1409.

Borggreven JMPM, Driessens FCM, Van Dijk JWE. Diffusion through bovine tooth enamel as related to the water structure in its pores. Arch Oral Biol 1980; 25: 345–348.

Borggreven JMPM, Van Dijk JWE, Driessens FCM. A quantitative radiochemical study of ionic and molecular transport in bovine dental enamel. Arch Oral Biol 1977; 22:467–472.

Braden M, Duckworth R, Joyston-Bechal S. The uptake of 24Na by human dental enamel. Arch Oral Biol 1971; 16: 367–374.

Byers MR, Yoon Lin KJ. Patterns of fluoro-gold entry into rat molar enamel, dentine, and pulp. J Dent Res 2003; 82: 312–317.

Chersoni S, Suppa P, Breschi L, Ferrari M, Tay FR, Pashley DH, Prati C. Water movement in the hybrid layer after different dentin treatments. Dent Mater 2004; 20: 796–803.

Dibdin GH. The water in human dental enamel and its diffusional exchange measured by clearance of tritiated water from enamel slabs of varying thickness. Caries Res 1993; 27: 81–86.

Fearnhead RW, Kawasaki K, Inoue K. Comments on the porosity of human tooth enamel. J Dent Res 1982; 61: 1524–1530.

Featherstone JDB. Diffusion phenomena and enamel caries development. In: Guggenheim, B, ed. Cariology Today. Basel: Karger, 1983; 259–268.

Flaitz CM, Hicks MJ, Siverstone LM. Radiographic, histologic, and electronic comparison of occlusal caries: an in vitro study. Pediatr Dent 1986; 8: 24–28.

Hoppenbrouwers PMM, Scholberg HPF, Borgreven JMPM. Measurement of the permeability of dental enamel and its variation with depth using an electrochemical method. J Dent Res 1986; 65: 154–157.

Huysmans MC, Verdonschot EH, Rondel P. Electrical conductance and electrode area on sound smooth enamel in extracted teeth. Caries Res 1995; 29: 88–93.

Ie YL, Verdonschot EH, Schaeken MJ, Vant'Hof MA. Electrical conductance of fissure enamel in recently erupted molar teeth as related to caries status. Caries Res 1995; 29: 94–99.

Itthagarun A, Tay FR. Self-contamination of deep dentin by dentin fluid. Am J Dent 2000; 13: 195–200.

Joyston-Bechal S, Duckworth R, Braden M. Diffusion of radioactive ions into human dental enamel. Arch Oral Biol 1971; 16: 375–384.

Kotsanos N, Darling AI. Influence of posteruptive age of enamel on its susceptibility to artificial caries. Caries Res 1991;25: 241–250.

Kuhar M, Cevc P, Schara M, Funduk N. In vitro permeability and scanning electron microscopy study of acid-etched and ground enamel surfaces protected with dental adhesive coating. J Oral Rehabil 1999; 26: 722–730.

Lindén LA, Bjo¨rkman S, Hattab F. The diffusion in vitro of fluoride and chlorhexidine in the enamel of human deciduous and permanent teeth. Arch Oral Biol 1986; 31: 33–37.

Lindén LA. Microscopic observations of fluid flowing through enamel in vitro. Odontol Rev 1968; 19: 349–365.

Moreno EC, Zahradnik RT. The pore structure of human dental enamel. Arch Oral Biol 1973; 18: 1063–1068.

Pashley DH. Dynamics of the pulpo-dentine complex. Crit Rev Oral Biol Med 1996; 7: 104–133.

Poole DF, Tailby PW, Berry DC. The movement of water and other molecules through human enamel. Arch Oral Biol 1963; 38: 771–772.

Poole DFG, Newman HN, Dibdin GH. Structure and porosity of human cervical enamel studied by polarizing microscopy and transmission electron microscopy. Arch Oral Biol 1981; 26:977–982.

Ricketts DN, Kidd EA, Liepins PJ, Wilson RF. Histological validation of electrical resistance measurement in the diagnosis of occlusal caries. Caries Res 1996; 30: 148–155.

Rock WP, Kidd EA. The electronic detection of demineralization in occlusal fissures. Br Dent J 1988; 164: 243–247.

Shellis RP, Dibdin GH. Enamel microporosity and its functional implications. In: Teaford MF, Ferguson MJ, Smith MM, eds. Teeth: development, evolution and function. Cambridge:Cambridge University Press, 2000; 242–251.

Shellis RP. A scanning electron-microscopic study of solubility variation in human enamel and dentine. Arch Oral Biol 1996; 41: 473–484.

Shellis RP. Transport processes in enamel and dentine. In: Addy M, Embery G, Edgar WM, Orchardson R, eds. Tooth wear and sensivity. London: Martin Duniz, 2000; 19–24.

Tarbet WJ, Forsick LS. Permeability of human dental esame to acriflavine and potassium fluoride. Arch Oral Biol 1971; 16:951–961.

Ten Bosch JJ, Fennis IEY, Verdonschot EH. Time-dependent decrease and seasonal variation of the porosity of recently erupted sound dental enamel in vivo. J Dent Res 2000; 79: 1556–1559.

Ten Cate M. Remineralization of caries lesions extending into dentine. J Dent Res 2001; 80: 1407–1411.

Van Dijk JW, Borggreven JM, Driessens FC. Diffusion in mammalian tooth enamel in relation to the caries process. Arch Oral Biol 1983; 28: 591–597.

Wang J, Someya Y, Inaba D, Longbottom C, Miyazaki H. Relationship between electrical resistance measurement and microradiographic variables during remineralization of softened enamel lesions. Caries Res 2005; 39: 60–64.

Whittaker DK. Structural variations in the surface zone of human tooth enamel observed by scanning electron microscopy. Arch Oral Biol 1982; 27: 383–392.

Chapter 5

In vivo fluid release from primary enamel

A relationship between caries susceptibility and enamel permeability has been proposed for permanent teeth by detecting *in vivo* outward fluid flow on tooth enamel surface.

The aim of this study was to reveal *in vivo* the occurrence of fluid release from primary tooth enamel.

Four primary upper canines with no visual signs of caries, cracks, erosion or restorations from 6 to 10 years old subjects and two retained primary upper canines from 33 and 40 years old subjects were included in the study. The enamel surface was gently polished and air dried for 10 s. An impression was immediately obtained by vinyl polyxiloxane. Replicas were then obtained by polyether impression material, gold coated and inspected under SEM. The hydrophobic vinyl polyxiloxane material enabled to obtain *in situ* a morphological image of the presence of droplets, most likely resulting from outward fluids flow through outer enamel.

Primary enamel showed a substantive permeability as confirmed by droplets presence on its surface. Droplets distribution covered the entire enamel surface in all the samples, without any specific localization. No signs of post-eruptive maturation with changes in droplets distribution were observed in samples from adult subjects.

SEM evaluation of droplets distribution on enamel surface indicated a substantive permeability in primary teeth, accordingly with histological features, without changes during aging and suggested a strong relationship between enamel permeability and caries susceptibility.

Introduction

Primary enamel is less-mineralized (81.3-94.2 wt%), more porous, contains more organic matter, more water, and shows a greater diffusion coefficient than enamel of permanent teeth (Lindén *et al.*, 1986; Cuy *et al.*, 2002; Wang *et al.*, 2006; Lussi *et al.*, 2000). Moreover, overall mineral density is lower in the outermost layers but shows no significant differences closed to enamel-dentine junction (Wang *et al.*, 2006; Wilson and Beyond, 1989). The primary enamel is not more susceptible to erosion even though it is reported to be statistically significantly softer and less elastic and was reported that dissolves considerably faster than permanent enamel (Lussi *et al.*, 200; Lippert *et al.*, 2004).

The structured nature of enamel allows transport of ions, molecules and water (Lindén, 1968; Lindén *et al.*, 1986; Shellis, 1996; Ten Bosh *et al.*, 2000). Fluid flowing through enamel is related to its water content and its permeability. As clinical studies showed caries formation and progression to be faster in primary than in permanent teeth (Kostanos and Darling, 1991; Sønju Clasen *et al.*, 1997; Issa *et al.*, 2003), an eventual correlation between caries susceptibility and fluid flow might be existing. Moreover enamel fluid flow has been correlated with post-eruptive maturation as permanent teeth showed a decreasing permeability with age (Bertacci *et al.*, 2007).

The aim of this study was to visualize *in vivo* fluid flow through tooth enamel in primary teeth with a replica technique and SEM observations to investigate the effects of intraoral staying on primary enamel.

Materials and Methods

Patient and tooth selection

Four primary upper canines with no visual signs of caries, cracks, erosion or restorations from six to 10 years old subjects and 2 retained upper primary canines from 2 adult subjects (aged 33 and 40 years) were included in this study. The study has been conducted in full accordance with ethical principles of the World Medical Association Declaration of Helsinki. Parents for all subjects aged 6 to 10 years old included in the study, gave written consensus to the procedure that was non-invasive, and did not create any risk for the patients.

Enamel treatment procedures

Each tooth was brushed with a prophylactic paste for 10 s, gently washed and finally air dried for 10 s with a dental chair air syringe (Castellini, Italy). The method used to investigate the morphology of enamel detecting the presence of droplets was previously described (Bertacci *et al.*, 2007).

Immediately after enamel preparation as previously described, an impression of the surface was made using a polyvinilsiloxane impression material (Affinis® light body COLTENE, Alstatten, Switzerland). After 4 min, the material was removed from the enamel surface and was degassed for 48 h and poured out in polyether impression material (Permadyne Garant®, 3M ESPE, St. Paul, MN, USA).

Samples were gold-sputtered and inspected by scanning electron microscope (SEM, JEOL, Model 5400, Tokyo, Japan).

Evaluation and statistical analysis

The evaluations on presence of droplets were performed at x 2000 and x 3500 magnification by two double blind operators examining randomly for each sample three different points representative of the enamel in the cervical, medium and incisal third.

Results

SEM replicas observation showed that all enamel surfaces release equal droplet formation. All the samples from patients aged 6-10 yr showed many droplets that covered the entire enamel surface (Fig 1). Enamel of primary teeth from adult subjects showed no difference in droplets distribution: droplets covered the entire surface without any specific localization (Fig.2).

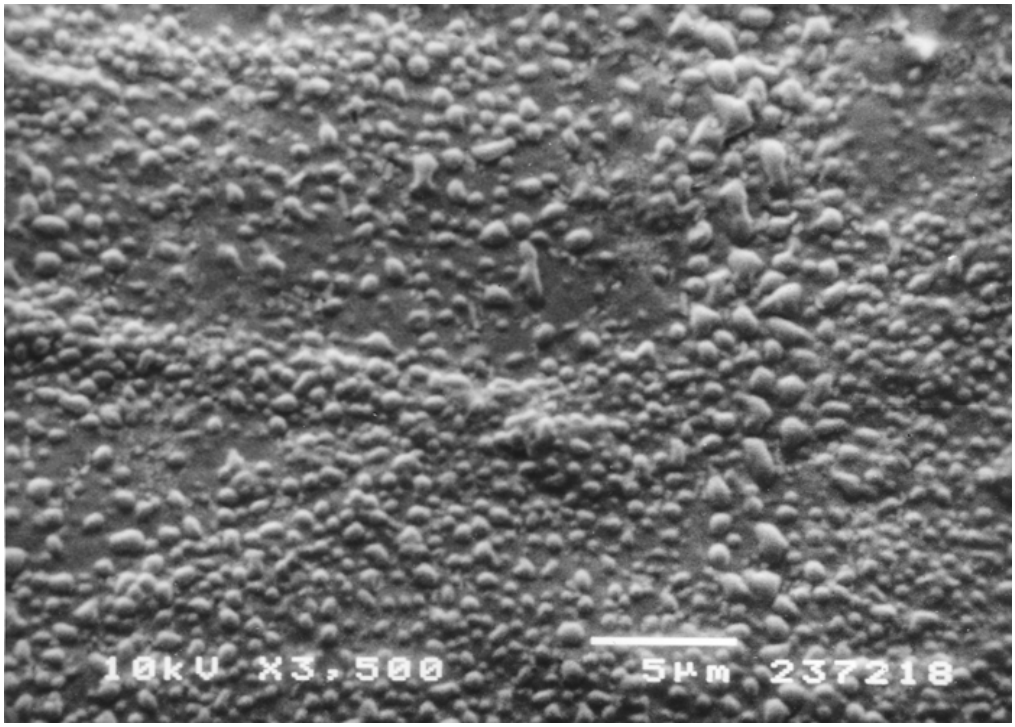


Fig. 1 SEM photomicrograph of 10-yr-old patient upper canine shows the typical droplets arrangement. Many droplets cover the whole enamel surface.

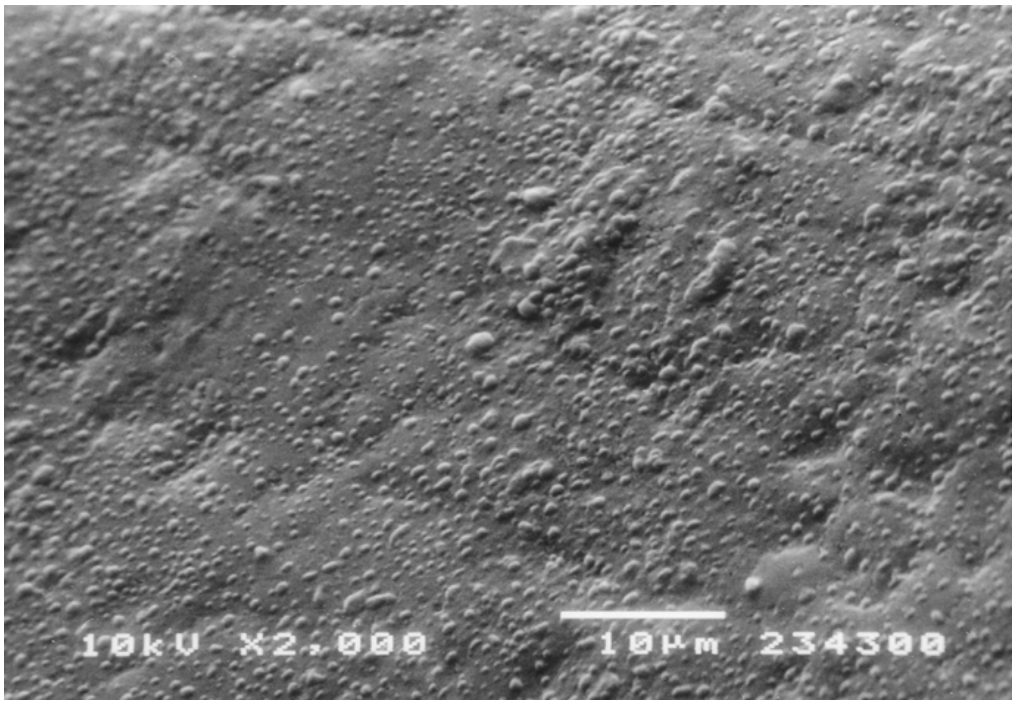


Fig. 2 SEM photomicrograph of 33-yr-old patient enamel surface. According to increasing subject's age no difference in droplets distribution were observed.

Discussion

The aim of this study was to visualize *in vivo* outward fluid flow from tooth enamel surfaces in order to predict susceptibility of the tissue for diverse influences such as caries, and intra-oral environment and to correlate these influences with enamel permeability.

The composition and structure of enamel tissues are inhomogeneous and change with post-eruptive maturation and also with caries progression (Wang *et al.*, 2006). Primary and permanent enamel showed different features that arise from differences in structure (Wang *et al.*, 2006; Sønju Clasen *et al.*, 1997; Shellis, 1984). Prism-junction density and volume fraction of inter-prismatic enamel are significantly greater in primary enamel than in fully matured enamel, whereas the degree of microcrystal arrangement is lower (Shellis, 1984; Wilson and Beynon, 1989). It has been demonstrated that matrix proteins are essentially removed during mature enamel formation (Wang *et al.*, 2006). Changes in ion transport, induced by local diffusion coefficients with changing porosity and the relative mineral contents could set the accessible pore volume in partially demineralised enamel and influence the distribution of mineral loss (Wang *et al.*, 2006; Dowker *et al.*, 2003).

The histological features of primary enamel are related with permeability and both of them are involved in caries pathogenesis.

Regarding permanent teeth, it has been demonstrated that enamel surface of recently erupted teeth showed more discharge of droplets than mature enamel presumably due to the presence of open structures containing proteins produced during tooth development that presumably form diffusion pathways as inter-crystalline spaces that will alter with dwell time in the oral cavity predominantly due to mineral deposition (Bertacci *et al.*, 2007; Tjäderane, 2007).

Primary enamel showed a great droplet forming (permeability) that remained unchanged despite the prolonged staying in the oral cavity, as the exam of primary teeth in adult subjects demonstrated. Primary enamel appeared to be not affected by surface alterations, such as deposition of calcium-phosphates in the outer layer of the tooth surface, due to intra-oral maturation. This could be ascribed to differences in enamel structure and to a metabolic inertia that prevent the mineral deposition and the exchange with salivary ions.

The results of this *in vivo* study confirmed that primary teeth are more permeable than permanent mature teeth as well studies on caries formation showed that primary enamel have a great susceptibility to demineralization than permanent teeth (Wang *et al.*, 2006)

This study suggested that caries susceptibility and enamel permeability are mutually related, and that enamel permeability depends on surface alterations essentially affecting the outer layer of enamel.

This study confirmed the relationship between caries susceptibility and enamel permeability in primary teeth. These innovative results open the way to further study about caries prevention and therapy.

References

Bertacci A, Chersoni S, Davidson CL, Prati C. In vivo enamel fluid movement. Eur J Oral Sci 2007; 115: 169-173..

Cuy JL, Mann AB, Livi KJ, Teaford MF, Weihs TP. Nanoindentation mapping of the mechanical properties of human molar tooth enamel. Arch Oral Biol 2002; 47: 281-291.

Dowker SE, Elliott JC, Davis GR, Wassif HS. Longitudinal study of the three-dimensional development of subsurface enamel lesions during in vitro demineralization. Caries Res 2003; 37: 237-245.

Issa AI, Preston KP, Preston AJ, Toumba KJ, Duggal MS. A study investigatine the formation of artificial sub-surface enamel caries-like lesions in deciduous and permanet teeth in the presence and absence of fluoride. Arch Oral Biol 2003; 48: 567-571.

Kotsanos N, Darling AI. Influence of posteruptive age of enamel on its susceptibility to artificial caries. Caries Res 1991; 25: 241-50.

Lindén LÅ, Bjorkman S, Hattab F. The diffusion in vitro of fluoride and chlorhexidine in the enamel of human deciduous and permanent teeth. Arch Oral Biol 1986; 31: 33–37.

Lindén LÅ. Microscopic observations of fluid flowing through enamel in vitro. Odontol Revy 1968; 19: 349-345.

Lippert F, Parker DM, Jandt KD. Susceptibility of deciduous and permanent enamel to dietary acid-induced erosion studied with atomic force microscopy nanoindentation. Eur J Oral Sci 2004; 112:61-66.

Lussi A, Kohler N, Zero D, Schaffner M, Megert B.A comparison of the erosive potential of different beverages in primary and permanent teeth using an in vitro model. Eur J Oral Sci 2000; 108: 110–114.

Schellis RP. Relationship between human enamel structure and the formation of caries-like lesions in vitro. Arch Oral Biol 1984; 29: 975-981.

Shellis RP. A scanning electron-microscopic study of solubility variation in human enamel and dentine. Arch Oral Biol 1996; 41: 473-484.

Sønju Clasen AB, Øgaard B, Duschner H, Ruben J, Arends J, Sønju T. Caries development in fluoridated and non fluoridated deciduous and permanent enamel in situ examined by microradiography and confocal laser scanning microscopy. Adv Dent Res 1997; 11: 442-7.

Ten Bosch JJ, Fennis-IE Y, Verdonschot EH. Time-dependent decrease and seasonal variation of the porosity of recently erupted sound dental enamel in vivo. J Dent Res 2000; 79: 1556-1559.

Tjäderhane L. The origin of enamel fluid. Eur J Oral Sci 2007;115:522-3.

Wang LJ, Tang R, Bonstein T, Bush P, Nancollas GH. Enamel demineralization in primary and permanent teeth. J Dent Res 2006; 85: 359-363.

Wilson PR, Beynon AD. Mineralization differences between human deciduous and permanent enamel measured by quantitative microradiography. Arch Oral Biol 1989; 34: 85–88.

Wilson PR, Beynon AD. Mineralization differences between human deciduous and permanent enamel measured by quantitative microradiography. Arch Oral Biol 1989; 34:85-88.

Chapter 6

Fluoride: in vivo effects on enamel permeability

The objective of this *in vivo* study was to evaluate the effects of topical fluoride application on enamel permeability to water.

Impressions of buccal enamel were obtained before and after a 2 min application of fluoride (NaF and APF, acidulate phosphate fluoride). Baseline replicas showed the presence of water droplets on enamel surfaces. Immediately after fluoride application CaF_2 globules and no water droplets were observable. After professional brushing and CaF_2 globules removal, water droplets were present again within 1 hour for NaF while APF treated samples still showed no droplets after 7 days. Control groups demonstrated that CaF_2-like globules were formed *in vivo* and that could be removed by professional toothbrushing, sonically and chemically by KOH. Fluoride treatments temporarily reduced enamel water permeability when CaF_2-like globules were removed. The *in vivo* permanence of decreased enamel permeability after CaF2 globules removal has been demonstrated for 1 h for NaF treated teeth and for at least 7 days for APF treated teeth. The caries-preventing action of fluoride application may be due, in part, to its ability to decrease enamel water permeability. CaF_2 like-globules seem to be indirectly involved in enamel protection over time maintaining low permeability.

Introduction

Fluoride-tooth interactions following topical fluoride application in mature permanent teeth involves enamel surfaces, oral fluids, and fluids flowing through dental hard tissues (ten Cate and Featherstone, 1991).

It has been demonstrated that most of the cariostatic effect of topical fluoride can not be attributed to the incorporation of fluoride in the hydroxyapatite crystal lattice (Øgaard *et al.*, 1988), despite a recent study that showed an increase of fluoride concentration on the treated enamel surface (Jeng *et al.*, 2008). CaF_2 globular complexes, also called phosphate contaminated calcium fluoride (Christoffersen *et al.*, 1988) are a major reaction product following topical treatment of dental hard tissues coating dental enamel (Nelson et al., 1984; Dijkman *et al.*,1988; Cruz *et al.*, 1992; Øgaard, 2001). Previous stuidies reported that calcium fluoride-like deposits is affected by pH (Saxegaard and Rølla, 1988), by the type of fluoride applied, and by the length of application (Nelson *et al.*,1984; Dijkman *et al.*, 1988). It has been suggested that CaF_2 acts as a pH-controlled reservoir of fluoride due to high solubility and release of a large amount of fluoride during dissolution (Nelson *et al.*, 1983; Øgaard, 2001; Jeng *et al.*, 2008). However, the deposited surface fluoride coating is not permanent and, depending on the topical fluoride agent and oral conditions, most of the fluoride can be lost over a few days to a few weeks (Brudevold *et al.*, 1967; Dijkman *et al.*, 1983, Caslavaska *et al.*, 1991). The effect of these CaF_2 globules in fluoride preventing action is still unclear. Few *in vivo* studies has been carried out to evaluate the retention of CaF_2 globules and the effect of fluoride treatment on human enamel physical properties and only one evaluated the effect on permeability (Brewer et. al, 1956; Melleberg et al., 1977; Arends et al., 1988; Øgaard et al., 1996).

The aim of this study was to evaluate *in vivo* changes in enamel water permeability produced by topical fluoride application on mature permanent enamel by SEM inspection of sequential replicas made *in vivo*. The tested null hypothesis was that enamel water permeability was not affected by fluoride treatment.

Materials and Methods

Enamel Preaparation

Forty permanent sound upper central incisors from 20 six subjects aged 25–40 yr were selected. All subjects enrolled in the study gave their informed consent to the procedure, which was non-invasive and risk-free. All the subjects did not use any fluoridated product at least in the previous week. Each tooth was brushed with a prophylactic brush (Prophy minicups, Westpoint-Perident, Firenze, Italy) mounted on a rotary micro-motor handpiece (4000 rpm) for 30 s and air-dried for 10 s.

Two different fluoride treatments were examined: two minutes rines with 0.2% NaF at neutral pH (Oral-B Fluorinse®, Procter & Gamble, Cincinnati, Usa) and two minutes of 1.23% acidulated phosphate fluoride gel application (APF gel, Dental Medical, Conegliano, Italy).

Baseline impressions of buccal enamel of both central incisor of each subject were made using polyvinylsiloxane (Affinis light body). Patients group 1 (n=10) were given 10 mL of Oral-B Fluorinse® and asked to agitate the mouthrinse in their mouth for 2 min. After expectoration, the right central incisor was brushed, washed and air-dried, while the left central incisor was only washed and air-dried before taking second impressions. Then the patients were dismissed and asked to return in 1 hr. After they returned, left incisors were brushed and air-dried while right incisors were only washed and air dried before taking third impressions. Patients of group 2 (n=10) applied 2 min of 1.23% acidulated phosphate fluoride on buccal surface of both central incisors. After water rinse and expectoration, the same time-impression sequence of the first experimental group was carried out.

Control Groups

The first *in vivo* control group consisted of two upper incisors that separately received both topical fluoride treatments, as previously described, followed by 30 s of water-spray sonication (Castellini, Castel Maggiore, Bologna, Italy) with ultrasonic tips (EMS, Geneva, Switzerland) before taking an impression. Likewise the second *in vivo* control group, consisted of tow upper incisors, was treated with topical fluoride application. After taking baseline impression of the two upper incisors, the incisor enamel surface was adjusted to be parallel to the floor and a 2 μL droplet of 1 N KOH was placed on one-half of the fluoride-treated surface to determine if such treatment (Caslavska *et al.*, 1975) could extract the surface globules. After 5 min, the drop of KOH was carefully removed by absorbent paper and the surface rinsed with water, air-dried, and then subjected to a

second impression. Additional samples (n=4) were obtained from 2 upper central incisors fluoride-treated as previously described and brushed with an electric toothbrush (Oral B triumph, Oral B, Procter & Gamble, Cincinnati, Usa) and from 2 APF treated incisors impressed 24 h and a week after treatment (application and professional toothbrushing).

Enamel Surface Replica

During each impression, the material was allowed to set on the tooth for 4 min. Then the material was removed from the enamel surface, degassed for at least 48 h and later cast in polyether impression material (Permadyne Garant; 3M ESPE, St Paul, MN, USA). After separation, the casts were gold-sputtered and inspected by scanning electron microscope (Model 5400; JEOL, Tokyo, Japan).

Results

Baseline replicas of incisors before fluoride-treatment showed the presence of water droplets on enamel surfaces that represent water outflow from enamel during the 4 min setting time of the material (Fig, 1). The second replica of the left incisor (unbrushed group) after fluoride treatment revealed extensive deposition of globular aggregates (well recognized as CaF_2) (Figs. 2A-C) except in the approximal area where a single bristle of the toothbrush partially removed CaF_2 gluobules (Fig. 2D). The second replica of the right tooth (brushed group) did not show any water droplets or globular aggregates (Figs. 3A-B). In fluoride-treated enamel surfaces that were not immediately brushed, when the patients returned after 1 h for the third impression of left tooth, replicas failed to reveal either water droplets or CaF_2 globules (Figs. 3C-D) on the enamel surfaces.

In contrast, when impressions were taken of the right incisor 1 h after fluoride treatment and immediate brushing, the NaF fluoride-treated surface showed water droplets again (Figs. 4 A-B).

Different results were obtained after 1.23% acidulated fluoride treatment (Figs 5 A-D). The permeability remained low from 24 h until a week after the removal of CaF_2 globules by professional tooth brushing.

When fluoride-treated enamel was treated with a droplet of KOH, of the strong base removed the CaF_2 globules, while the surrounding area remained covered with such globules (Figs. 6A-B). Sonicated teeth that had been treated with topical fluoride applica-

tion showed no CaF_2 aggregates on enamel surface and no water droplets (images not shown).

Teeth brushed with the electric toothbrush after fluoride treatment still showed CaF_2 globules (Fig. 7).

Thus, topical fluoride treatment temporarily reduced enamel permeability and produced deposition of globular aggregates that were easily removed by brushing, ultrasonical or chemical extraction with KOH. In the presence of CaF_2 globules in the unbrushed incisors, the enamel permeability remained low after 1 h. After removal of CaF_2 globules by toothbrushing, the water permeability remained very low initially, but returned to pre-treatment levels after 1 hr in NaF treated samples while remained low until 7 days, for 1.23% acidulated fluoride treatment.

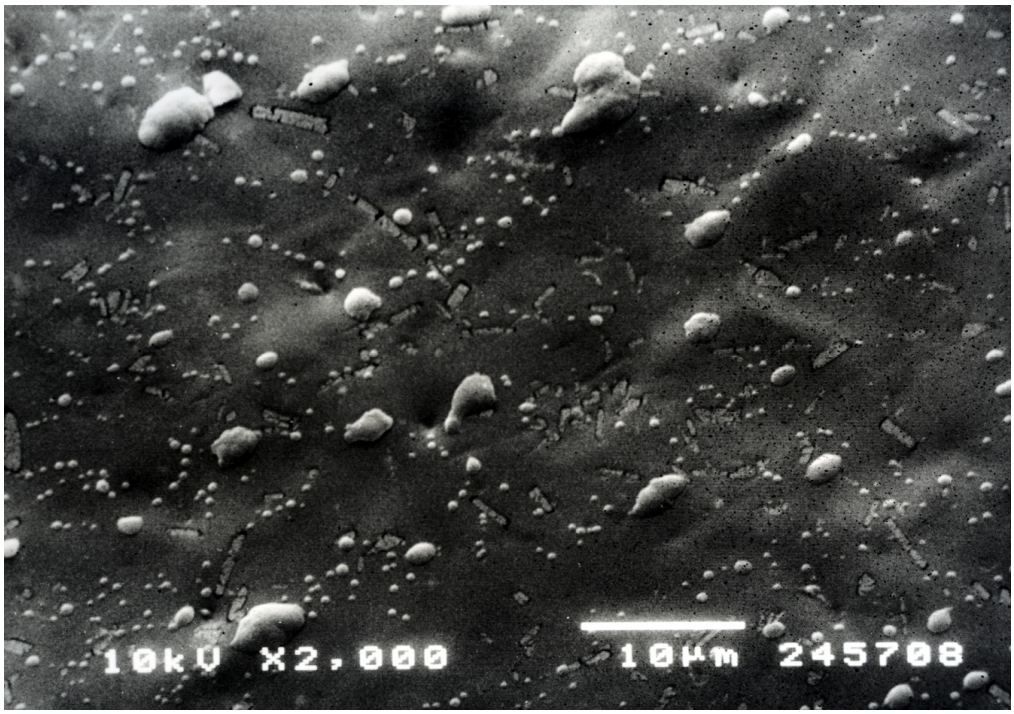


Fig. 1 Scanning electron microscopy (SEM) photomicrographs of baseline enamel control replicas from 30-yr-old (1) patient, showing many water droplets, mainly localized along the interprismatic (perikymata) enamel. These droplets measured approximately 1 µm or less in diameter. No topical fluoride was applied in the control condition.

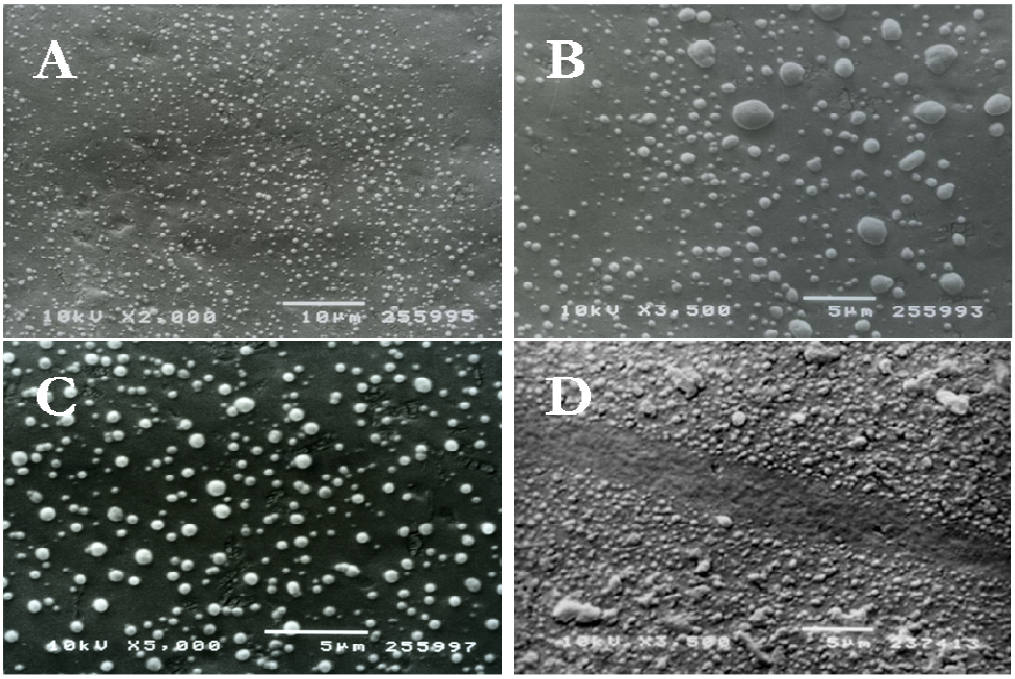


Fig. 2 Scanning electron microscopy (SEM) photomicrographs (A,B,C) of unbrushed fluoride-treated enamel replicas showing a deposition of globular aggregates that ranged in size from 0.4-4 µm in diameter. No fluid droplets are observable. The globules are CaF_2 aggregates. Scanning electron microscopy (SEM) photomicrograph (D) of approximal enamel replica showing the detail of partially removed CaF_2 globules by a single bristle of the brush.

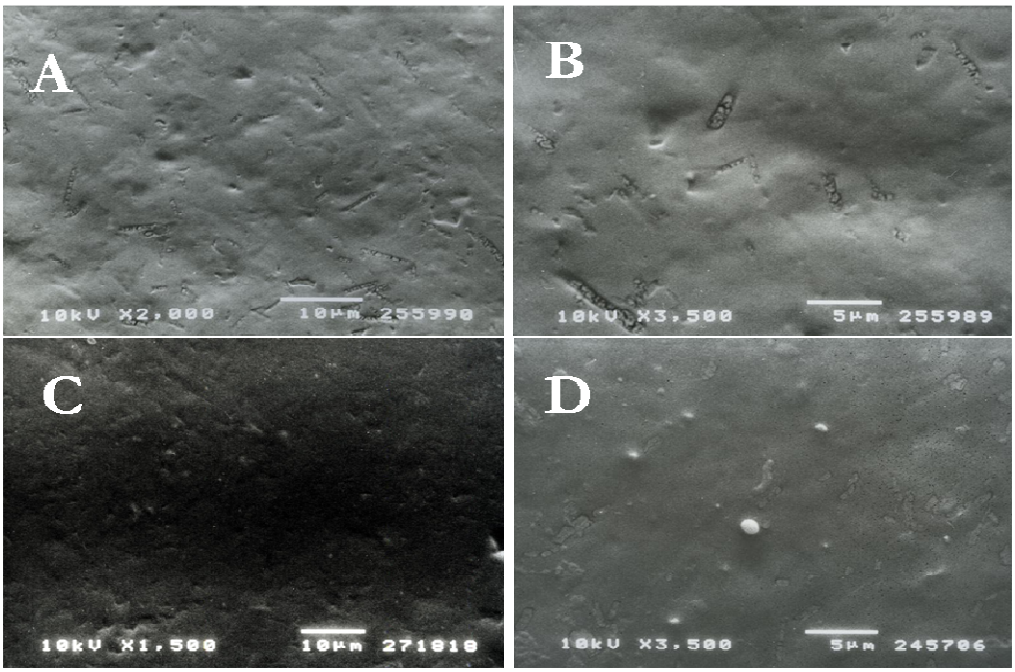


Fig. 3 Scanning electron microscopy (SEM) photomicrographs (A,B) of fluoride-treated and brushed enamel replicas showing no CaF_2 aggregates and no water droplets were observable. Scanning electron microscopy (SEM) photomicrograph (C) of fluoride-treated enamel brushed after 1 h replicas showing no CaF_2 aggregates and no droplets observable. Only very few CaF_2 aggregates were present in a smple (D).

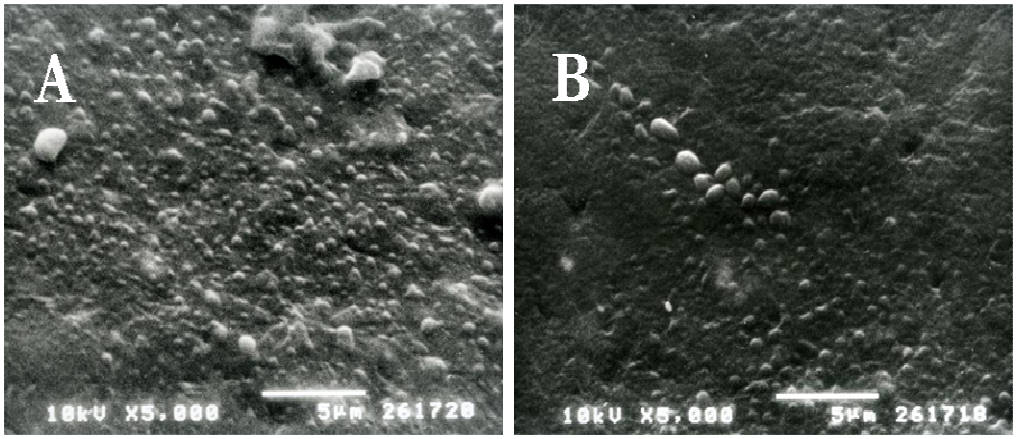


Fig. 4 Scanning electron microscopy (SEM) photomicrographs of enamel replicas after fluoride treatment and immediate toothbrushing 1h later showing many fluid droplets observable as baselines replicas showed. The patient was dismissed for 1 h. Upon their return, the tooth was rinsed and air-dried and impressions replicas were made. The previously impermeable enamel was now permeable to water and formed water droplets.

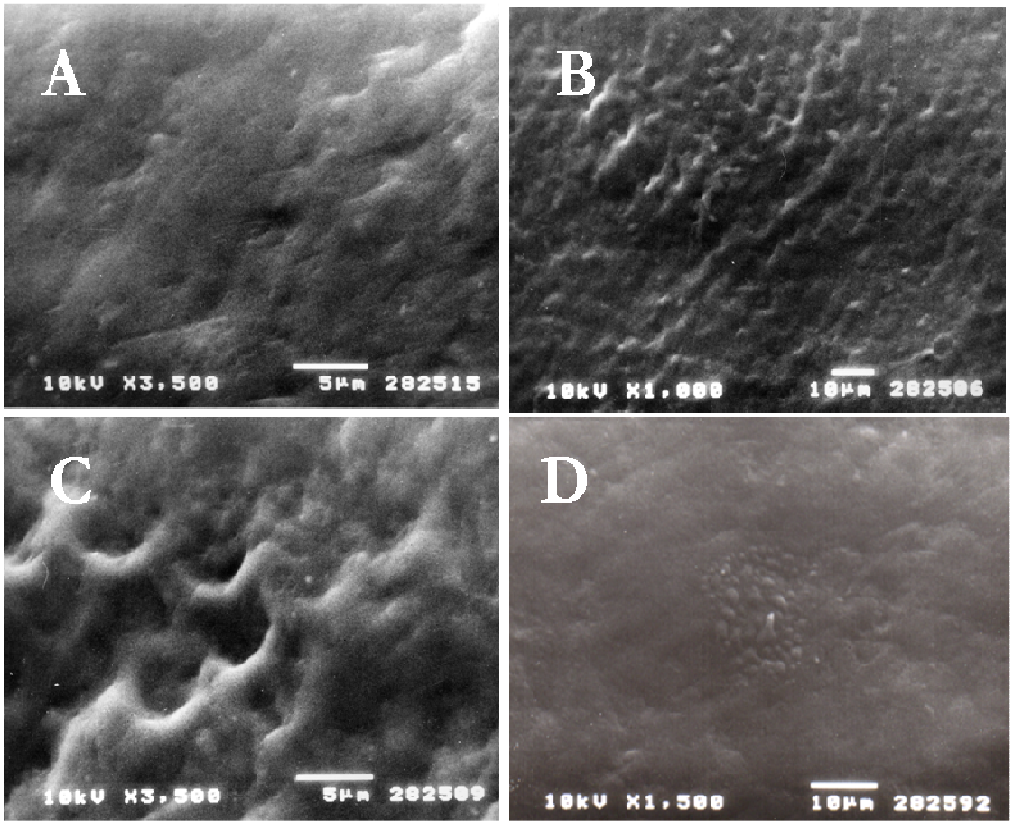


Fig. 5 SEM photomicrographs of APF treated teeth. The permeability remained low from 1h, 24 h until a week (D) after the removal of CaF_2 globules by professional tooth brushing.

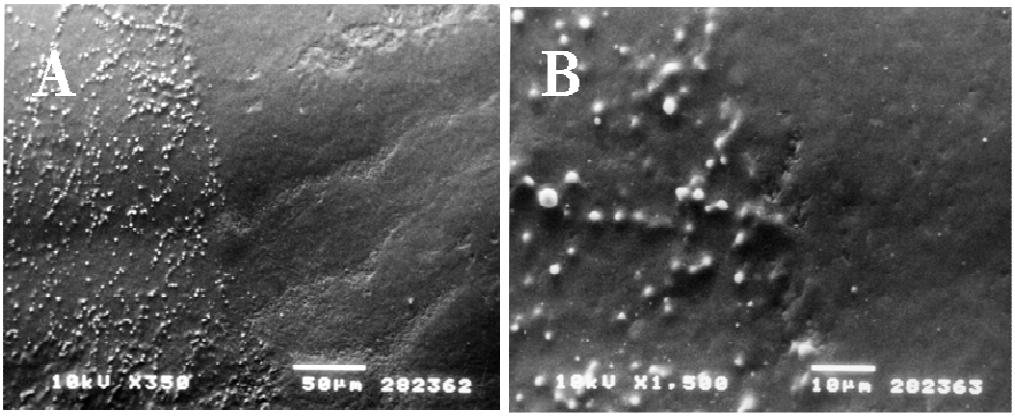


Fig. 6 Low power (350X) micrograph (5A) of replicas of human incisor enamel after fluoride treatment. After rinsing and air-drying a tiny drop of 1 N, KOH was placed in the center of the fluoride-treated area, and left in place for 5 min. The KOH was then removed with absorbent paper, rinsed with water and air-dried. Then an impression was taken and a replica made.
Note the globules of CaF_2 on the left side of Fig. 5A, and their absence on the right side that was treated with KOH. Fig. 5B shows a higher power (1500X) view of the CaF_2 globules showing that their diameters were 1-3 μm.

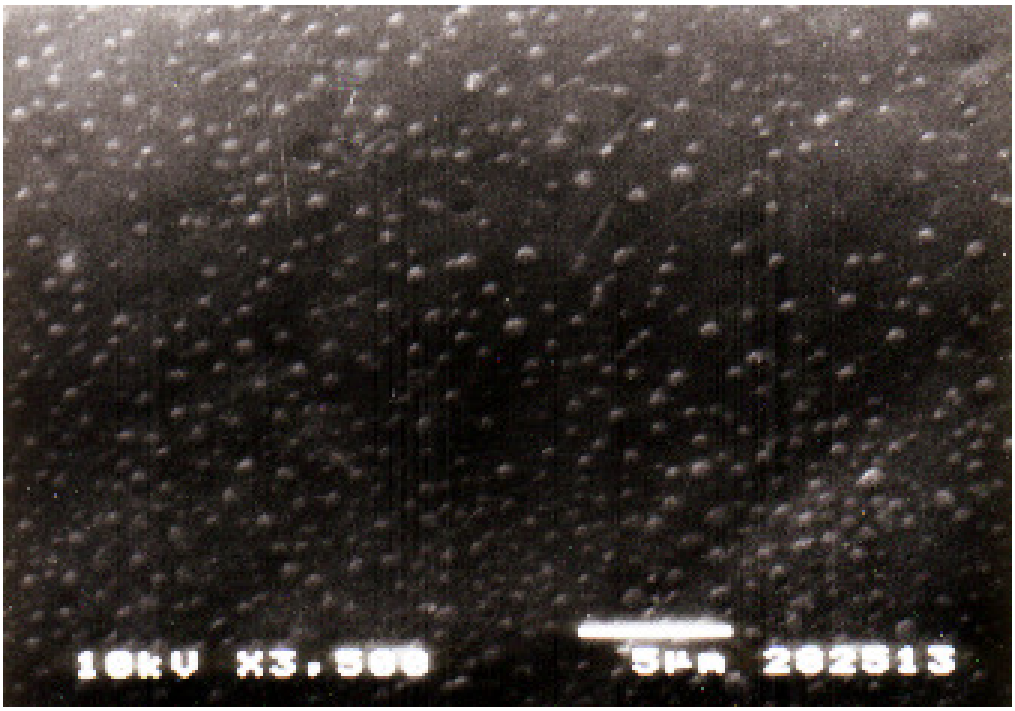


Fig. 7 SEM photomicrograph of a fluoride treated tooth after brushing with an electric tooth brush: CaF_2 gluobules were still present on enamel surfaces.

Discussion

When enamel surfaces were treated with 0.2% NaF mouthrinse and with 1.23% acidulate fluoride gel the CaF_2 globules deposited on the surface blocked outward water movement through interprismatic enamel and this result requires rejection of the test null hypothesis that enamel water permeability was not affected by fluoride treatment.

This globular surface material often becomes coated with phosphates or proteins and is regarded as being relatively insoluble (Øgaard, 1999), but is reported to be lost from enamel surface over time, ranging from days to weeks (Dijkman *et al.*,1983; Dijkman and Arends,1988; Jeng *et al*, 2008) as result of daily brushing and mastication (Jeng *et al.*, 2008). Therefore, some researchers have argued that these deposits provide no more than a limited protective capability (Jeng *et al.*, 2008). The present study confirmed that these surface deposits are easily removed mechanically and cannot be responsible of the long-term effectiveness of topical fluoride treatment. It has been suggested that CaF_2 is formed not only on surfaces but also to some extent in the underlying enamel (Rølla, 1988; Øgaard, 2001; Jeng *et al.*, 2008), but if TEM examination did not reveal any recrystallized layer of fluorapatite beneath the CaF_2 layer (Nelson *et al.*, 1983; White and Nancollas, 1990), on the other hand a recent study showed that the underlying enamel surface showed a 22% increase in the fluoride content after removal of surface CaF_2 using KOH compared with control enamel (Jeng *et al.*, 2008). Regardless of these consideration it has been demonstrated that only KOH-soluble fluoride inhibited caries lesion development significantly (Øgaard *et al.*, 1990), while the increase in KOH-insoluble fluoride (firmly-bound or apatitically-incorporated fluoride) had apparently no clinical significance and that even pure fluorapatite has been shown to have a limited cariostatic potential in intra-oral models (Øgaard *et al.*,1988).

In the present study, fluoride-containing globules were removed *in vivo* by a sonic scaler, by professional toothbrushing and by KOH. We speculate that although surface CaF_2 deposits are mechanically removable, their anti-caries action could be ascribed to transient decreases in subsurface enamel permeability and increases in plaque CaF_2. The fact that enamel that had been exposed to the fluoride and immediately brushed had neither CaF_2 globules nor water droplets meant that treatment of enamel reduced the water-permeability even in the absence of CaF_2 globules.

It is likely that there were relatively insoluble salts of fluoride in subsurface interprismatic porosities that blocked water movement but that solubilised with 1 h for NaF

treatment. On the other hand 1.23% acidulated fluoride reduced enamel permeability for 24 hour until 7 days.

The effectiveness of a topical fluoride agent may be related to the changes it produces in enamel permeability and these changes could be related to the pH of the fluoride solution, with a more prolonged effect in permeability reduction demonstrated for APF gel in this study.

Calcium fluoride deposition may be the outward manifestation of relatively insoluble subsurface blockage of enamel surface that may block enamel pores (ten Cate and Featherstone 1991) and other diffusion pathways (Nelson *et al.*, 1984; Shellis and Dibdin 2000) probably also realated to the pH of the fluoride solution as unpublished results on acid treatment on sound enamel suggest.

Since there is an outward fluid movement from the tooth to the enamel surface, it is likely that water-soluble materials could move from the surface into the enamel. If this material includes hydrogen ions, it might promote caries development. It has been speculated that specific occlusion of the pathways of fluid outflow may increase caries resistance (Bertacci *et al.*, 2007).

The major effect of topical fluoride treatment is the formation of CaF_2 on the enamel surface or in decalcified enamel lesions (Øgaard *et al.*, 1990; Rølla and Saxegaard 1990).

The results of this study showed that the deposition of CaF_2 globular aggregates on enamel surfaces is associated with a transient decrease of enamel permeability and that mechanical removal of these globules by ultrasonication and professional toothbrushing allows the return of baseline water permeability within 1 for NaF h and much longer (at least 7 days) for acidulate fluoride treatment.

However, teeth that were not brushed with professional tooth-brush, showed very low enamel permeability for at least 1 h. So professional toothbrushing could reduce the efficacy of fluoride treatment by removing CaF_2 globules deposited, that remains only in the approximal areas where the bristles do not reach.

During the carious process, demineralization occurs on and under enamel surfaces and involves ionic exchange between the lesion and the enamel surface. The cariostatic effect of fluoride could achieve by blocking these demineralizating episodes, by transient reductions in enamel permeability, rather than the reinforcing of the enamel surface, in as much as the deposited layer is potentially lost in a short time. Previous studies have shown that acid etching of enamel caries-like lesions enhances reminaralization *in vitro* (Yamazaki and Margolis, 2008). It has been proposed that organic acid buffers contain-

ing fluoride can simultaneously promote enamel dissolution and the deposition of fluorapatite-like phases within enamel (Yamazaki and Margolis, 2008).

The caries-preventing action of fluoride application to enamel may be due to its ability to modify the permeability of enamel and associated ionic and osmotic fluid fluxes. Calcium-fluoride like-globules seem to be indirectly involved in enamel protection over time since their removal did not immediately restore enamel permeability to outward fluid flow. The presence of these globular deposits of CaF_2 may protect underlying fluoride by delaying its solubilization.

Recently a three layer structure model of enamel treated with amine fluorides (pH=4) has beeen proposed (Gerth *et al.*, 2007). The authors assumed that top surface CaF_2 layer acts as a fluoride reservoir and covered a layer of $Ca(OH)_2$ (Gerth *et al.*, 2007). This $Ca(OH)_2$ layer could be responsible for reduced enamel demineralization and as our results on permeability allowed to speculate.

A similar layer or a calcium phosphate acid induced could be responsible for decreasing enamel permeability.

Enamel permeability could be involved in caries pathogenesis and the caries prevention action of fluoride could be ascribed to both decreasing enamel permeability and to promoting remineralization of demineralized enamel.

The null hypothesis was rejected because fluoride is able to temporarily block enamel permeability.

These results suggest a new mechanism of fluoride action, opening the way to further studies. If the caries preventive action of fluoride depends on decreasing enamel permeability, new substances with the same effects but with longer retention could be introduced for caries prevention. The proposed mechanism of fluoride caries-preventing action could explain the evidence that the primary effect of topical fluoride is post-eruptive (Cury and Tenuta, 2008).

References

Arends J, Reintsma H, Dijkman TG. "Calcium fluoride-like" material formed in partially demineralised human enamel in vivo owing the cation to fluoridated toothpastes. Acta Odontol Scand 1988;46:347-353.

Bertacci A, Chersoni S, Davidson CL, Prati C. In vivo enamel fluid movement. Eur J Oral Sci 2007;115:169–173.

Brewer HE, Muhler C, Fischer RB. Effects of fluorides on the permeability of human dental enamel to inorganic ions. J Dent Res 1956;35:59-64.

Brudevold F, MCCann HG, Nilsson R, Richardson B, Coklica V. The chemistry of caries inhibition, problems and challenger in topical treatments. J Dent Res 1967;46:37-45.

Caslavaska V, Gron P, Kent RL, Joshipura K, DePaola PF. Caf_2 in enamel biopsies 6 weeks and 18 months after fluoride treatment. Caries Res 1991;25:21-6.

Caslavska V, Moreno EC, Brudevold F. Determination of calcium fluoride formed in vitro exposure of human enamel to fluoride solutions. Arch Oral Biol 1975:20:333-9.

Christoffersen J, Christoffersen MR, Kibalczyc W, PerdokWG. Kinetics of dissolution and growth of calcium fluoride and effects of phosphate. Acta Odontol Scand 1988; 46:325-336.

Cruz R, Øgaard B, Rølla G. Acquisition of alkali-soluble fluoride by enamel through treatment with naf-containing toothpastes in vitro. Scand J Dent Res 1992;100:81-87.

Cury JA, Tenuta LMA. How to maintain a cariostatic fluoride concentration in the oral environment. Adv Dent Res 2008;20:13-16.

Dijkman AG, De Boer P, Arends J. In vivo investigation on the fluoride content in and on human enamel after topical application. Caries Res 1983;17:392-402.

Dijkman TG, Arends J. The role of caf_2-like material in topical fluoridation of enamel in situ. Acta Odontol Scand 1988;46:391-397.

Duschner H, Uchtman H. Degradation of surface enamel and formation of precipitate after topical applications of sodium fluoride solutions in vitro. Acta Odontol Scand 1988;46:265-374.

Gerth HUV, Dammaschke T, Shäfer E, Züchner H. A three layer structure of fluoridated enamel containing CaF_2, $Ca(OH)_2$ AND FAp. Dental Mat 2007;231521-28.

Jeng YR, Lin TT, Wong TY, Chang HJ, Shieh DB. Nano-mechanical properties of fluoride-treated enamel surface. J Dent Res 2008;87:381-385.

Lagerlöf F, Saxegaard E, Barkvoll P, Rølla G. Effects of inorganic orthophosphate and pyrophosphate on dissolution of calcium fluoride in water. J Dent Res 1988;67:447-49.

Melleberg JR, Nicholson CR, Franchi GJ, Englander HR, Mosley GW. Enamel fluoride uptake and retention from intensive APF gel applications in vivo. J Dent Res 1977;56:716-721.

Nelson DG, Jongebloed WL, Arends J. Morphology of enamel surfaces treated with topical fluoride agents: SEM considerations. J Dent Res 1983;62:1201-1208.

Nelson G, Jongebloed WL, Arends J. Crystallographic structure of enamel surfaces treated with topical fluoride agents: TEM and XRD consideration. J Dent Res 1984;63:6-12.

Øgaard B, Rølla G, Ruben J, Arends J. Relative cariostatic effects of KOH-soluble and KOH-insoluble fluoride in situ. J Dent Res 1990;69:1505-1507.

Øgaard B, Rølla G, Helgeland K. Uptake and retention of alkali soluble and insoluble fluoride in sound enamel in vivo after mouth-rinses with 0.05% or 0.2% naf. Caries Res 1983;17:520-524.

Øgaard B, Rølla G, Ruben J, Dijkman T, Arends J. Microradiographic study of demineralization of shark enamel in a human caries model. Scand J Dent Res 1988;96:209-211.

Øgaard B, Seppä L, Rølla G. Professional topical fluoride applications clinical efficacy and mechanism of action. Adv Dent Res 1994;8:190-121.

Øgaard B. CaF(2) formation: cariostatic properties and factors of enhancing the effect. Caries Res 2001;35:40-4.

Øgaard B. The cariostatic mechanism of fluoride. Compend Contin Educ Dent 1999;20:10-7.

Øgaard R, Duschner H, Rubens J, Arends J. Microradiographic and confocal laser scanning microscopy applied to enamel lesions formed in vivo with and without fluoride treatment. Eur J Oral Sci 1996;104:378-383.

Rölla G, Bowen WH. Surface adsorption of fluoride and ionic exchange reactions on hydroxyapatite. Acta Odontol Scand 1978;36:219-24.

Rølla G, Saxegaard E. Critical evaluation of the composition and use of topical fluorides, with emphasis on the role of calcium fluoride in caries inhibition. J Dent Res 1990;69:780-5.

Rølla G. On the role of calcium fluoride in the cariostatic mechanism of fluoride. Acta Odontol Scand 1988;46:341-345.

Saxegaard E, Rølla G. Fluoride acquisition on and in human enamel during topical application in vitro. Scand J Dent Res 1988;96:523-535.

Shellis RP, Dibdin GH. Microporosity of enamel and its functional implications. In: Teeth: Development, Evolution and Function, ed. By MF Teafold, MM Smith and MJ Ferguson. Cambridge University Press, Cambridge, 2000, pp. 242-251.

Takagi S, Chow LC, Yamada EM, Brown WE. Enhanced enamel F uptake by monocalcium phosphate monohydrate gels. J Dent Res 1987;66:1523-1526.

Ten Cate JM, Featherstone J. Mechanistic aspects of the interactions between fluoride and dental enamel. Crit Rev Oral Biol Med 1991;2:283-296.

White DJ, Nancollas GH. Physical and chemical considerations of the role of firmly and loosely bound fluoride in caries prevention. J Dent Res 1990;69:587-594.

Yamazaki H, Margolis HC. Enhanced enamel remineralization under acidic conditions in vitro. J Dent Res 2008;87:569-74.

Chapter 7

Effects of fluoride release from an orthodontic bondig agent on enamel demineralization

The aim of this study was to compare *in vitro* a traditional orthodontic bonding agent with a fluoride-releasing one. The treated enamel surface around the bracket was investigated through MOM and SEM/EDX analysis after acid attack.

Two groups of 6 elements were respectively bonded with Transbond XT (group A) and Transbond Plus, fluoride-releasing (group B).

A template calibrated on bracket size (3x3 mm), with an excess of 3 mm from one side was placed on each sample in order to delimit an etched and primer-painted enamel surface.

The samples were exposed to a demineralizing solution (lactic acid) for three days, then incorporated in resin cylinders and finally smoothed and polished.

In all the enamel surfaces investigated, samples of group B showed an average depth of demineralization which was at least 40% lower compared to samples of group A. The differences in demineralization marks and in calcium and phosphorus content between the two groups are likely due to the different chemical characteristics (fluoride-release) of the bonding agents examined.

Fluoride particles have been found on the enamel surface of samples of group B at 150 μm from the bonding system until the maximum depth of approximately 300 μm.

In the present study the amount of fluoride contained in the examined bonding agents appeared to reduce the demineralization marks and to modify the chemical composition of the enamel in the treated area.

Introduction

The appearance of enamel demineralization around bonded brackets represents a remarkable clinical problem in orthodontic treatment with fixed appliances. Moreover the presence of bands/brackets and of others orthodontic devices such as elastics makes the patient's dental hygiene hard to be maintained and facilitates plaque accumulation.

Clinically demineralization marks appear as white lesions (white spot lesions) around the brackets (Al-Khateeb *et al.*, 1998; Buren *et al.*, 2008). These lesions constitute early forms of enamel caries that possess the potential for own remineralization in the presence of fluoride (Gorelick *et al.*, 1982; Geiger *et al.*, 1992). Consequently many authors suggest topical applications of fluoride during orthodontic treatment thorough dental hygiene procedures (Geiger *et al.*, 1992; Todd *et al.*, 1999; Wenderoth *et al.*, 1999).

Adjunctive fluoride therapy is commonly used to prevent demineralization and to promote enamel remineralisation. As fluoride has been also shown able to reduce plaque formation by inhibiting bacterial enzymatic acid production its use appears suitable in clinical conditions that make plaque removal difficult such as the presence of orthodontic appliances (Stratemann and Shannon, 1974; Arends *et al.*, 1975; Geiger *et al.*, 1992; Basdra *et al.*, 1996; Vorhies *et al.*, 1998; Todd *et al.*, 1999; Prati *et al.*, 2001; Foley *et al.*, 2002; Chadwick *et al.*, 2005; Farrow *et al.*, 2007; Wiegand *et al.*, 2007; Buren *et al.*, 2008).

Moving from the good results achieved in several *in vitro* studies that showed the efficacy of fluoride in inhibiting white spot lesions and enamel demineralization and the lower but clinically acceptable bond strengths (Geiger *et al.* 1992; Basdra *et al.*, 1996; Vorhies *et al.*, 1998; Todd *et al.*, 1999; Foley *et al.*, 2002) new bonding agents fluoride releasing have been subsequently introduced on the market (Buren *et al.*, 2008).

The quoted *in vitro* studies investigated enamel decalcification through SEM (Scanning Electron Microscope), PLM (Polarized Light Microscope), QLF (Quantitative Light-induced Fluorescence), sonic digitizer (Basdra *et al.*, 1996; Al-Khateeb *et al.*, 1998; Foley *et al.*, 2002; Benson *et al.*, 2003; Sudjalim *et al.*, 2007; Buren *et al.*, 2008). Other authors carried out similar experimentations, using EDX (Energy Dispersive X-ray Analysis), an instrumental analytic methodical that takes advantage from X-rays emission and allows an accurate semiquantitative analysis of the chemical composition of the materials through X-ray detector system attached to a SEM (Takagi *et al.*, 2000; Arnold *et al.*, 2003; Mahoney *et al.*, 2004; Arnold *et al.*, 2006). Only one study in the literature applied such methodical to analyze orthodontic adhesives (Scougall Vilchis *et al.*, 2008). Moreover there are no published studies that evaluate the demineralization and the fluoride con-

tent in the enamel areas adjacent to brackets applied with a fluoride-releasing adhesive comparing with a not fluoride-releasing one.

The aim of this study was to investigate the morphology and the depth of demineralization of the enamel surface surrounding bonded orthodontic brackets exposed to an artificial caries solution (lactic acid). The fluoride content and the differences in the concentration of calcium and phosphate were also evaluated.

Two different orthodontic resins Transbond Plus, fluoride-releasing and Transbond XT, not fluoride-releasing, were compared to evidence the differences in depth and morphology of enamel lesion and to verify the presence of the fluoride.

Materials and Methods

Sample preparation

Twelve extracted healthy and caries-free human molars collected and kept in water until thirty days were randomly divided into two groups: group A composed of 6 teeth bonded with Transbond XT (3M Unitek, Monrovia, CA, USA) and group B of 6 elements bonded with Transbond Plus (3M Unitek, Monrovia, CA, USA). The teeth were also cross-sectioned axially with a carbide tungsten bur Komet H245 (ISO 233006) on the turbine at high speed, thus eliminating the root component of teeth.

Each sample was rinsed and cleaned with a rubber prophylaxis cup at slow speed for 15 seconds. Samples of both groups were etched with 35% phosphoric acid gel (Scotchbond 3M Unitek, Monrovia, CA, USA) for 30 seconds and before bracket bonding the same primer was applied (Transbond XT, light cure adhesive primer. 3M Unitek, Monrovia, CA, USA). On each sample was placed a template calibrated on bracket size (3x3 mm), with an excess of 3 mm from a single side, in order to delimit an etched and primer-painted enamel surface.

After the application of a thin and uniform layer of the composite resin Transbond XT (group A) on the brackets were positioned with firm pressure. Likewise on group B samples brackets were applied with adhesive paste Transbond Plus fluoride-releasing. The excess bonding material was carefully removed with a scaler before the set of the adhesive and then started the polymerization of 20 seconds with a visible curing light (L.E. Demetron I) at the constant intensity of 350-400 mW/cm2. Before demineralization simulation procedure, a layer of varnish (colored nail polish) was applied on each

sample over the end of the template in order to define, making it visible, the area to examine.

Storage and perfusion

All the teeth were subjected to an artificial caries solution consisting of 7.44 ml of 99% lactic acid at pH 4.4, taken with a graduated pipette and placed in a container having a capacity of 1000ml and brought to volume with deionized water.

This was to reproduce *in vitro* the effects of bacterial metabolism and to simulate the factors stimulating the the oral microflora reaction.

All the samples prepared as previously described were placed in 25 ml of this solution and stored in an incubator model ICT 70 (Falc Instruments s.r.l. BG) at 37°.

Teeth were cycled between artificial caries solution and deionized water for three days, as shown in table 1.

After the three day-storage, samples were washed and copiously rinsed with deionized water to completely remove any cariogenic solution remaining and finally dried.

All specimens were embedded in methylmetacrylate (Technovit ®2060, Italy) in plastic cylinders (diameter 0.2), left to harden for 20-30 minutes at room temperature.

Sample preparation for microscope analyses

After polymerization reaction of the resin, specimens were flattened by passing through paper of abrasive particle size decreasing from 220 to 2000 Grit and polished on discs of tissue (Polilap n°. 10), with a suspension of alumina powder N2-3 from 3 to 0.1 μm to obtain a mirror surface of the sample.

This preparation made possible the complete removal of abrasion from the samples' surfaces allowing a better observation on metallographic optical microscope (Reichert MeF3), on SEM (Zeiss EVO 50) and on EDX (INCAX-sight Oxford Instruments).

All samples were subjected to morphological evaluations of the enamel in the following areas:

- central with bracket bonded area (zone 1);
- etched and primer-painted area (zone 2);
- not-treated area (zone 3).

More specifically the areas have been divided into 4 different positions of observation, as shown in Figure 1.

Results

Metallographic optical microscope (MOM) analysis

Images obtained by the MOM showed micrometric lesions below the surface of the bracket of approxiamtely 10μm in depth in 4 of 6 samples of group A while, in all the samples of Group B, the enamel below the bracket-adhesive system appeared intact: this indicates a higher protective effect from acid attack.

The mean depth and area, standard deviations, ranges and maximum measurements recorded in the different positions of the analyzed samples are summarized in table 2.

In all the enamel positions investigated, samples of group B (Transbond Plus) showed average depths of demineralization at least 40% lower compared to samples of group A (Transbond XT).

In a comparison between the treated and untreated areas in both groups (zones 2 and 3), the demineralization depth marks appeared lower in position 3 and 4 than in positions 1 and 2, as well as a significant difference in the depth of lesion was detected by the comparison between groups in zones 2 (position 3 and 4) and 3 (positions 1 and 2).

The distance between the edge of the adhesive on the enamel surface and the border of the demineralized zone was significantly longer for the fluoride-releasing samples: 368.44 μm (348.33 μm in position 1 and 2; 342.5 μm in position 3 and 4) recorded in sample of group B, compared to an average distance of - 23.33μm * (- 26.67 μm in position 1 and 2; - 20 μm in position 3 and 4) in the samples of group A.

*negative values (sign "-" before the values) indicate that the demineralization zone started under the bracket.

SEM analysis

SEM morphological analysis revealed the presence of different degrees of demineralization highlighted in the form of bars, explained as the progressive loss of substance in the most superficial area of hydroxyapatite crystals.

Although these bands were visible in the enamel of both groups, the lesions presented different morphologies: SEM evaluation of the enamel demineralization in samples of group A (Transbond XT) showed a marked corrosion rate and a line of fracture ("crack") within the lesion (Figs 2-4). Corrosion rate and gap appeared less pronounced in samples of group B (Transbond Plus) and the "crack" of the enamel was not detected (Figs5-6).

EDX analysis

The EDX analysis at positions 3 and 4 allowed to detect differences in calcium and phosphorus content in correspondence to the previously mentioned bands. These differences were more pronounced in the samples of group A, in fact it has been possible to highlight the peaks of reduction of the concentration of these elements. (Figs.7 -8).

To confirm MOM data, EDX analysis at positions 3 and 4 also detected the presence of fluoride at approximately 150 μm away from the bracket and about 300 μm deep from the surface (Figs 9-12).

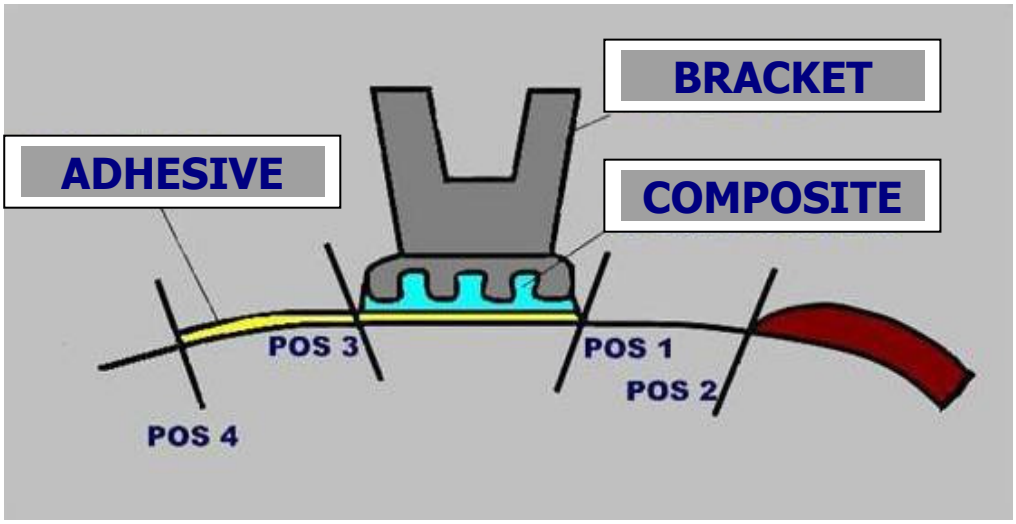


Fig. 1 Analyzed positions for the evaluation of demineralization area:
- Position 1= 100μm from the orthodontic bonding agent in the untreated area.
- Position 2= greatest demineralization depth in the untreated area.
- Position 3=100μm from the orthodontic bonding agent in the etched and primer-painted area.
- Position 4=greatest demineralization depth in the etched and primer-painted area.

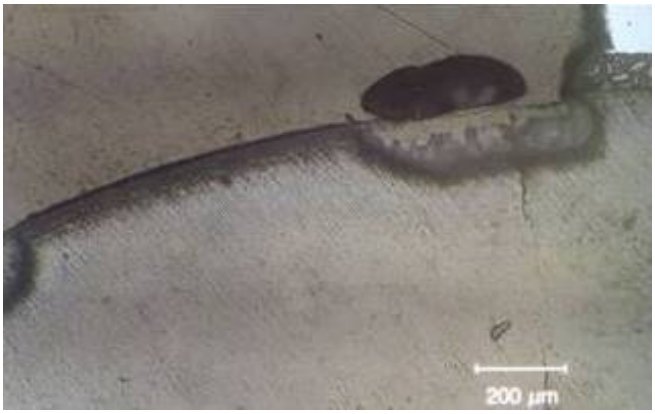


Fig. 2 Enamel demineralization observed in a sample of group A.

Fig. 3 Detail of the enamel "void" in the previous sample.

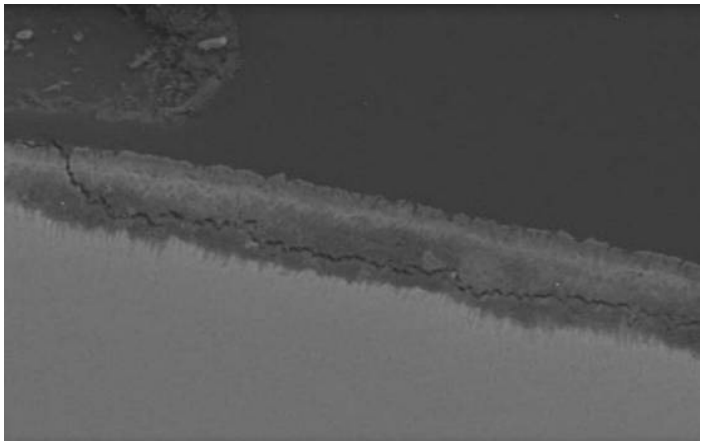


Fig. 4 SEM evaluation of the enamel demineralization in a sample of Group A: a line of fracture within the lesion.

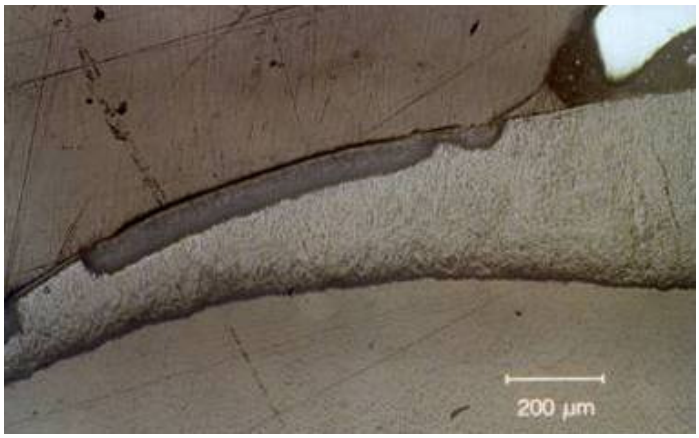


Fig. 5 Enamel demineralization observed in a sample of group B.

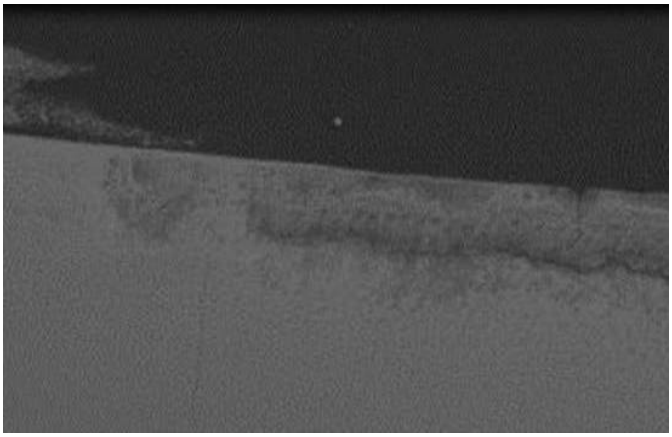


Fig. 6 SEM evaluation of the enamel demineralization in a sample of group B: corrosion rate and gap appeared less marked.

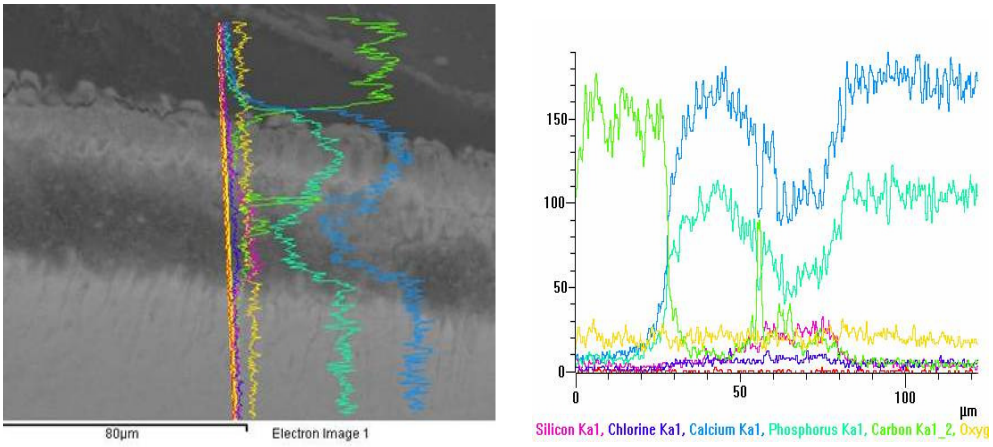


Fig. 7 EDX evaluation of the enamel demineralization in a sample of group A : line-scan in the various bars within the lesion showed a decrease of calcium and phosphorus content in the enamel.

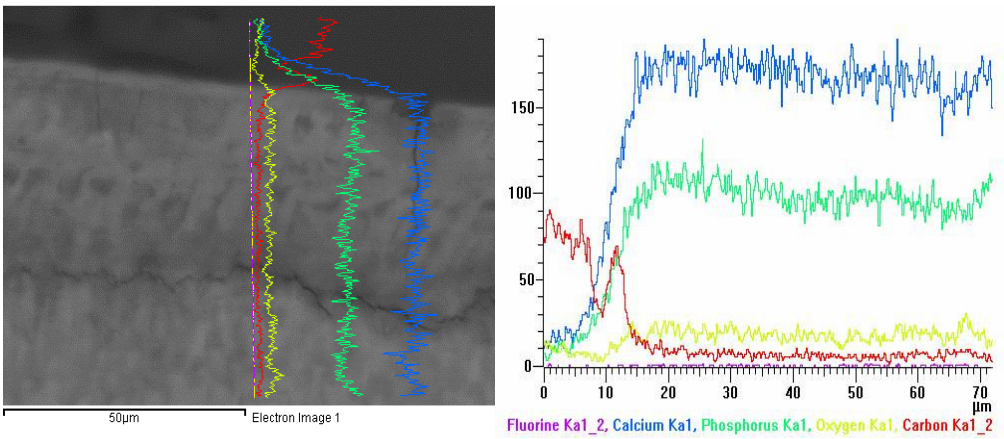
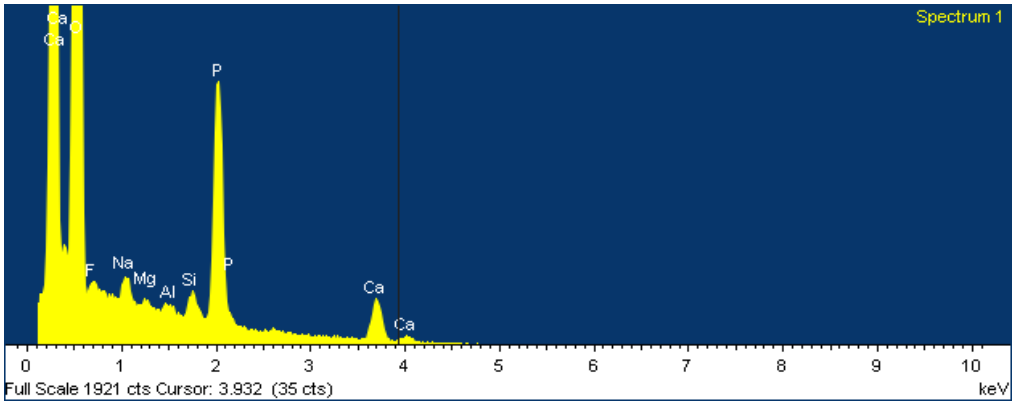
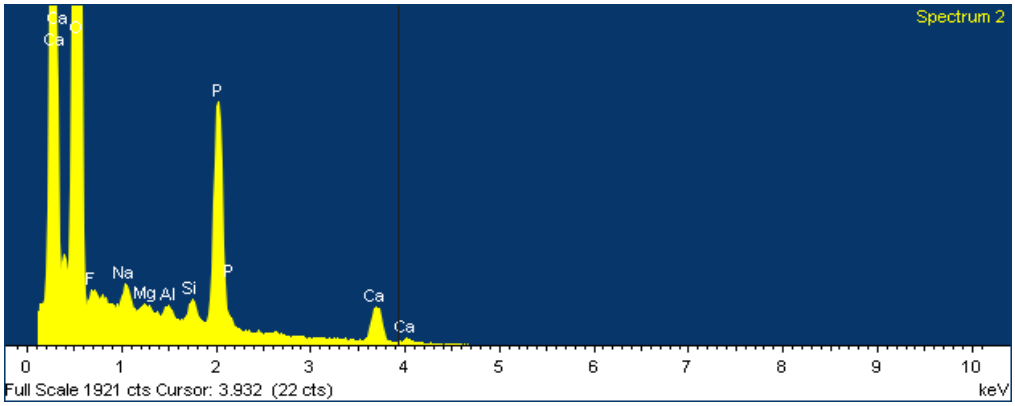


Fig. 8 EDX mapping of the enamel demineralization in a sample of group B showed a homogeneous distribution of calcium and phosphorus within the lesion.



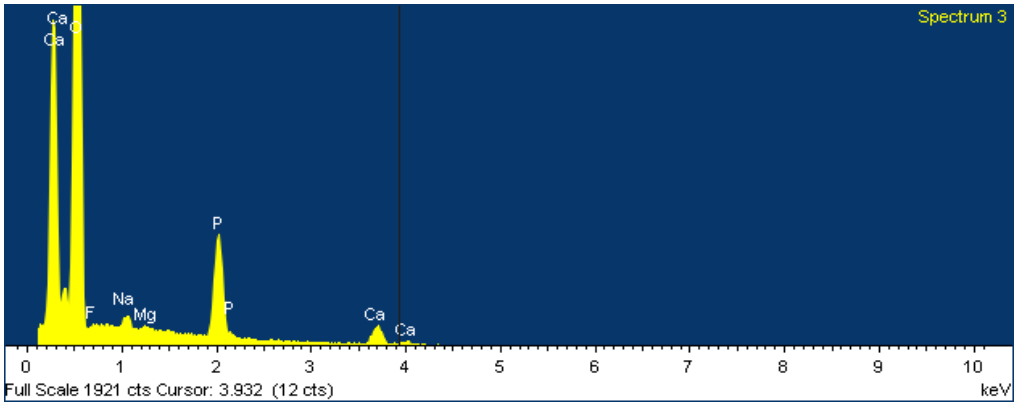
Element	Weight %	Atomic %
OK	48.76	68.13
FK	0.63	0.74
NaK	0.50	0.48
AlK	0.16	0.13
SiK	0.51	0.41
PK	15.45	11.15
CaK	34.00	18.96
Total	100.00	

Fig. 9 XRD patterns of demineralized enamel in a sample of group B at about 30 μm deep from the surface and approximately 150 μm away from the bracket.



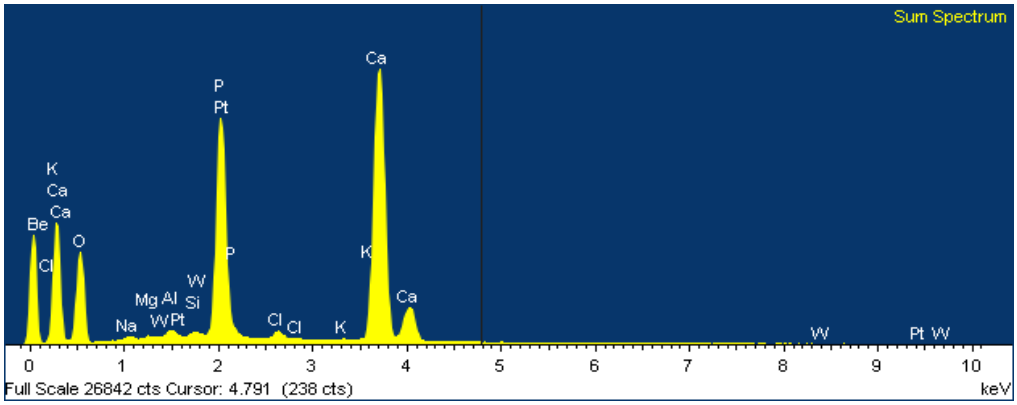
Element	Weight %	Atomic %
OK	48.85	68.07
FK	0.56	0.65
NaK	0.51	0.50
MgK	0.19	0.18
SiK	0.82	0.65
PK	15.67	11.28
CaK	33.03	18.37
Total	100.00	

Fig. 10 XRD patterns of demineralized enamel in the previous sample at about 80 μm deep from the surface.



Element	Weight %	Atomic %
OK	49.40	68.92
FK	0.00	0.00
NaK	0.68	0.66
MgK	0.23	0.21
PK	15.44	11.13
CaK	34.26	19.08
Total	100.00	

Fig. 11 XRD patterns of demineralized enamel in the previous sample at about 300 µm deep from the surface: only a not-significant amount of fluoride was found.



Element	Weight %	Atomic %
O K	46.96	66.72
Na K	0.46	
Al K	0.13	
P K	17.42	
Cl K	0.84	
Ca K	34.19	
Total	100.00	

Fig. 12 XRD patterns of demineralized enamel in a sample of group A at about 30 µm deep from the surface and 150 µm away from the bracket: the presence of fluoride was not detected.

Time	Fluid
8.00→8.30	Deionized water
8.30→12.30	Lactic acid
12.30→13.00	Deionized water
13.00→16.30	Lactic acid
16.30→17.00	Deionized water
17.00→8.00	Lactic acid

Tab. 1 Cycles artificial caries solution and deionized water.

	Position 1	Position 2	Position 3	Position 4
Transbond XT	108.33± 44.46	135.00±57.18	75.00±8.37	78.33±11.69
Transbond Plus	40.00± 27.66	63.33± 32.66	30.00± 29.66	55.00±36.19

Tab.2 Summarizing table of the different values of demineralization depth in all the positions examined.

Discussion

The presence of enamel demineralization and white spot lesions adjacent to fixed orthodontic appliances still represents a relevant clinical problem that could compromise both esthetic and oral health, overshadowing the positive effects of orthodontic treatment (Erikson *et al.*, 1995; Al-Khateeb *et al.*, 1998; Benson *et al.*, 2003; Benson *et al.*, 2005; Alessandri Bonetti *et al.*, 2009). Because of the increased difficulty in adequately removal bacterial plaque around orthodontic appliances, adjunctive fluoride therapy is commonly used to prevent demineralization. Some authors suggested the use of a daily sodium fluoride rinses program (Marinelli *et al.*, 1997; Benson et al., 2005; Sudjalim *et al.*, 2007), fluoride varnishes and sealers surrounding orthodontic appliances (Todd *et al.*, 1999; Buren *et al.*, 2008). However, effective protection with fluoride could require appropriate patient compliance and the inconsistency of patient cooperation is a challenging aspect of orthodontic care. One of the different methods proposed to prevent and decrease enamel demineralization that do not require patient compliance is the use of fluoride-releasing bonding systems.

Clinical studies exhibited conflicting data about the efficacy of these materials in prevention or inhibition of white spot lesions and in affecting the growth of caries-associated bacteria compared to non-fluoritated sealants or adhesives (Trimpeneers *et al.*, 1996; Wenderoth *et al.*, 1999; Farrow *et al.*, 2007).

The aim of this *in vitro* study was to examine the fluoride release and its effects on enamel demineralization (depth and morphology) and to investigate the alterations on the enamel surface after the use of two different orthodontic bonding system.

The fluoride content in the bonding agent tested in this study (Transbond Plus) showed ability to reduce "white spots" both in depth and in extension, changing the morphology of the demineralization.

The comparison between zones 2 and 3 of both groups showed demineralization depths lower in positions 3 and 4 than in positions 1 and 2: this result is due to a protective, mechanical, action of the primer on the enamel. On the other hand the differences in demineralization marks observed between groups A and B in zones 2 and 3 are due to the different chemical (release of F^-) features of the two adhesives tested.

It has been demonstrate that acid attack causes loss of calcium and other minerals, including phosphate (Arnold *et al.*, 2006). In this experimental model, acid attack resulted in an increase of the enamel porosity and permeability, both in the surface and in deeper layers over 100 μm as showed by SEM.

The EDX analysis confirmed that the calcium/phosphate ratio of hypo-demineralised enamel was significantly lower than the unaffected enamel (Mahoney *et al.* 2004).

The lower concentrations of calcium and phosphorus, observed in the present study, within the lesions on enamel treated with a bonding agent not fluoride-releasing and the higher concentrations in the samples bonded with a fluoride releasing one are in agreement with previous studies (Basdra *et al.*, 1996; Trimpaneers *et al.*, 1996; Wenderoth *et al.*, 1999; Benson *et al.*, 2005; Sudjalim *et al.*, 2007).

As shown in several study, calcium fluoride-like particles are present on the enamel surface of the fluoride-releasing bonding systems, forming a potential protective deposit on the enamel surface (Basdra *et al.*, 1996). The dissociation of fluoride ions from calcium fluoride crystals and their diffusion into enemel pores may occurred, either during the initial intense release or later during the slow but regular exposure to fluoride, followed by incorporation into the enamel apatite crystals during demineralization and remineralization procedures, finally forming larger, more acid resistant crystals (Basdra *et al.*, 1996).

Fluoride ions released on the enamel surface from Transbond Plus may act as a potential reservoir, available for remineralization or deposition into demineralized areas, or as a diffusion barrier during acid attacks. The purpose of this *in vitro* study was to evaluate the effectiveness of an orthodontic bonding agent fluoride-releasing on inhibiting enamel demineralization and early caries by the anlysis of depth and extension of enamel demineralization and surface appearance.

The conclusion achieved were that fluoride released from the adhesive system provided significant reduction in enamel demineralization marks and changed the morphology of

the enamel lesions: our results qualitatively and quantitatively confirmed the existence of an inhibitory effect of fluoride (found up to 300 μm depth) on enamel demineralization induced by exposition to an artificial caries solution.

Clinically, these results encourage the choice of fluoride-releasing materials for bands and brackets application in order to prevent the onset of caries in a subject at risk as the orthodontic one: these products can be applied directly by orthodontists and are not bound to the domestic use, allowing to overcome one of the major limitations of prevention with fluoride, the patient's compliance.

References

Alessandri Bonetti G, Zanarini M, Incerti Parenti S, Marchionni S, Checchi L. In vitro evaluation of casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) effect on stripped enamel surfaces. A SEM investigation. J Dent 2009; 37: 228-32.

Al-Khateeb S, Forsberg CM, De Josselin De Jong E. A longitudinal laser fluorescence study of white spot lesions in orthodontic patients. Am J Orthod Dentofacial Orthop 1998; 113: 595-602.

Arends J, Schuthof J. Fluoride content in human enamel after fluoride application and washing: an in vitro study. Caries Res 1975; 9: 363-72.

Arnold WH, Cerman K, Neuhaus K, Gaengler P. Volumetric assessment and quantitative element analysis of the effect of fluoridated milk on enamel demineralization. Arch Oral Biol 2003; 48: 467-73.

Arnold WH, Gaengler P. Quantitative analysis of the calcium and phosphorus content of developing and permanent human teeth. Ann Anat 2006; 189: 183-90.

Basdra EK, Huber H, Komposch G. Fluoride released from orthodontic bonding agents alters the enamel surface and inhibits enamel demineralization in vitro. Am J Orthod Dentofacial Orthop 1996; 109: 466-72.

Benson EP, Pender N, Higham SM. Quantifying enamel demineralization from teeth with orthodontic brackets—a comparison of two methods. Part 1: repeatability and agreement. Eur J Orthod 2003; 25: 149-58.

Benson EP, Pender N, Higham SM. Quantifying enamel demineralization from teeth with orthodontic brackets—a comparison of two methods. Part 2: validity. Eur J Orthod 2003; 25: 159-65.

Benson EP, Shah AA, Millett DT, Dyer F, Parkin N, Vine RS. Fluorides, Orthodontics and demineralization: a systematic review. J Orthod 2005; 32: 102–14.

Buren JL, Staley RN, Wefel J, Qian F. Inhibition of enamel demineralization by an enamel sealant, Pro Seal: An in vitro study. Am J Orthod Dentofacial Orthop 2008; 133: S88-94.

Chadwick BL, Roy J, Knox J, Treasure ET. The effect of topical fluorides on decalcification in patients with fixed orthodontic appliances: a systematic review. Am J Orthod Dentofacial Orthop 2005; 128: 601-6.

Farrow ML, Newman SM, Oesterle LJ, Shellhart WC. Filled and unfilled restorative materials to reduce enamel decalcification during fixed-appliance orthodontic treatment. Am J Orthod Dentofacial Orthop 2007; 132: 577.e 13-577.e 18.

Foley T, Aggarwal M, Hatibovic-Kofman S. A comparison of in vitro enamel demineralization potential of 3 orthodontic cements. Am J Orthod Dentofacial Orthop 2002; 121: 526–30.

Geiger AM, Gorelick L, Gwinnett AJ. The effect of a fluoride program on white spot formation during orthodontic treatment. Am J Orthod Dentofacial Orthop 1992; 101: 403-7.

Gorelick L, Geiger AM, Gwinnett AJ. Incidence of white spot formation after bonding and banding. Am J Orthod 1982; 81: 93-8.

Mahoney EK, Rohanizadeh R, Ismail FSM, Kilpatrick NM, Swain MV. Mechanical properties and microstructure of hypomineralised enamel of permanent teeth. Biomat 2004; 25: 5091-100.

Marinelli CB, Donly KJ, Wefel JS, Jakobsen JR, Denehy GE. An in vitro comparison of three fluoride regimens on enamel remineralization. Caries Res. 1997; 31: 418-22.

Prati C, Zanarini M, Marchionni S, Ruggeri O. Ability of glass-ionomer restorative material to prevent secondary caries and to modify the de- and remineralization process of tooth structures. Atti del Congresso Clinical Alternatives in Restorative Dentistry marzo 8-9/ 2001, Certosa di Pontignano pp.45-56.

Schougall Vilchis RJ, Hotta Y, Yamamoto K. Examination of Six Orthodontic Adhesives with Electron Microscopy, Hardness Tester and Energy Dispersive X-ray Microanalyzer. Angle Orthodontist 2008; 78: 655-61.

Stratemann NW, Shannon IL. Control of decalcification patients by daily self-administered application of a water free 0.4 percent stannous fluoride gel. Am J Orthod 1974; 66: 273-79.

Sudjalim TR, Woods GM, Manton DJ, Reynolds EC. Prevention of demineralization around orthodontic brackets in vitro. Am J Orthod Dentofacial Orthop 2007; 131: 705-9.

Takagi S, Liao H, Chow LC. Effect of tooth-bound fluoride on enamel demineralization/ remineralization in vitro. Caries Res 2000; 34: 281-8.

Todd MA, Stanley RN, Kanellis MJ, Donly KJ, Wefel JS. Effect of a fluoride varnish on demineralization adjacent to orthodontic brackets. Am J Orthod Dentofacial Orthop 1999; 116: 159-67.

Trimpaneers LM, Dermaut LR. A clinical evaluation of the effectiveness of a fluoride-releasing visible light-activated bonding system to reduce demineralization around orthodontic brackets. Am J Orthod Dentofac Orthop 1996; 110: 218-22.

Vorhies AB, Donly KJ, Staley RN. Enamel demineralization adjacent to orthodontic brackets bonded with hybrid glass ionomer cements: an in vitro study. Am J Orthod Dentofacial Orthop 1998; 114: 668-74.

Wenderoth CJ, Weinstein M, Borislow AJ. Effectiveness of a fluoride-releasing sealant in reducing decalcification during orthodontic treatment. Am J Orthod Dentofacial Orthop 1999; 116: 629-34.

Wiegand A, Buchalla W, Attin T. Review on fluoride-releasing restorative materials—Fluoride release and uptake characteristics, antibacterial activity and influence on caries formation. Dental Mat 2007; 23: 343–62.

Chapter 8

Acid treatments modify enamel permeability

The aim of the present study was to evaluate the acid-induced structural transformations in enamel after two different treatments by means of Raman and IR spectroscopy analysis and to correlate these findings with enamel permeability.

Two different treatments were investigated: 3 slices were etched with 15 % HCl for 120 s and 3 slices with 37% phosphoric acid gel (H_3PO_4) for 30 s, rinsed for 30 s and then air-dried for 20 s. Powders of enamel treated as previously described were produced. Replicas of enamel subjected to the same treatments were obtained.

Raman and IR spectroscopy showed that the treatment with both hydrochloric and phosphoric acids induced a decrease in the carbonate content of the enamel apatite. At the same time, both acids induced the formation of HPO_4^{2-} ions. After H_3PO_4 treatment the bands due to the organic component of enamel decreased in intensity, while increased after HCl treatment.

Replicas of H_3PO_4 treated enamel showed a strongly reduced permeability with no droplets detectable. Replicas of HCl 15% treated samples showed a maintained permeability, with smaller droplets covering the enamel surface with a different distribution in comparison with baseline replicas. A decrease of the enamel organic component, as resulted after H_3PO_4 treatment, involves a decrease in enamel permeability, while the increase of the organic matter (achieved by HCl treatment) still maintains enamel permeability. These results suggested a correlation between the amount of the organic matter, enamel permeability and caries.

Introduction

Since its introduction by Buonocore (Buonocore, 1955) acid etching of dental enamel has been usually employed in bonding procedures increasing microscopic roughness, by selectively removing mineral crystal. This procedure creates microscopic porosities in the enamel surface and hardens in tag-like projections that attach the material to the tooth structure according to a micromechanical retention principle to improve the resin-enamel bond in composite restoratives (Vicente *et al.*, 2006; Fjeld and Øgaard., 2006; Yousseff *et al.*, 2006). To achieve this a relatively strong acid, generally 37% phosphoric acid, is used for 30 seconds to clean the surface and dissolve the minerals (Ajlouni *et al.*, 2004; Kim *et al.*, 2006; Fjeld and Øgaard , 2006).

The effects of phosphoric acid concentration and duration of etching on enamel surface morphology and bond strength have been investigated by several authors (Ajlouni *et al.*, 2004; Vicente *et al.*, 2006). It has been supposed that routine etching with phosphoric acid removes irreversibly several microns of the enamel surface although not to a uniform depth (Kuhar *et al.*, 1997; Fjeld and Øgaard , 2006) and induces surface softening, with the resultant increased porosity of the outer dental layers (Kuhar *et al.*, 1997). The mineral loss could make the acid-etched region vulnerable to successive acid attacks in the oral environment because of persisting of the characteristic etch patterns for several months (Kim *et al.*, 2006; Kuhar *et al.*, 1997). As a result, recent studies suggest a shorter duration of acid etching (Kim *et al.*, 2006).

Acid etching might perforate or even remove the surface layer and thus enhance resin infiltration of the more porous lesion body. Hydrochloric acid produced a small demineralised layer with brief erosion (Wiegand *et al.*, 2007) and has been applied in aesthetic dentistry to remove superficial discolorations by enamel microabrasion (Meyer-Lueckel *et al.*, 2007).

Recently it has been demonstrated that HCl 15% gel for 90-120 s led to a virtually complete removal of the surface layer and therefore seems to be more suitable for the pre-treatment of natural enamel lesion prior to resin infiltration (Paris *et al.*, 2007).

The aim of the present study was to evaluate the acid-induced structural transformations in enamel after two different treatments (15 % HCl for 120 s and 37% phosphoric acid gel (H_3PO_4) for 30 s) by means of Raman and IR spectroscopy analysis and to correlate these findings with enamel permeability evaluated by SEM inspection of replicas.

Materials and Methods

Raman and IR spectroscopy

Nine slices of enamel (1mm thick) were cut from sound human molars by means of a diamond bur. Two different treatments were investigated: 3 slices were etched with 15 % HCl for 120 s and 3 slices with 37% phosphoric acid gel (H_3PO_4) for 30 s, rinsed for 30 s and then air-dried for 20 s. Three slices of untreated enamel were used as control.

Powders for IR analysis were obtained by grinding with a multilama bur 9 slices of enamel treated and not as previously described.

The Raman spectra were recorded on a Bruker IFS66 spectrometer equipped with a FRA-106 FT-Raman module and a cooled Ge-diode detector. The excitation source was a Nd^{3+}-YAG laser (1064 nm) in the backscattering (180°) configuration. The focused laser beam diameter was about 100 μm, the spectral resolution was 4 cm^{-1}, and the laser power at the sample was about 140 mW.

The IR spectra were recorded on a Nicolet 5700 FTIR spectrometer, equipped with a Smart Orbit diamond attenuated total reflectance (ATR) accessory and a DTGS detector; the spectral resolution was 4 cm^{-1}.

Replica tecnique

Four permanent teeth (premolars), extracted for orthodontic reasons from young patients (range age 20–40 yr), were prepared for replica technique at baseline and after the two treatments previously described. An impression of the buccal surface was made using polyvinylsiloxane impression material (Affinis ligth body; Coltene, Alstatten, Switzerland). After 4 min, the material was removed from the enamel surface, degassed for at least 48 h and later cast in polyether impression material (Permadyne Garant; 3M ESPE, St Paul, MN, USA). Samples were gold-sputtered and inspected by a scanning electron microscope.

Results

The IR spectrum of control enamel showed the presence of both A- and B- types carbonated apatites; in fact, the former and the latter are characterised by the bands at about 1545 and 1410 cm^{-1}, respectively (Nelson and Featherstone, 1982; Apfelbaum *et al.*, 1992).

The Raman spectrum as well revealed the presence of both A- and B- types carbonated apatites; in fact, the former and the latter are characterised by the bands at about 1100 and 1073 cm^{-1}, respectively (Nelson and Featherstone, 1982; Penel *et al.*, 1998). Upon treatment with both hydrochloric and phosphoric acids the carbonate content of enamel decreased. In fact, the relative intensity of the bands at 1073 cm^{-1} (Raman, B-type), 1411 cm^{-1} (IR, B-type) and 1541 cm^{-1} (IR, A-type) decreased upon treatment. From a quantitative point of view, the A_{1411}/A_{560} (Featherstone *et al.*, 1984) and A_{1411}/A_{600} (Sønju-Clasen *et al.*, 1997) absorbance ratios were identified as marker of B-type carbonate content; as can be seen from Table 1, their values decreased upon treatment with both acids. The same behaviour was observed for the A_{1541}/A_{600} absorbance ratio (see Table 1), which was identified as marker of A-type carbonate (Sønju-Clasen *et al.*, 1997). Also the downshift of the IR band at 1013 cm^{-1} revealed the decrease of the carbonate content, according to other authors (Antonakos *et al.*, 2007).

At the same time, both acids induced the formation of HPO_4^{2-} ions, as revealed by the increase in relative intensity of the bands at 587 cm^{-1} (Raman) and 525 cm^{-1} (IR) (Casciani and Condrate, 1980; Rey *et al.*, 1990).

The hydrogen bonding system as well was affected by the acid treatment; the IR bands due to a free OH group at about 3570 and 630 cm^{-1} became visible, probably due to the breaking of the hydrogen bonds typical of the enamel structure (Rey *et al.*, 1990).

The position of the bands at about 600 and 560 cm^{-1} as well as their A_{560}/A_{600} absorbance ratio has been identified as marker of the maturation degree of the minerl hydroyapatite-lke phase of bone. In the spectra of the acid-treated enamel this ratio decreased and the bands shifted to lower wavenumbers, i.e. to a situation which would be typical of a less mature mineral stage (Rey *et al.*, 1990). Upon treatment with hydrochloric acid, strong bands due to the organic component were observed in both Raman (2940, 1670, 1455, 1275-1248 cm^{-1}) and IR (2951, 2922, 2852 cm^{-1}) spectra. On the contrary a decrease in intensity of the Raman organic bands was observed upon treatment with phosphoric acid.

SEM photomicrographs of H_3PO_4 treated enamel showed the typical appearance of enamel (Fig.s 4 A-D).

SEM analysis of replicas confirmed that baseline replicas showed many droplets covering the whole surface in several areas and mainly localized along the perikymata as previously described (Bertacci *et al.*, 2007). Replicas of H_3PO_4 treated enamel showed a strongly reduced permeability with no droplets detectable (Figs. 5 A-B). On the other

hand replicas of HCl 15% treated samples showed a maintained permeability, with smaller droplets covering the enamel surface with a different distribution in comparison with baseline replicas (Figs. 5 C-D).

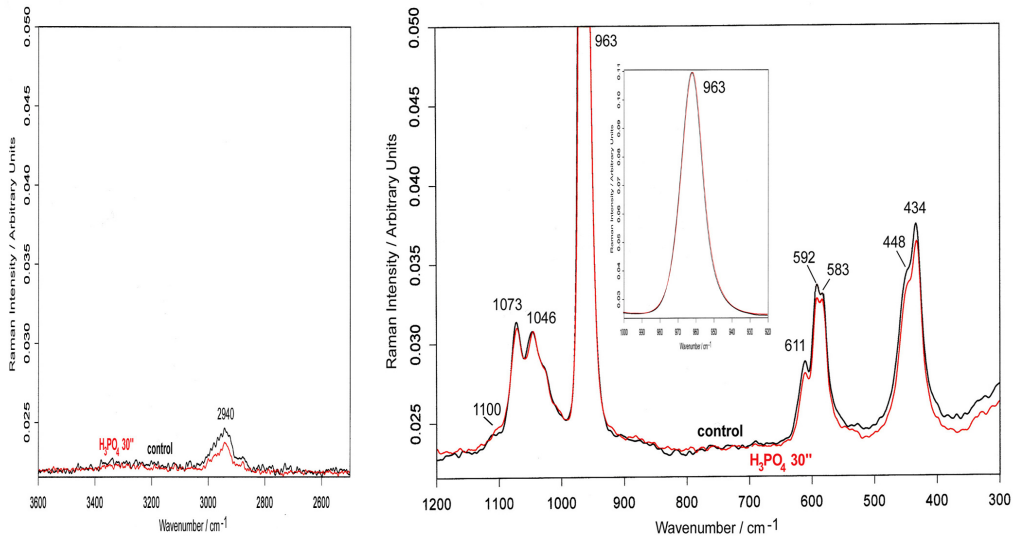


Fig. 1 Raman spectra of enamel before (control) and after treatment with H_3PO_4 for 30".

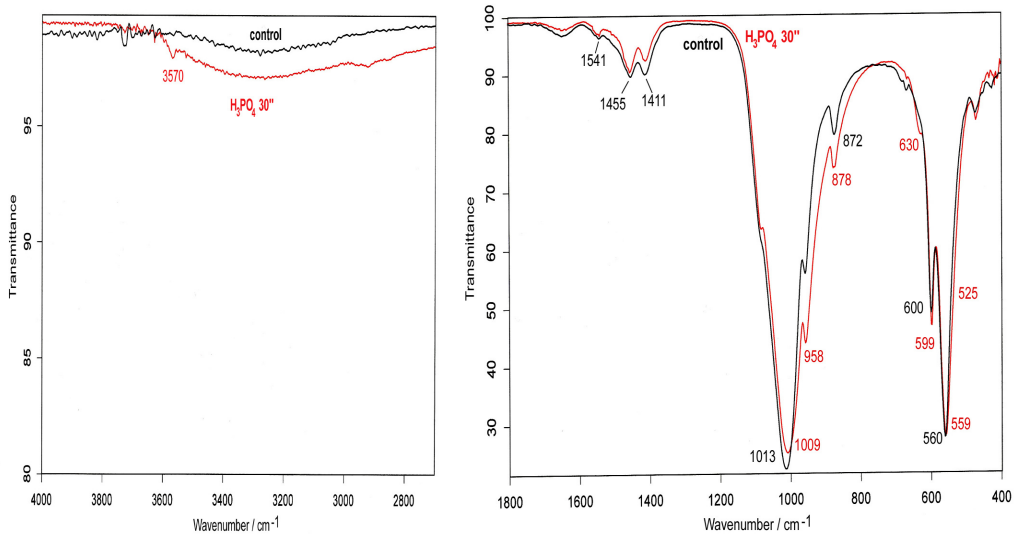


Fig. 2 IR spectra of enamel before (control) and after treatment with H_3PO_4 for 30".

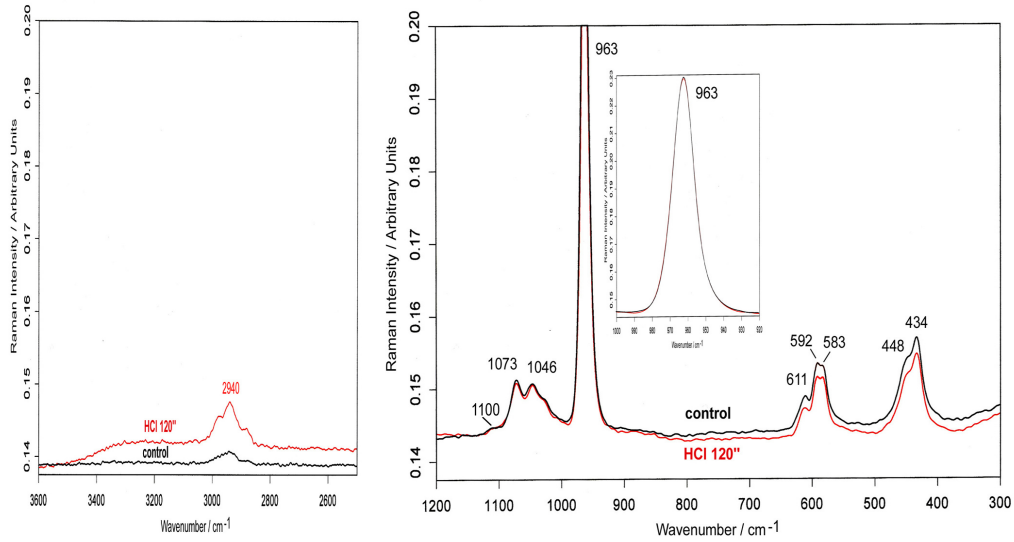


Fig. 3 Raman spectra of enamel before (control) and after treatment with HCl for 120".

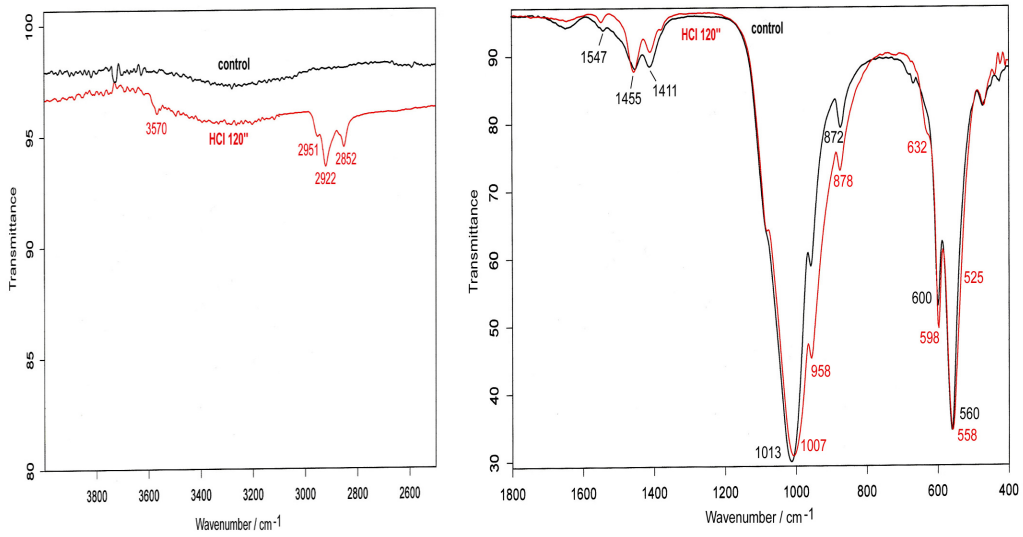


Fig. 2 IR spectra of enamel before (control) and after treatment with HCl for 120".

Table 1. A_{1411}/A_{560}, A_{1411}/A_{600} and A_{1541}/A_{600} absorbance ratios (mean value on three spectra ± standard deviation) obtained from the IR spectra of the analysed powders.

Sample	A_{1411}/A_{560}*	A_{1411}/A_{600}*	A_{1541}/A_{600}**
Control	0.144 ± 0.001	0.211 ± 0.002	0.079 ± 0.005
H_3PO_4 30"	0.112 ± 0.003	0.161 ± 0.005	0.044 ± 0.009
HCl 120"	0.108 ± 0.006	0.151 ± 0.003	0.061 ± 0.002

* marker of B-type carbonated apatite content

** marker of A-type carbonated apatite content

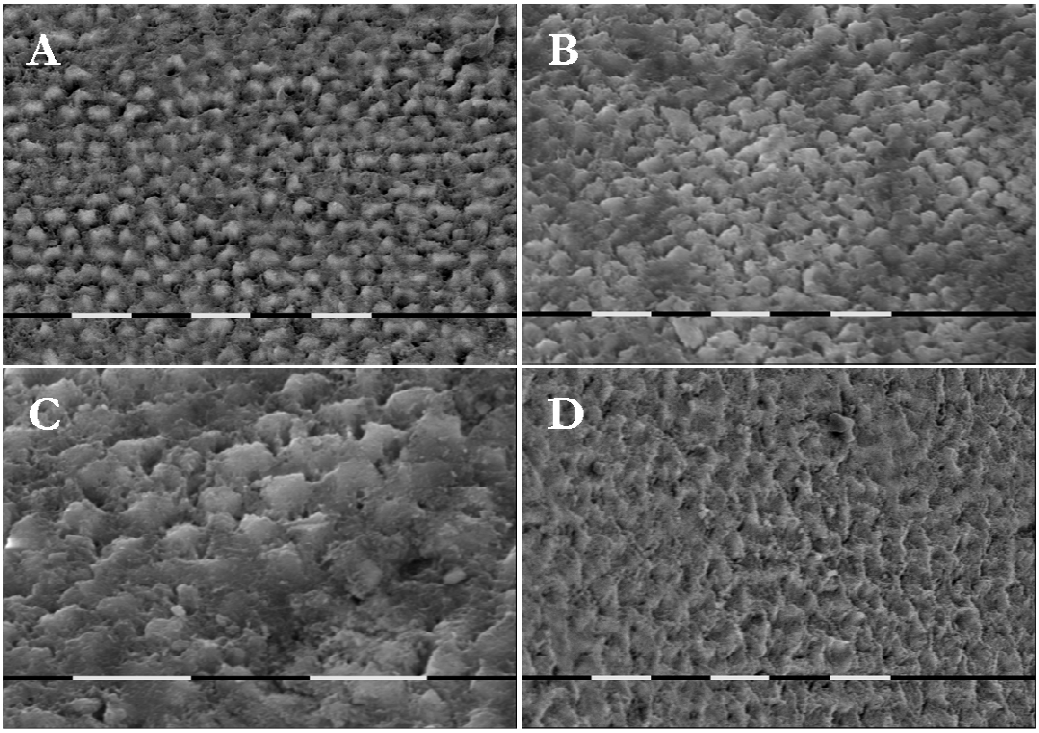


Fig. 4 Scanning electron photomicrographs of H_3PO_4 trated enamel. The acid dissolved both interprismatic and prismatic enamel resulting in the typical honeycomb appearance.

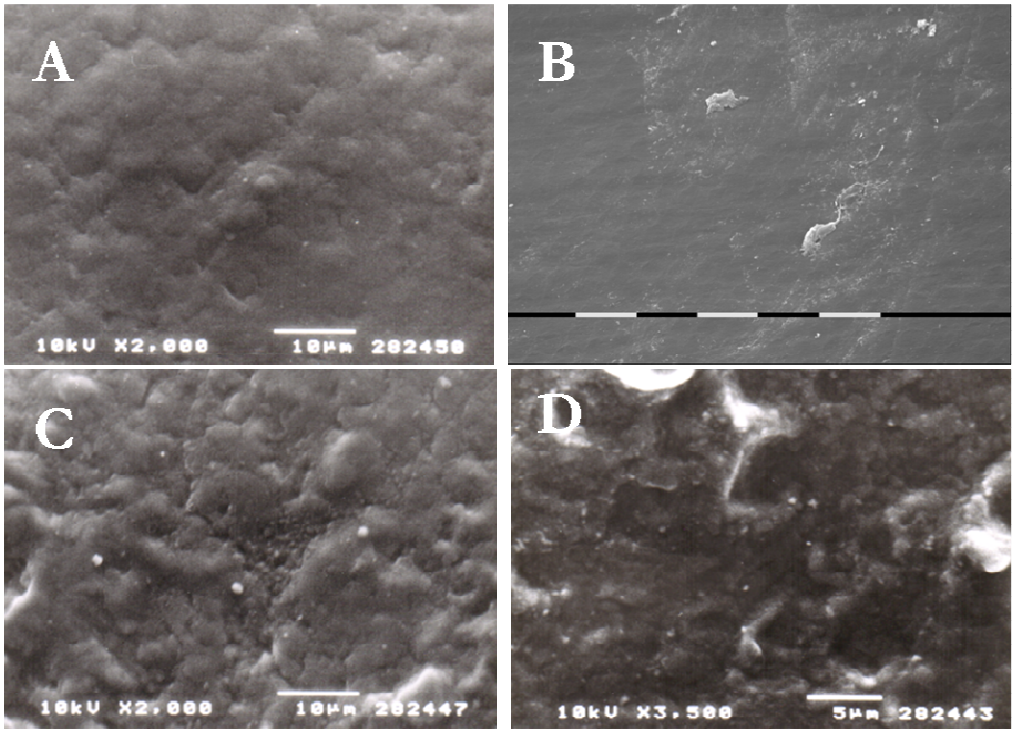


Fig. 5 Scanning electron photomicrographs of replicas of H_3PO_4 treated enamel showed a strongly reduced permeability with no droplets detectable (A,B) Replicas of HCl 15% treated samples showed a maintained permeability, with smaller droplets covering the enamel surface (C,D) Decrease of the enamel organic component, as resulted after H_3PO_4 treatment, involves a decrease in enamel permeability, while the increase of the organic matter (achieved by HCl treatment) still maintains enamel permeability.

Discussion

The surface morphology resulting from etching treatment and the induced chemical alterations are important for evaluation and further improvement of the adhesion systems. Different etching treatments showed different effects on enamel structure due to their specific characteristics (pH, type of acid, proton concentration) (West *et al.*, 2001; Hanning *et al.*, 2005).

Surface erosion is a diffusion-controlled process with a strong relationship between losss of enamel and etching duration (Hermsen *et al.*, 1993; Meyer-Lueckel *et al.*, 2007).

It has been demonstrated that HCl caused linear release of Ca and P, phosphoric acid of Ca. The different erosive effects could be due to specific interactions of organic acids and hydroxyapatite. Mono-, di-, and tri-carboxylic acids are chemisorbed and bonded to hydroxyapatite via ionic interactions (Hanning *et al.*, 2005).

Carboxylic groups are assumed to substitute hydroxyl or phosphate groups on the surface of enamel or hydroxyapatite respectively (Hanning *et al.*, 2005).

In several studies an increased remineralization of initial subsurface lesions could be observed after acid etching (Flaitz and Hicks 1994, Al-Khateeb *et al.*, 2002) thus suggesting and increased access of ions required for mineralization (Meyer-Lueckel *et al.*, 2007).
Another study that investigated whether full remineralization would occur in white spot lesions when the surface porosity was increase by acid-etching concluded that full remineralization was not achieved by etching, by the addition of fluoride, nor by the combination of both treatments in this in vitro study (Al-Khateeb *et al.*, 2000).
It has been demonstrated that the surface layer of enamel caries lesions has a lower pore volume compared with that of the lesion body underneath (Bergman and Linde 1966; Paris *et al.*, 2007) and that pores in enamel, which are filled with organic material and water, act as a diffusion pathway (Kuhar *et al.*, 1997; Dibdin, 1993).
The accessible pore volume in partially demineralised enamel influences the distribution of subsequent mineral loss. The effects might be mediated by changes in ion transport, induced by local diffusion coefficients with changing porosity (Dowker *et al.*, 2003) and the relative mineral content (Naujoks *et al.*, 1967).
In the *in vivo* situation the effects of acids on dental hard tissue are modulated by several factors, such as tissue related factors (local porosities, crystal quality, presence of prismatic enamel) and others as components of acidic beverages like buffering agents, fluoride, calcium and phosphate (Fjeld and Øgaard., 2006; Hobson *et al.*, 2002; Hanning *et al.*, 2005). Also salivary flow rate, mode of drinking and especially the acquired salivary pellicle have great impact on erosive effects (Hanning *et al.*, 2005).
Conventional enamel etching performed with 37% phosphoric acid for 30 seconds involves the loss of mineral crystals (Kim *et al.*, 2006) dissolving the prismless enamel (Johnston *et al.*, 1998) and provides retentive enamel surface (Ajloundi *et al.*, 2004).
Recently it has been demonstrated that 15% hydrochloric acod gel proved to erode surface layer more effectively than 37% phosphoric acid gel (Meyer-Luekel *et al.*, 2007) and that HCl 15% gel for 90-120 s seems to be more suitable for the pre-treatment of natural enamel lesion prior to resin infiltration (Paris *et al.*, 2007).

In this study Raman and IR spectroscopy showed that the treatment with both hydrochloric and phosphoric acids induced a decrease in the carbonate content of the enamel apatite. At the same time, both acids induced the formation of HPO_4^{2-} ions. After H_3PO_4 treatment the bands due to the organic component of enamel decreased in intensity, while increased after HCl treatment.

The results of Raman and IR spectroscopy could be related with the data obtained by monitoring enamel permeability. A decrease of the enamel organic component, as resulted after H_3PO_4 treatment involves a decrease in enamel permeability, while the increase of the organic matter (achieved by HCl treatment) still maintains enamel permeability. The innovative results obtained in this study about the effects of acid etching on fluid outflow allow improving the knowledge on etching and adhesion mechanisms. It has been previously demonstrated that organic components inhibit crystal dissolution and that matrix proteins are essentially removed during mature enamel formation (Veis, 2004) with a post eruptive maturation that decreases enamel permeability and caries susceptibility (Bertcci *et al.*, 2007). The results of this study confirmed that organic matter and permeability are correlated. The organic matter and permeability resulted increased in HCl treated samples in contrast with other results reported in the literature that suggest such treatment for the pre-treatment of enamel prior to resin infiltration (Paris *et al.*, 2007).

Enamel permeability that is related to the porous structure of enamel, could reflect caries susceptibility (Bertacci *et al.*, 2007) and these results suggested a correlation between the amount of the organic matter, enamel permeability and caries.

References

Ajlouni R, Bishara SE, Oonsombat C, Denehy GE. Evaluation of modifying the bonding protocol of a new acid-etch primer on the shear bond strength of orthodontic brackets. Angle Orthod 2004;74:410-3.

Al-Khateeb S, Exterkate R, Angmar-Månsson B, ten Cate JM. Effect of acid-etching on remineralization of enamel white spot lesions. Acta Odontol Scand 2000;58:31-6.

Antonakos A, Liarokapis E, Leventouri T. Micro-Raman and FTIR studies of synthetic and natural apatites. Biomaterials 2007;28:3043-3054.

Apfelbaum F, Diab H, Mayer I, Featherstone JBD. An FTIR study of carbonate in synthetic apatites. J Inorg Biochem 1992;45:277-282.

ASDC J Dent Child 1994;61:21-8.

Bergman G, Lindén L. Techniques for microscopic study of enamel fluid in vivo. J Dent Res 1965; 44: 1409.

Bertacci A, Chersoni S, Davidson CL, Prati C. In vivo enamel fluid movement. Eur J Oral Sci 2007;115:169–173.

Buonocore MG. A simple method of increasing the adhesion of acrylic filling materials to enamel surfaces. J Dent Res 1955;34:849-53.

Casciani FS, Condrate RA in: C. Ean (Ed.), Proceedings of the Second International Congress on Phosphorous Compounds, Phosphorous Publications, Paris, 1980, p. 175.

Dibdin GH. The water in human dental enamel and its diffusional exchange measured by clearance of tritiated water from enamel slabs of varying thickness. Caries Res 1993; 27: 81–86.

Dowker SE, Elliott JC, Davis GR, Wassif HS. Longitudinal study of the three-dimensional development of subsurface enamel lesions during in vitro demineralization. Caries Res. 2003;37:237-45.

Featherstone JDB, Pearson S, LeGeros RZ. An infrared method for quantification of carbonate in carbonated apatites. Caries Res 1984;18:63-66.

Fjeld M, Øgaard B. Scanning electron microscopic evaluation of enamel surfaces exposed to 3 orthodontic bonding systems. Am J Orthod Dentofacial Orthop. 2006;130:575-81.

Flaitz CM, Hicks MJ. Role of the acid-etch technique in remineralization of caries-like lesions of enamel: a polarized light and scanning electron microscopic study.

Hanning C, Hamkens A, Becker K, Attin R, Attin T. Erosive effects of different acids on bovine enamel: release of calcium and phosphate in vitro. Arch Oral Biol 2005;50:541-552.

Hermsen RJ, Vrijhoef MMA. Loss of enamel due to etching with phosphoric or maleic acid. Dental Mat 1993 9;332-336.

Hobson RS, Rugg-Gunn AJ, Booth TA. Acid-etch patterns on the buccal surface of human permanent teeth. Arch Oral Biol. 2002;47:407-12.

Johnston CD, Burden DJ, Hussey DL, Mitchell CA. Bonding to molars--the effect of etch time (an in vitro study). Eur J Orthod. 1998;20:195-9.

Kim JH, Kwon OW, Kim HI, Kwon YH. Acid resistance of erbium-doped yttrium aluminum garnet laser-treated and phosphoric acid-etched enamels. Angle Orthod 2006;76:1052-6.

Kuhar M, Cevc P, Schara M, Funduk N. Enhanced permeability of acid-etched or ground dental enamel. J Prosthet Dent 1997;77:578-82.

Meyer-Lueckel H, Paris S, Kielbassa AM. Surface layer erosion of natural caries lesions with phosphoric and hydrochloric acid gel in preparation for resin infiltration. Caries Res 2007;41:223-230.

Naujoks R, Schade H, Zelinka F. Chemical composition of different areas of the enamel of deciduous and permanent teeth. (The content of Ca, P, CO2, Na and N2). Caries Res. 1967;1:137-43.

Nelson and J.D.B. Featherstone. Preparation, analysis and characterization of carbonated apatites. Calcif. Tissue Int. 1982, 34, S69-S81.

Paris S, Meyer-Lueckel H, Kielbassa AM. Resin infiltration of natural caries lesions. J Dent Res 2007;86:662-666.

Penel G, Leroy G, Bres G. Micro-Raman spectral study of the PO_4 and CO_3 vibrational modes in synthetic and biological apatites. Calcif Tissue Int 1998, 63, 475-481.

Rey C, M. Shimizu M, Collins B, Glimcher MJ. Resolution-enhanced Fourier Transform Infrared spectroscopy study of the environment of phosphate ions in the early deposits of a solid phase of calcium phosphate in bone and enamel, and their evolution with age. Investigation in the ν_4 PO_4 domain. Calcif Tissue Int 1990; 46:384-394.

Sønju Clasen AB, Øgaard B, Duschner H, Ruben J, Arends J, Sønju T. Caries development in fluoridated and non fluoridated deciduous and permanent enamel in situ examined by microradiography and confocal laser scanning microscopy. Adv Dent Res 1997; 11: 442-7.

Veis A. Biomineralization: on the trail of the phosphate. Part II: Phosphophoryn, the DMPs, and more.J Dent Res. 2004;83:6-10.

Vicente A, Bravo LA, Romero M. Self-etching primer and a non-rinse conditioner versus phosphoric acid: alternative methods for bonding brackets. Eur J Orthod. 2006;28:173-8.

West NX, Hughes JA, Addy M. Erosion of dentine and enamel in vitro by dietary acids: the effect of temperature, acid character, concentration and exposure time. J Oral Rehabil 2000;27:875-80.

Wiegand A, Köwing L, Attin T. Impact of brushing force on abrasion of acid-softened and sound enamel. Arch Oral Biol 2007;52:1043-7.

Youssef MN, Youssef FA, Souza-Zaroni WC, Turbino ML, Vieira MM. Effect of enamel preparation method on in vitro marginal microleakage of a flowable composite used as pit and fissure sealant. Int J Paediatr Dent. 2006;16:342-7.

Chapter 9
Appendix

Thesis Abstract

This thesis evaluated *in vivo* and *in vitro* enamel permeability in different physiological and clinical conditions by means of SEM inspection of replicas of enamel surface obtained from polyvinyl siloxane impressions subsequently later cast in polyether impression material. This technique, not invasive and risk-free, allows the evaluation of fluid outflow from enamel surface and is able to detect the presence of small quantities of fluid, visualized as droplets. Fluid outflow on enamel surface represents enamel permeability. This property has a paramount importance in enamel physiolgy and pathology although its effective role in adhesion, caries pathogenesis and prevention today is still not fully understood.

The aim of the studies proposed was to evaluate enamel permeability changes in different conditions and to correlate the findings with the actual knowledge about enamel physiology, caries pathogenesis, fluoride and etchinhg treatments. To obtain confirmed data the replica technique has been supported by others specific techniques such as Raman and IR spectroscopy and EDX analysis.

The first study carried out visualized fluid movement through dental enamel *in vivo* confirmed that enamel is a permeable substrate and demonstrated that age and enamel permeability are closely related. Examined samples from subjects of different ages showed a decreasing number and size of droplets with increasing age: freshly erupted permanent teeth showed many droplets covering the entire enamel surface. Droplets in permanent teeth were prominent along enamel perikymata.

These results obtained through SEM inspection of replicas allowed innovative remarks in enamel physiology. An analogous testing has been developed for evaluation of enamel permeability in primary enamel. The results of this second study showed that primary enamel revealed a substantive permeability with droplets covering the entire enamel surface without any specific localization accordingly with histological features, without changes during aging signs of post-eruptive maturation. These results confirmed clinical data that showed a higher caries susceptibility for primary enamel and suggested a strong relationship between this one and enamel permeability.

Topical fluoride application represents the gold standard for caries prevention although the mechanism of cariostatic effect of fluoride still needs to be clarified. The effects of topical fluoride application on enamel permeability were evaluated. Particularly two different treatments (NaF and APF), with different pH, were examined. The major product of topical fluoride application was the deposition of CaF_2-like globules. Replicas inspection before and after both treatments at different times intervals and after specific additional clinical interventions showed that such globule formed *in vivo* could be removed by professional toothbrushing, sonically and chemically by KOH. The results obtained in relation to enamel permeability showed that fluoride treatments temporarily reduced enamel water permeability when CaF_2-like globules were removed. The *in vivo* permanence of decreased enamel permeability after CaF2 globules removal has been demonstrated for 1 h for NaF treated teeth and for at least 7 days for APF treated teeth.

Important clinical consideration moved from these results. In fact the caries-preventing action of fluoride application may be due, in part, to its ability to decrease enamel water permeability and CaF_2 like-globules seem to be indirectly involved in enamel protection over time maintaining low permeability.

Others results obtained by metallographic microscope and SEM/EDX analyses of orthodontic resins fluoride releasing and not demonstrated the relevance of topical fluoride application in decreasing the demineralization marks and modifying the chemical composition of the enamel in the treated area.

These data obtained in both the experiments confirmed the efficacy of fluoride in caries prevention and contribute to clarify its mechanism of action.

Adhesive dentistry is the gold standard for caries treatment and tooth rehabilitation and is founded on important chemical and physical principles involving both enamel and dentine substrates.

Particularly acid etching of dental enamel enamel has usually employed in bonding procedures increasing microscopic roughness. Different acids have been tested in the literature suggesting several etching procedures. The acid-induced structural transformations in enamel after different etching treatments by means of Raman and IR spectroscopy analysis were evaluated and these findings were correlated with enamel permeability. Conventional etching with 37% phosphoric acid gel (H_3PO_4) for 30 s and etching with 15 % HCl for 120 s were investigated.

Raman and IR spectroscopy showed that the treatment with both hydrochloric and phosphoric acids induced a decrease in the carbonate content of the enamel apatite. At the same time, both acids induced the formation of HPO_4^{2-} ions. After H_3PO_4 treatment the bands due to the organic component of enamel decreased in intensity, while increased after HCl treatment.

Replicas of H_3PO_4 treated enamel showed a strongly reduced permeability while replicas of HCl 15% treated samples showed a maintained permeability. A decrease of the enamel organic component, as resulted after H_3PO_4 treatment, involves a decrease in enamel permeability, while the increase of the organic matter (achieved by HCl treatment) still maintains enamel permeability. These results suggested a correlation between the amount of the organic matter, enamel permeability and caries.

The results of the different studies carried out in this thesis contributed to clarify and improve the knowledge about enamel properties with important rebounds in theoretical and clinical aspects of Dentistry.

Acknowledgments

This thesis is respectfully submitted to prof. Pier Ugo Calzolari, Rector of the University of Bologna, to prof. Sergio Stefoni, Dean of the Faculty of Medicine, University of Bologna, to prof.ssa Marialuisa Zerbini coordinator of the PhD Project, to prof. Carlo Prati, Head of the Department of Oral Sciences, University of Bologna.
This research has been carried out in the Department of Oral Sciences, University of Bologna, in co-operation with other Departments of the University of Bologna.

I wish to thank everyone who has been involved in this project for contribution, assistance and suggestions that increased the quality of this thesis and made my work easier.

First of all I would like to thank prof. Carlo Prati and dott. Stefano Chersoni as promoter and co-promoter of the entire project without whose help and support this experience would not have been possible.
Other important contributors that I would to thank are: prof. Paola Taddei, prof. Carel Leon Davidson, prof. David H.Pashley, prof. Oddone Ruggeri, prof. Giulio Alessandri Bonetti, Paolo Ferrieri, dott. Matteo Zanarini and Elisabetta Pazzi.

I want also to thank Paolo Signorini, my family and all the colleagues of Endodontics Unit for their support through these three years.

Scientific papers published as part of this thesis

Bertacci A, Chersoni S, Davidson CL, Prati C. In vivo enamel fluid movement. Eur J Oral Sci 2007;115:169-173.

Bertacci A, Chersoni S, Davidson CL, Prati C. The double origin of enamel fluid. Eur J Oral Sci 2007;115:523-24.

Chersoni S, Bertacci A, Gandolfi MG, Iacono F, Tay FR, Pashley DH, Prati C. Fluoride and enamel permeability in vivo. In: IADR 86th General Session & Exhibition. Toronto, July 2-5, 2008.

Bertacci A, Taddei P, Prati C, Pashley DH, Tay FR, Gandolfi MG, Chersoni S. Acid treatments modify enamel permeability. In: IADR 87th General Session & Exhibition. Miami, April 1-4, 2009.

Zanarini M, Ruggeri O, Pazzi E, Bertacci A, Alessandri Bonetti G, Prati C. Demineralizzazione dello smalto dopo esposizione ad una soluzione di acido lattico. Valutazione al SEM/EDX ed al microscopio ottico metallografico di due sistemi adesivi ortodontici. Giornale Italiano di Conservativa (in press).

Eur J Oral Sci 2007; 115: 169–173
Printed in Singapore. All rights reserved

European Journal of
Oral Sciences

In vivo enamel fluid movement

Angelica Bertacci[1], Stefano Chersoni[1], Carel Leon Davidson[2], Carlo Prati[1]

[1]Endodontics Unit, Department of Oral Sciences, Alma Mater Studiorum-University of Bologna, Italy; [2]Dental Materials Science, University of Amsterdam, the Netherlands

Bertacci A, Chersoni S, Davidson CL, Prati C. In vivo *enamel fluid movement. Eur J Oral Sci 2007; 115: 169–173. © 2007 The Authors. Journal compilation* © 2007 Eur J Oral Sci

The aim of this study was to visualize fluid movement through dental enamel *in vivo*. Fifty permanent upper central incisors, from subjects aged 10–70 yr, and 5 permanent central just-erupted incisors, from subjects aged 6–7 yr, were included in the study. An impression was obtained by vinyl polyxiloxane, and replicas were then obtained by polyether impression material. The hydrophobic vinyl polyxiloxane material yielded a morphological image *in situ* of outward fluid flow through tooth enamel. The study confirmed *in vivo* that enamel is a permeable substrate, as shown by the presence of droplets on its surface, and demonstrated that age and enamel permeability are closely related. Samples from subjects of different ages showed a decreasing number and size of droplets with increasing age: freshly erupted permanent teeth showed many droplets covering the entire enamel surface. Droplets in permanent teeth were prominent along enamel perikymata.

Dr Angelica Bertacci, Endodontics Unit, Department of Oral Sciences, Alma Mater Studiorum-University of Bologna, via San Vitale 59, 40125 Bologna, Italy

Telefax: + 39–051–225208
E-mail: angelica.bertacci2@unibo.it

Key words: caries; enamel; permeability

Accepted for publication March 2007

Enamel is not a completely dense inorganic material as its prismatic structure also contains water and organic material (1–4). Many studies on enamel have focused on caries research to explain the morphology of demineralization and remineralization (5, 6). Despite what is known about enamel permeability in caries, the efficacy of restorative materials and pulp–dentine–enamel interactions remain unresolved (7).

Throughout the last century, enamel permeability was investigated in different ways, including dye penetration (8), diffusion of organic components (9), inorganic ions (7) or radioactive tracers (10, 11), and water (1, 12, 13). Studies have applied *in vitro* and/or *in vivo* monitoring techniques, ranging from scanning electron microscopy (SEM) (14) and transmission microscopy (15), to the measurement of diffusion coefficients (1, 16), electrical resistance (17, 18) or conductance (4, 19).

The diffusion rate of cariogenic and cariostatic substances, ions and molecules through the aqueous phase in the enamel and pores plays a crucial role in the dynamics of the caries process (20–22) and fluoride treatment (23). These transport processes are significantly affected by enamel porosity and the amount of water available in the tissue (24).

Fluid flowing through enamel is related to permeability: it is important to correlate enamel permeability to age and the extent of enamel demineralization, as caries susceptibility decreases with age (25). In addition, 'post-eruptive (continuing) maturation' (5, 25) could reduce the permeability of enamel, making it clinically important to determine enamel permeability *in situ*, despite the dearth of information currently available (1, 26, 27).

The aim of this study was to visualize fluid flow through tooth enamel *in vivo* in permanent immature and mature teeth using a replica technique and SEM observations to test the effect of enamel 'posteruptive maturation'. The test null hypothesis was that patient age did not affect enamel permeability.

Material and methods

Fifty permanent upper central incisors, with no visual signs of caries, cracks, erosion or restorations, in subjects aged 10–70 yr, and 5 permanent central just-erupted incisors, in subjects aged 6–7 yr, were selected for this study.

Four permanent teeth (premolars), extracted for orthodontic reasons from young patients (range age 20–40 yr), were used as controls. The extractions were carried out with great care to prevent any type of alteration to the enamel surface.

All subjects enrolled in the study (parents for subjects aged 6–17 yr) gave their informed consent to the procedure, which was non-invasive and risk-free.

Enamel surface replica

Each tooth was brushed with a prophylactic paste for 10 s, gently washed and finally air dried for 10 s with a dental chair air syringe (Castellini, Castel Maggiore, Bologna, Italy).

The method used to investigate the morphology of enamel, by detecting the presence of droplets, has been described previously (28,29).

Immediately after enamel preparation, as previously described, an impression of the surface was made using polyvinylsiloxane impression material (Affinis ligth body; Coltene, Alstatten, Switzerland). After 4 min, the material was removed from the enamel surface, degassed for at least 48 h and later cast in polyether impression material (Permadyne Garant; 3M ESPE, St Paul, MN, USA). Samples were gold-sputtered and inspected by a scanning electron microscope (Model 5400; JEOL, Tokyo, Japan).

Evaluation and statistical analysis

High, moderate, and low numbers of droplets were evaluated at ×2000 magnification by two operators, randomly examining, in a double-blind manner, three different points representative of the enamel in the cervical, medium and incisal thirds of each sample.

The following visual scale was employed:

- high: more than 75% of the entire enamel surface was covered with droplets;
- moderate: less than 75% but more than 5% of the entire enamel surface was covered with droplets; and
- low: less than 5% of entire enamel surface was covered with droplets

Statistical analysis was performed by the chi-square test.

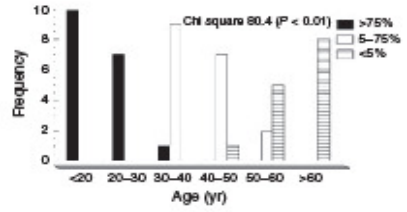


Fig. 1. Percentage distribution of enamel area covered with droplets related to age. Barchart for groups by score.

Results

Figure 1 summarizes the statistical analysis and shows the results related to healthy teeth.

The percentage distribution revealed a strong relationship ($P < 0.01$) with age: data showed that all the samples from subjects aged 6–20 yr presented more than 75% of the enamel surface covered with droplets. Samples from older subjects showed a decreasing percentage: samples from the 30–50 yr age group predominantly presented a moderate (5–75%) percentage, whereas in the 50–60 yr age group the number of samples with a low (< 5%) percentage of enamel area covered with droplets increased up to the last group (age > 60 yr), where all the samples showed less than 5% of the enamel surface covered with droplets (Fig. 2A–D and 3A–D). Figure 4A–D shows details of an enamel pore, an enamel crack, and white spot lesions, respectively.

The number of droplets disclosed by SEM observation confirmed that enamel is a permeable substrate. Our results demonstrated that permeability was related to age: freshly erupted permanent teeth showed more droplets covering the entire enamel surface. Samples from subjects of different ages showed a decreasing number and size of droplets.

Permanent mature teeth showed many droplets mainly localized along the perikymata, and only a few droplets were detected away from these.

In vitro testing on extracted teeth showed a similar morphology. Droplets were still present along the perikymata.

Discussion

Enamel permeability has been demonstrated *in vivo* and *in vitro* (1,4,7). Permeability is more substantial in teeth

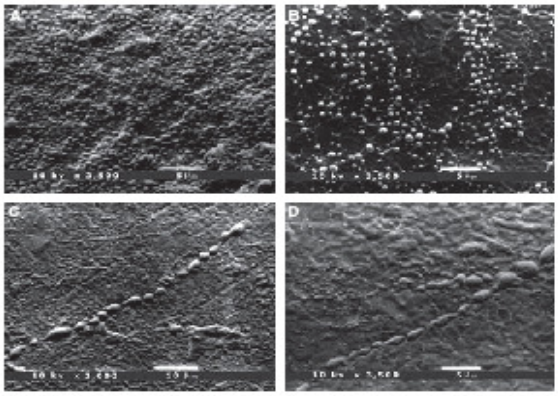


Fig. 2. The arrangement of droplets in samples according to increasing age of the subject. Scanning electron microscopy (SEM) photomicrographs of enamel from 6-yr-old (A) and 17-yr-old (B) patients, showing many more droplets on the enamel surface, covering the whole surface in several areas. Permanent teeth showed many droplets, mainly localized along the perikymata. SEM photomicrograph of 28-yr-old (C) and 30-yr-old (D) patients, showing typical droplet distribution along the perikymata. These droplets measured approximately 1 μm or less in diameter.

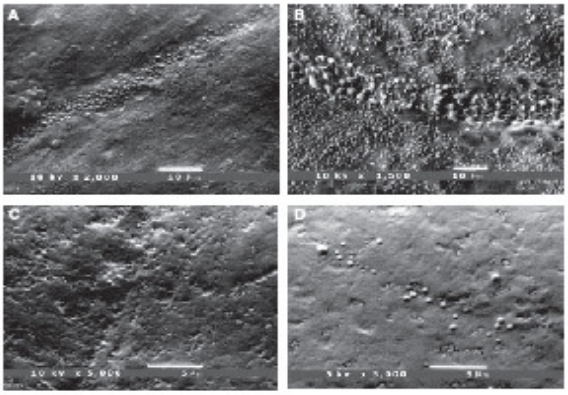


Fig. 3. Scanning electron microscopy (SEM) photomicrograph of 33-yr-old (A) and 39-yr-old (B) patients, showing the perikymata covered with droplets that appeared to be much larger than those of the adjacent enamel. SEM photomicrograph of 67-yr-old (C) and 70- yr-old (D) patients, showing only a few small droplets, probably as a result of the reduced enamel water content.

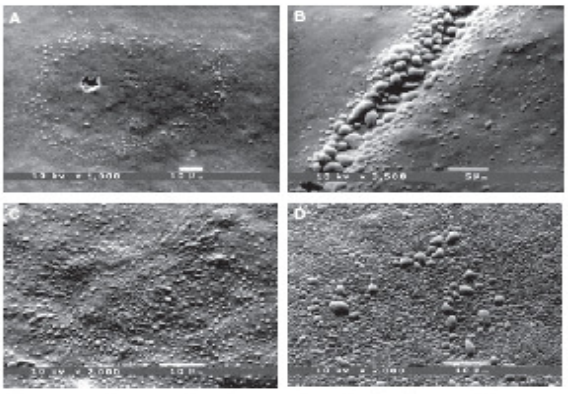


Fig. 4. Details of an enamel pore (A) and an enamel crack (B), and samples of white spot lesions (C,D).

with immature enamel and appears to require a partnership with dentine (7, 30). Permeability is also correlated with enamel pores, which may cause water uptake and release (3). Most permeability studies recorded electrical variables, such as electrical resistance (17, 18) or conductance (4, 19), providing an indirect evaluation of enamel thickness, mineral loss and uptake (4), and enamel porosity (31–34).

The present study yielded qualitative and quantitative findings on outward fluid flow on tooth enamel surfaces *in vivo* by means of scanning electron microscopy inspection of polyether replicas. This technique uses droplet formation to display the discharge of liquid from enamel during the setting time of the impression material, as demonstrated *in vitro* by BARNES (35).

The fluid forming these droplets may come from free, unbound water in blind outer enamel porosities and partly in deeper structures, as suggested by the droplet distribution on enamel surface related to age.

Presumably, the mechanism of droplet formation is simply diffusion. When a water-free impression material is applied to hydrated enamel, water diffuses out of the enamel down its concentration gradient and accumulates over the pores, without wetting or spreading, on the light-bodied hydrophobic material.

Droplet formation appeared to be typical in its location on the enamel surface of permanent mature teeth, with a strong preference for the perikymata.

The enamel surface of recently erupted teeth presents these and other open structures containing proteins produced during tooth development. Indeed, the enamel of freshly erupted permanent teeth showed more discharge of droplets than mature enamel. It is likely that these structures and interprismatic spaces form diffusion pathways, which alter with time in the oral cavity as a result of intermittent pH shift, traumas, and mineral deposition (30).

The results of this study appear to predict that the water content of outer enamel decreases with age. Moreover, increasing enamel maturation and age involve a progressive localization of outward fluid flow on the enamel surface along perikymata that are anatomically correlated to deep enamel structures.

The results of this *in vivo* study, obtained with a new, non-invasive technique, could be correlated to epidemiological data on caries. Recently erupted teeth are more prone to dental caries than teeth that have remained free from caries lesions for a few years after eruption (4), as confirmed by experiments in which artificial caries lesions were produced in extracted teeth of different posteruptive ages (4, 25). This may be ascribed to differences in enamel porosity dependent on intra-oral maturation, presumably caused by congestion of the pathways by deposition of calcium-phosphates in the outer layer of the tooth surface (4).

Therefore, enamel surface alterations, interpreted as posteruptive maturation and, consequently, enamel permeability, are of paramount importance for caries pathogenesis.

Enamel fluid could also interfere with adhesive procedures. On the other hand, clinical procedures, such as acid etching and reshaping of teeth by grinding off some of the enamel outer surface, will increase the permeability of dental enamel. Partial recovery from such damage takes several months *in vivo*, and in the meantime the tooth is more susceptible to carious decay (36).

The replica procedure described identified the location of the pathway openings in the outer surface of tooth enamel *in vivo* by demonstrating fluid outflow, namely along the perikymata. Furthermore, the null hypothesis was rejected; the enamel of freshly erupted teeth presented higher outflow than mature enamel. We speculate that this outflow reflects both enamel permeability and, possibly, caries susceptibility. Specific obstruction of these pathways may increase caries resistance.

Acknowledgements – The authors wish to thank Mr Paolo Ferrieri for SEM assistance and Mr Vincenzo Giordano for planning the figures.

References

1. Lindén LA. Microscopic observations of fluid flowing through enamel *in vitro*. *Odontol Rev* 1968; **19**: 349–365.
2. Pashley DH. Dynamics of the pulpo-dentine complex. *Crit Rev Oral Biol Med* 1996; **7**: 104–133.
3. Shellis RP. A scanning electron-microscopic study of solubility variation in human enamel and dentine. *Arch Oral Biol* 1996; **41**: 473–484.
4. Ten Bosch JJ, Finnis IEY, Verdonschot EH. Time-dependent decrease and seasonal variation of the porosity of recently erupted sound dental enamel *in vivo*. *J Dent Res* 2000; **79**: 1556–1559.
5. Fearnhead RW, Kawasaki K, Inoue K. Comments on the porosity of human tooth enamel. *J Dent Res* 1982; **61**: 1524–1530.
6. Ten Cate M. Remineralization of caries lesions extending into dentine. *J Dent Res* 2001; **80**: 1407–1411.
7. Byers MR, Yoon Lin KJ. Patterns of fluoro-gold entry into rat molar enamel, dentine, and pulp. *J Dent Res* 2003; **82**: 312–317.
8. Tarbet WJ, Forsick LS. Permeability of human dental enamel to acriflavine and potassium fluoride. *Arch Oral Biol* 1971; **16**: 951–961.
9. Borggreven JMPM, Van Dijk JWE, Driessens FCM. A quantitative radiochemical study of ionic and molecular transport in bovine dental enamel. *Arch Oral Biol* 1977; **22**: 467–472.
10. Braden M, Duckworth R, Joyston-Bechal S. The uptake of ^{24}Na by human dental enamel. *Arch Oral Biol* 1971; **16**: 367–374.
11. Joyston-Bechal S, Duckworth R, Braden M. Diffusion of radioactive ions into human dental enamel. *Arch Oral Biol* 1971; **16**: 375–384.
12. Poole DF, Tailby PW, Berry DC. The movement of water and other molecules through human enamel. *Arch Oral Biol* 1963; **38**: 771–772.
13. Shellis RP. Transport processes in enamel and dentine. In: Addy M, Embery G, Edgar WM, Orchardson R, eds. *Tooth wear and sensitivity*. London: Martin Duniz, 2000; 19–24.
14. Whittaker DK. Structural variations in the surface zone of human tooth enamel observed by scanning electron microscopy. *Arch Oral Biol* 1982; **27**: 383–392.
15. Poole DFG, Newman HN, Dibdin GH. Structure and porosity of human cervical enamel studied by polarizing microscopy and transmission electron microscopy. *Arch Oral Biol* 1981; **26**: 977–982.
16. Borggreven JMPM, Driessens FCM, Van Dijk JWE. Diffusion through bovine tooth enamel as related to the water structure in its pores. *Arch Oral Biol* 1980; **25**: 345–348.
17. Hoppenbrouwers PMM, Schoilberg HPF, Borggreven JMPM. Measurement of the permeability of dental enamel and its variation with depth using an electrochemical method. *J Dent Res* 1986; **65**: 154–157.
18. Wang J, Someya Y, Inaba D, Longbottom C, Miyazaki H. Relationship between electrical resistance measurement and microradiographic variables during remineralization of softened enamel lesions. *Caries Res* 2005; **39**: 60–64.
19. Ie YL, Verdonschot EH, Schaeken MJ, Vant'Hof MA. Electrical conductance of fissure enamel in recently erupted molar teeth as related to caries status. *Caries Res* 1995; **29**: 94–99.
20. Van Dijk JW, Borggreven JM, Driessens FC. Diffusion in mammalian tooth enamel in relation to the caries process. *Arch Oral Biol* 1983; **28**: 591–597.
21. Featherstone JDB. Diffusion phenomena and enamel caries development. In: Guggenheim, B, ed. *Cariology Today*. Basel: Karger, 1983; 259–268.
22. Lindén LA, Björkman S, Hattab F. The diffusion *in vitro* of fluoride and chlorhexidine in the enamel of human deciduous and permanent teeth. *Arch Oral Biol* 1986; **31**: 33–37.
23. Dibdin GH. The water in human dental enamel and its diffusional exchange measured by clearance of tritiated water from enamel slabs of varying thickness. *Caries Res* 1993; **27**: 81–86.
24. Moreno EC, Zahradnik RT. The pore structure of human dental enamel. *Arch Oral Biol* 1973; **18**: 1063–1068.
25. Kotsanos N, Darling AI. Influence of posteruptive age of enamel on its susceptibility to artificial caries. *Caries Res* 1991; **25**: 241–250.
26. Bergman G, Lindén L. Techniques for microscopic study of enamel fluid *in vivo*. *J Dent Res* 1965; **44**: 1409.

27. Bakhos Y, Brudevold F, Aasenden R. *In vivo* estimation of the permeability of surface human enamel. *Arch Oral Biol* 1977; **22**: 599–603.
28. Ithagarun A, Tay FR. Self-contamination of deep dentin by dentin fluid. *Am J Dent* 2000; **13**: 195–200.
29. Cheroni S, Suppa P, Breschi L, Ferrari M, Tay FR, Pashley DH, Prati C. Water movement in the hybrid layer after different dentin treatments. *Dent Mater* 2004; **20**: 796–803.
30. Shellis RP, Dibdin GH. Enamel microporosity and its functional implications. In: Teaford MF, Ferguson MJ, Smith MM, eds. *Teeth: development, evolution and function*. Cambridge: Cambridge University Press, 2000; 242–251.
31. Flaitz CM, Hicks MJ, Silverstone LM. Radiographic, histologic, and electronic comparison of occlusal caries: an *in vitro* study. *Pediatr Dent* 1986; **8**: 24–28.
32. Rock WP, Kidd EA. The electronic detection of demineralization in occlusal fissures. *Br Dent J* 1988; **164**: 243–247.
33. Huysmans MC, Verdonschot EH, Rondel P. Electrical conductance and electrode area on sound smooth enamel in extracted teeth. *Caries Res* 1995; **29**: 88–93.
34. Ricketts DN, Kidd EA, Liepins PJ, Wilson RF. Histological validation of electrical resistance measurement in the diagnosis of occlusal caries. *Caries Res* 1996; **30**: 148–155.
35. Barnes IE. The adaptation of composite resins to tooth structure. Part 1. Study 1: introduction and the adaptation of composite resins to the unetched enamel cavity wall. *Br Dent J* 1977; **142**: 122–129.
36. Kuhar M, Civc P, Schara M, Funduk N. *In vitro* permeability and scanning electron microscopy study of acid-etched and ground enamel surfaces protected with dental adhesive coating. *J Oral Rehabil* 1999; **26**: 722–730.

Eur J Oral Sci 2007; 115: 522–524
Printed in Singapore. All rights reserved

European Journal of
Oral Sciences

Letter to the Editor

The origin of enamel fluid

Dear Editor,

I read with interest the recent article by BERTACCI *et al.* (1) in which the authors described the fluid droplets on the enamel surface and demonstrated that the amount of fluid is age dependent. The article certainly is a welcome addition to the field. The authors speculated in the Discussion that the reason behind the observed droplet formation is presumably diffusion. This may be the case; however, there is another possible explanation that the authors do not present in the article – the droplets may represent dentinal fluid.

The authors refer to an excellent article by BYERS & YOON LIN (2), in which the permeability of enamel was demonstrated with external fluoro–gold enamel labelling. This article demonstrated label penetration through enamel and dentin all the way to the odontoblast layer, once again showing that instead of being solid, dental hard tissues are merely semipermeable, allowing influx of label and, presumably, bacterial acids and toxins, among others. There is at least one article showing that fluid transportation also occurs in the other direction, all the way from the pulp tissue to the enamel surface. SOGNNAES *et al.* (3) demonstrated that injected radioisotopes may penetrate dentin and enter enamel from the pulp–dentin complex.

The odontoblast cell layer has been suggested to form a functional barrier between pulp tissue and dentin, controlling at least the transport of molecules into the dentinal fluid (4, 5). In the case of caries and cavity preparation, during which the tight junctions between odontoblasts may be altered (4, 5), pulpal cells might produce fluid diffusion into the extracellular space and into dentin. Therefore, the fluid would be a physiologic response to trauma (5, 6). However, even in intact teeth, studies with radioisotopes (3, 7) and fluorescent dyes (6, 8, 9) have shown a transport mechanism to exist between the blood circulation and dentin. The rate of dentinal fluid flow is affected by several factors, including, for example, dentinal tubule size and patency, reparative dentin formation (reviewed by PASHLEY, ref. 10), and high dietary sucrose levels (6, 8, 9). Decrease in tubule size and patency with time could lead to the age-related decrease in enamel fluid observed by BERTACCI *et al.* (1). There is also some evidence that an endocrine system, controlling the rate of dentinal fluid flow, exists (9, 11).

BERTACCI *et al.* (1) also demonstrated that the enamel of freshly erupted teeth presented higher fluid outflow than mature enamel, and they speculated that this outflow reflects both enamel permeability and, possibly, caries susceptibility. This may very well be true, even if the fluid in question originates from the dentin–pulp complex. The role of dentinal fluid in dental caries has traditionally been thought to be protective: outward flow should protect the tooth from bacterial toxins and acidic challenge. Dentinal fluid in carious teeth has been indicated to have higher concentrations of mineral elements than dentinal fluid in intact dentin (12), and supersaturated pulpal fluid reduces dentinal caries progression *in vitro* (13). Reduction in dentinal fluid flow caused by a high-sucrose diet (6, 8, 9) would disturb this protective effect, thus predisposing the tooth to caries, especially in young teeth with wide and patent tubules. However, experimental data indicates that devitalization of teeth may actually reduce the caries caused by high dietary sucrose or desalivation (8, 14, 15). It has even been proposed that carbohydrates needed for hidden carious lesions might originate from blood and penetrate through dentinal tubules into the lesion area (16). Interestingly, BROWN & LEFKOWITZ (14) observed a significant reduction also in enamel caries after the devitalization of teeth.

It is surprising to see how little we actually know of the content, function, fate, and even origin of dentinal fluid, and what its role and importance is in caries. Hopefully, the article by BERTACCI *et al.* (1) will lead to increased interest in this fascinating component of the tooth.

References

1. BERTACCI A, CHERSONI S, DAVIDSON CL, PRATI C. *In vivo* enamel fluid movement. *Eur J Oral Sci* 2007; **115**: 169–173.
2. BYERS MR, YOON LIN KJ. Patterns of fluoro-gold entry into rat molar enamel, dentine, and pulp. *J Dent Res* 2003; **82**: 312–317.
3. SOGNNAES RF, SHAW JH, BOGGROCH R. Radiotracer studies on bone, cementum, dentin and enamel of rhesus monkeys. *Am J Physiol* 1955; **180**: 408–420.
4. TURNER DF, MARFURT CF, SATTELBERG C. Demonstration of physiological barrier between pulpal odontoblasts and its perturbation following routine restorative procedures: a horseradish peroxidase tracing study in the rat. *J Dent Res* 1989; **68**: 1262–1268.

5. Turner DF. Immediate physiological response of odontoblasts. *Proc Finn Dent Soc* 1992; **88**(Suppl I): 55–63.
6. Leonora J, Tjäderhane JM, Steinman RR. The effect of dietary factors on intradentinal dye penetration in the rat. *Arch Oral Biol* 1992; **37**: 733–741.
7. Potts TV, Cunningham T, Finkelstein MJ, Silverberg-Strumfeld L. The movement of radioactive molecules across dentine in vivo in the dog. *Arch Oral Biol* 1985; **30**: 353–357.
8. Steinman R R, Leonora J, Singh RJ. The effect of desalivation upon pulpal function and dental caries in rats. *J Dent Res* 1980; **59**: 176–185.
9. Leonora J, Tjäderhane JM, Steinman RR. Further evidence for a hypothalamus-parotid gland endocrine axis in the rat. *Arch Oral Biol* 1993; **38**: 911–916.
10. Pashley DH. Dynamics of the pulpo-dentin complex. *Crit Rev Oral Biol Med* 1996; **7**: 104–133.
11. Tjäderhane JM, Leonora J, Steinman RR. High-sucrose diet inhibits basal secretion of intradentinal dye penetration-stimulating parotid hormone in pigs. *J Appl Physiol* 1994; **76**: 218–222.
12. Larmas M, Häyrynen H, Lajunen L. Sodium, potassium, calcium, magnesium and phosphate contents of dentinal fluid and gingival crevicular fluid in health and disease. In: Leiner T, Cimasoni G, eds *The borderland between caries and periodontal disease III*. Geneve: Editions Médecine et Hygiéne, 1986; 105–110.
13. Shellis RP. Effects of a supersaturated pulpal fluid on the formation of caries-like lesions on the roots of human teeth. *Caries Res* 1994; **28**: 14–20.
14. Brown LR, Lefkowitz W. Influences of dentinal fluids on experimental caries. *J Dent Res* 1966; **45**: 1493–1498.
15. Steinman RR. Physiologic activity of the pulp-dentin complex. *Quintessence Int* 1985; **16**: 723–726.
16. de Soet JJ, Weerheijm KL, van Amerongen WE, de Graaff J. A comparison of the microbial flora in carious dentine of clinically detectable and undetectable occlusal lesions. *Caries Res* 1995; **29**: 46–49.

Leo Tjäderhane
Institute of Dentistry, University of Helsinki,
Helsinki, Finland
E-mail: leo.tjaderhane@helsinki.fi

Response

The double origin of enamel fluid

Dear Editor,

We thank Prof. Tjäderhane for his interest in our study and for his comments on it. The actual understanding of tooth enamel still does not allow the unambiguous identification of the real origin of fluid flow. However, some conclusions can be derived from the results of our experiments (1) and from the literature on age changes and permeability in dental tissues. Our observations of the distribution of droplets on the enamel surface confirmed that interprismatic spaces, tufts, and lamellae form diffusion pathways, which are changed with time in the oral cavity. We suggested that the fluid which forms these droplets may originate partly from the diffusion of free, unbound water in outer enamel porosities and partly from deeper structures.

The role of dentinal fluids in droplet formation on enamel was not assessed in our experiments. As droplet formation was also present on enamel samples from which dentin had been removed (unpublished results), dentinal fluids are unlikely to be the only source of fluid giving the demonstrated effect.

Moreover, it has been shown that enamel behaves as a permeable membrane to small ions and as a semipermeable membranes to large molecules (2), and that anions do not pass through enamel as readily as water or cations (3).

The osmotic pressure of saliva is about half that of blood and tissue fluid (4) so, under physiological conditions, water tends to be drawn into the tooth.

The *in vivo* enamel dehydration under a rubber dam may be caused by an inward flow of fluid at an osmotic gradient. If outward fluid flow from dentine to enamel existed, the evaporation process would absorb any underlying fluid and thus would not allow dehydration. However, when the outward osmotic pressure in enamel increases (e.g. in the presence of plaque or a concentrated sugar solution), fluid would be likely to move from dentin into enamel and through the enamel to the enamel surfaces that are immersed in saliva. It may be speculated that this type of osmotic change plays a role in caries pathogenesis.

As the permeability of dentin is far greater than that of enamel, a proportional age-related permeability decrease for dentin is required to explain the age-related droplet formation on enamel under physiological conditions, as observed in our experiments.

We can speculate that enamel fluids have a multiple, or at least a dual, origin that is dependent on the osmotic balance between dentinal fluids on one side and saliva on the other side, where the latter plays a protective role in posteruptive maturation.

Prof. Tjäderhane's suggestion of the significance of dentinal fluids will certainly be borne in mind in our further studies on identification of the origin of enamel fluids.

References

1. Bertacci A, Chersoni S, Davidson CL, Prati C. *In vivo* enamel fluid movement. *Eur J Oral Sci* 2007; **115**: 169–173.
2. Atkinson HF. An investigation into the permeability of enamel using osmotic methods. *Br Dent J* 1944; **83**: 205–214.
3. Poole DF, Tarlby PW, Berry DC. The movement of water and other molecules through human enamel. *Arch Oral Biol* 1963; **38**: 771–772.
4. Jenkins GN. Permeability and age changes in the dental tissues. In: Jenkins GN, ed. *The physiology and biochemistry of the mouth*, 4th edn. Oxford: Blackwell, 1978; 170–177.

Angelica Bertacci[1]
Stefano Chersoni[1]
Carel Leon Davidson[2]
Carlo Prati[1]
[1]Endodontics Unit, Department of Oral Sciences, Alma Mater Studiorum-University of Bologna, Italy;
[2]Dental Materials Science, University of Amsterdam, the Netherlands
E-mail: angelica.bertacci2@unibo.it

Other scientific papers published on journals with impact factor

Clin Oral Invest
DOI 10.1007/s00784-007-0127-y

ORIGINAL ARTICLE

The influence of smear layer in lateral channels filling

Angelica Bertacci · Chiara Baroni · Lorenzo Breschi · Mauro Venturi · Carlo Prati

Received: 20 June 2006 / Accepted: 14 May 2007

Abstract This in vitro study evaluated the ability of a warm gutta-percha obturation system Thermafil to fill lateral channels in presence/absence of smear layer. Forty single-rooted extracted human teeth were randomly divided into two groups for which different irrigation regimens were used: group A, 5 ml of 5% NaOCl + 2.5 ml of 3.6% H_2O_2; group B, 5 ml of 5% NaOCl 5% + 2.5 ml of 17% ethylenediamine tetraacetic acid. A conventional crown-down preparation technique was employed. Obturation was performed using epoxy resin-based cement (AH Plus) and a warm gutta-percha plastic carrier system (Thermafil). Specimens were cleared in methyl salicylate and analyzed under a stereomicroscope to evaluate the number, length, and diameter of lateral channels. Lateral channels were identified in both groups at medium and apical thirds. Additional samples were prepared for scanning electron microscopy inspection to confirm the presence of smear layer in group A, and the absence of smear layer in group B. All lateral channels resulted filled in both groups. No statistically significant differences regarding number, length, and diameter were observed between the two groups. Smear layer did not prevent the sealing of lateral channels.

A. Bertacci (✉) · C. Baroni · M. Venturi · C. Prati
Endodontics Unit, Department of Oral Sciences,
University of Bologna,
via San Vitale 59,
40125 Bologna, Italy
e-mail: angelica.bertacci2@unibo.it

L. Breschi
UCO of Dental Sciences,
University of Trieste,
Trieste, Italy

Keywords Smear layer · Sealing lateral channels · Warm gutta-percha · Thermafil · Irrigants

Introduction

The anatomical complexity of the endodontic canal system limits the likelihood of achieving a complete filling after chemo-mechanical treatment. Besides the infection of dentinal tubules [17], the presence of lateral channels represents a potential clinical issue related to the presence of bacteria colonizing their lumen [30, 31]. Harbored by pulpal or inorganic debris, many bacteria may reside in these areas and concur with pathological conditions. These areas may be repopulated by other bacteria, such as *Enterococcus faecalis*, and may be the principal reason for a secondary endodontic disease or refractory infection [13, 28, 30, 31]. Endodontic sealers and gutta-percha should close and fill lateral channels and dentinal tubules to prevent bacterial growth and percolation of bacteria and their by-products through the apex [5, 25, 33].

Among the various systems proposed to fill and seal endodontic channels after instrumentation, Thermafil represents a relatively straightforward and standardizable clinical choice [2, 4, 12, 26, 33].

A recent study [31] proposed a novel clearing technique which allows the adaptation of gutta-percha and sealer to endodontic dentinal walls to be evaluated and both filled and unfilled lateral channels to be observed. Type of sealer may influence the outcome of the injection of lateral channels [31] and its distribution in root canals [34].

Alone or in association with H_2O_2, NaOCl is frequently used as irrigant solution because of its well-known proteolytic activity [1] and its antimicrobial action [18]. Unfortunately, it is not able to remove smear layer [1].

Endodontic instrumentation creates a smear layer on the root canal walls that occludes dentinal tubules and may protect microorganism from the effects of NaOCl irrigation [5, 8]. Moreover, smear layer could support the growth of entrapped bacteria predisposing periapical reactions [5].

The influence of smear layer in the filling ability of a sealing technique is not fully clarified, as its presence might represent, at least in theory, an obstacle to the penetration of sealer and gutta-percha inside lateral channels [21, 29].

Several authors have suggested that ethylenediaminetetraacetic acid (EDTA) solutions or EDTA-based lubricants acting on the inorganic residue may contribute to removing smear layer [10, 16], and these are frequently used in endodontic therapy.

The aim of this study was to evaluate the extent to which smear layer prevents the filling of lateral channel with gutta-percha and sealer. The test null hypothesis was that the presence of smear layer impaired the filling of lateral channels.

Materials and methods

Sample preparation

Forty non-carious extracted single-root, single canal human teeth were used in this study.

All the teeth included in the study were necrotic, as confirmed radiographically by the presence of periapical lesions.

Teeth were equally divided in two groups with a homogeneous distribution regarding tooth type and apical diameter. Roots with resorption, fractures, or open apices were preventively discarded. The root canals selected for the study had an initial apical diameter of 0.20 to 0.30 mm.

Apical foramina were directly measured on the tooth using an optical microscope (Kaps SOM 62 standard, Karl Kaps GMBH åt KG, Asslar, Germany).

Calculus or debris on the root surface were removed before endodontic treatment using number 7/8 Gracey curettes (Hu-Friedy, Chicago, IL, USA). All specimens were prepared by the same clinician.

The crown of each tooth was removed using a tapered diamond bur (#845.314.012 Komet Brasseler, Lemgo, Germany) mounted on a contra-angle high-speed hand piece (Ceramic, Castellini, Bologna, Italy).

Root instrumentation was performed using conventional crown-down technique followed by a step-back technique. Stainless steel K-files (F.K.G. Dentaire, La Chaux-de-Fonds, Switzerland) and Gates Glidden drills (Dentsply-Maillefer, Baillagues, Switzerland) were used. The apical instrumentation was performed to obtain a final apical diameter of 0.30 or 0.40 mm depending on tooth anatomy.

Two different irrigation regimens were used in two designed experimental groups: group A, (n=20 roots) 5 ml of 5% NaOCl solution (Niclor-5, Ogna, Milan, Italy) followed by 2.5 ml of 3.6% hydrogen peroxide solution (Ogna, Milan, Italy); group B (n=20 roots) 5 ml of 5% NaOCl solution (Niclor-5, Ogna) followed by 2.5 ml of 17% EDTA (Ogna, Milan, Italy).

Irrigation was repeated after the use of each instrument and NaOCl solution operated for 20 min.

After canal preparation, a final 1 ml aliquot of 17% EDTA solution was left in situ for 2 min and replaced by 1 ml of 5% NaOCl for 3 min.

All irrigation procedures were delivered with a 25-gauge needle (Molteni, Firenze, Italy) inserted in the canal half-length.

Thermafil (Thermafil, Tulsa Dental, Tulsa, OK, USA) was used according to manufacturer instructions. The Thermafil carrier was selected according to the size of the gauging master apical file. A small amount of sealer (AH Plus, Dentsply DeTrey GmbH, Konstanz, Germany) was positioned inside each canal using a small K-file (no. 15). Immediately after the canal obturation, each sample was filled with Coltosol (Coltene, Switzerland) in the coronal aspect and immediately immersed in tap water for 24 h.

All teeth were then immersed for 14 days in a demineralizing solution composed of 9% formic acid, 8% hydrochloric acid, and 10% sodium citrate. The solution was changed every 3 days while specimens were kept under continuous agitation (using an agitator 722 by Asal srl, Milan, Italy) during the whole procedure. At the end of the demineralizing process, two specimens (a maxillary lateral incisor and a mandibular second bicuspid) revealed a longitudinal fracture and were discarded. The roots were then rinsed in running tap water for 2 h, immersed in 99% acetic acid overnight, rinsed again in distilled water, dehydrated in ascending ethanol from 25% to 100%, and finally cleared and stored in methyl salicylate (Sigma, St Louis, MO, USA).

Optical microscope was used with magnifications increasing from 5 to 40×, as performed in a previous study [31], and aided by the use of a micrometer to detect the number, the diameter, and the length of lateral channels. Diameter of lateral channels was measured at the opening along the wall of the root canal. Lateral channels were recorded, making a note of different filling within apical and middle third of the roots.

Statistical analysis

Data were analyzed by applying logistic regression analysis performed using STAT 7.0 (STATA, College Station, TX, USA).

Scanning electron microscopy preparation

Additional samples (n=12, six for each group) were prepared using conventional crown-down technique followed by a step-back technique as described above. No root canal filling was made so that the dentin surface could be observed after irrigation with NaOCl (n=6) or EDTA (n=6). Once prepared, each sample was immediately immersed in 4% glutaraldehyde in 0.2 M sodium cacodylate buffer solution before preparation for scanning electron microscopy (SEM) inspection. Each sample was then longitudinally fractured, dehydrated in graded concentration alcohol, dried in a critical point drier (E 3000; Polaron, West Sussex, UK), then gold-sputtered (Sputter Coater; SPI, Toronto, Canada) and observed under SEM (JEOL, JSM 5200, Tokyo, Japan). Two photomicrographs were obtained at a magnification of 2,000× at coronal, medium, and apical thirds.

Additional root samples were prepared according to the method described above (NaOCl: n=4; EDTA: n=4) then filled using the Thermafil system and immersed in tap water for 1 week at 37°C. Each sample was then transversally sectioned with a slow-speed diamond saw underwater to obtain three different root segments, approximately in the middle of each third. Each segment was conserved and fixed in 4% glutaraldehyde in 0.2 M sodium cacodylate buffer solution at 4°C, dehydrated in graded concentration alcohol, dried in a critical point drier (E 3000; Polaron, West Sussex, UK) then gold-sputtered (Sputter Coater; SPI, Toronto, Canada), and observed using SEM (JEOL, JSM 5200, Tokyo, Japan). Two photomicrographs were obtained at a magnification of 50×.

All images were saved digitally using specific software (SemAfore; JEOL) and scored in a double-blind manner by two trained operators.

Results

The clearing technique allows the observation of all the lateral channels (both filled and unfilled ones). The samples observed showed no unfilled channels: All lateral channels were at least partially filled even only by the sealer. The percentage of unfilled channels was nought in both groups despite the anatomical variation between the two groups.

Table 1 reports the number of lateral channels observed in the two groups according to the kind of teeth included in the study and to their locations in medium or apical third of both maxillary and mandibular teeth. A total of 130 lateral channels were identified along the roots, most of them being localized in the apical third. The presence of lateral channels was evenly distributed between maxillary and mandibular teeth.

Table 1 Number of lateral channels observed in the two experimental groups and distribution of observation according to their localization in the apical and medium thirds

	$NaOCl + H_2O_2$		NaOCl + EDTA		Total
	Apical third	Middle third	Apical third	Middle third	
Central maxillary incisors	9	6	2	5	22
Lateral maxillary incisors	0	0	1	14	15
Central mandibular incisors	8	0	15	2	25
Lateral mandibular incisors	0	0	4	1	5
Maxillary canines	8	9	4	0	21
Mandibular canines	1	0	0	0	1
Second maxillary premolars	1	9	0	0	10
First mandibular premolar	5	3	10	4	22
Second mandibular premolar	0	0	8	1	9
Total	32	27	44	27	130

Table 2 reports a descriptive statistic regarding the length (μm) and the diameter (μm) of lateral channels in the two groups at apical and medium thirds observed under optical microscope. Samples treated with NaOCl + EDTA compared to samples treated with NaOCl and H_2O_2 revealed a small increase (not statistically significant) in the number of lateral channels identified. Maximum, minimum, average,

Table 2 Length (μm) and diameter (μm) of lateral channels observed under optical microscope

Irrigation	$NaOCl/H_2O_2$		NaOCl/EDTA	
Length	Apical third	Medium third	Apical third	Medium third
Mean	161.72 (±228.11)	757.37 (±1519.67)	253.86 (±336.47)	373.89 (±540.42)
Min–max	30–1,200	20–6,500	20–1,500	60–2,000
Diameter	Medium third	Apical third	Medium third	Apical third
Mean	135.53 (±223.05)	54.79 (±64.56)	81.93 (±85.13)	71.77 (±62.47)
Min–max	20–1,000	3–300	4–400	4–300

and SD value of the length and the diameter of lateral channels were reported.

Lateral channels of the apical thirds were shorter and thinner compared with lateral channels at the middle thirds ($P<0.01$).

Table 3 reports lateral channel diameter opening for the two groups of irrigants tested. An interesting result was that the diameter of the majority of the lateral channels (81 out of 130) was less than 50 μm. A great number of lateral channels, with larger diameter and longer length, was reported in the apical third of specimens treated with NaOCl + EDTA compared to NaOCl + H_2O_2, which revealed higher values for the same parameters in the middle third, but both the results were not statistically significant.

Stereomicroscope analysis

Stereomicroscopic images of cleared roots showing the filling of lateral channels at different distances from the apex both in presence and in absence of smear layer are shown in (Figs. 1 and 2).

Scanning electron microscopy analysis

Specific areas were observed at a magnification of 50×. SEM evaluations confirmed the presence of the plastic carrier and of gutta-percha (Fig. 3). Several limited gaps were observed along the sections, probably due to preparation artifacts. Sealer thickness was observed at the interface between gutta-percha and dentin.

After treatment with NaOCl, dentin samples were completely covered by smear layer and smear plugs. The morphology of dentin was similar at coronal, medium, and apical thirds. In several samples, apical thirds were partially covered by dentin debris (dimensions: 3–45 μ) and presented limited grooves with small area of predentin partially covered by debris.

Table 3 Distribution of the diameter of the opening (orifices) of lateral channel in the two experimental groups

	NaOCl + H_2O_2	NaOCl + EDTA
≤10 μ	10	5
11–20 μ	9	7
21–50 μ	19	41
51–100 μ	9	18
101–150 μ	6	5
151–200 μ	0	1
201–300 μ	5	3
301–400 μ	0	1
>400 μ	1	0
Total	59	71

Fig. 1 Mandibular canine of the NaOCl–H_2O_2 group: the filling of apical bifurcation and lateral branches was detected

EDTA samples presented a smooth and smear layer free dentin with all dentinal tubules fully opened. Only apical thirds presented limited areas of compacted and partially layered smear layer islands.

Discussion

As no mechanical instrumentation can completely reach into all the root canal surface because of the complexity of the anatomy of the root canal system, the only clinical tools that can be used to reduce bacterial colonization are irrigants and filling materials [28, 30]. Rud and Andreasen [23] revealed that incomplete filling of lateral channels, as probably also the coronal leakage, may cause failure of endodontic treatment, as these empty areas represent pathways for bacteria and diffusion of toxins between endodontic and periodontal tissues. Moreover, endodontic failures that could be ascribed to incomplete sealing of lateral channels resulted in complete healing after filling of these areas [23].

Fig. 2 Second mandibular premolar of the NaOCl–EDTA group: apical branches of root canal were filled

Thus, the use of sealing materials able to penetrate and to fill lateral channels could be seen as the correct approach to prevent any further contamination and diffusion of bacteria present in the deepest part of dentinal tubules and lateral channels.

Previous studies indicated that filling techniques involving the use of thermoplasticized gutta-percha are very effective in filling the main root canal and lateral channels [9, 22]. Venturi et al. [31] described adequate filling of the lateral channels using a combined warm technique. In clinical use, Thermafil has been compared to cold lateral compaction technique when used with different endodontic sealers [3, 6, 9, 12, 26]. It still represents a simple and not clinician-sensitive method to fill root canals.

The clearing procedures used in this study allowed to be identified in both groups (NaOCl- and EDTA-treated samples) all lateral channels filled even only by the sealer. This data suggest that the insertion of Thermafil obturators toward the apical region may exert sufficient pressure to force endodontic sealer (AH Plus) and heated gutta-percha inside lateral channels. It is also plausible that smear layer and smear plugs may be pushed inside the lateral channels, especially in the NaOCl-treated root canals. The transparency induced by the clearing procedure could have been so good that it prevented the smear layer and smear plugs from being identified.

AH Plus was chosen for this study due to its low viscosity, which means it flows into thin spaces when used with warm gutta-percha obturation techniques [5, 13]. A previous study [31] found AH Plus to be a better sealer compared to other non-resinous endodontic sealers, agreeing with Haïkel et al. [11] who reported better performance of resin-based endodontic cements than non-resin-based ones.

Smear layer could be defined as a complex mixture of inorganic and organic particles constituting of dentinal collagen, pulpal debris, bacteria, and inorganic debris such as apatite [21, 27], created by all endodontic instruments [8]. Different endodontic procedures may produce a different amount of debris and a different morphology of smear layer [21] that may be greatly affected by design of instruments and methods of application and by the type of irrigation. Manual instrumentation with K-file produced a fine multilayered smear layer. Sodium hypochlorite solution is able to remove pulpal debris and dentin collagen, but leaves smear layer intact [20, 29].

There has been a considerable debate about smear layer impact on endodontic treatment outcome [27, 29]. The question about presence/absence of smear layer is still controversial, and it is a problem of primary importance considering the possible role of smear layer in preventing lateral channels sealing, apical sealing, and bacterial contamination of dentinal tubules [21]. Previous studies demonstrated that it may harbor microorganisms and support their survival and growth [5]. Smear layer could also prevent or delay diffusion of irrigants and medicaments

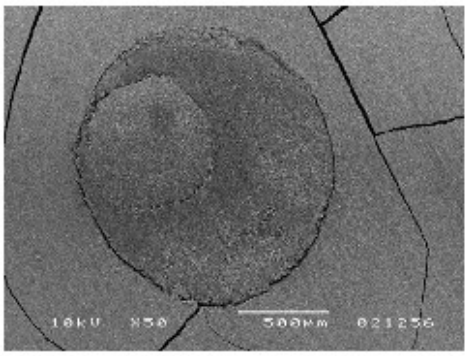


Fig. 3 Apical third section of a central maxillary incisor of the EDTA–NaOCl group with an off-center carrier, covered by a thin layer of gutta-percha, in absence of voids and gaps

into dentinal tubules and reduce the sealing ability of obturation materials [29]. On one hand, a detrimental effect of smear layer has been supposed by inducing bacteria contamination and preventing adequate adaption of sealers [21, 29]. On the other hand, smear plugs may be responsible for reduced permeability of root canal walls and for prevention of bacterial infiltration [21].

Two different irrigation regimens were selected and used in this study to evaluate the effective influence of smear layer in filling lateral channels. So, in group A, NaOCl and hydrogen peroxide were used as agents capable of leaving smear layer produced by K-files intact and unaltered, as confirmed by SEM analysis. In group B, in contrast, instead of hydrogen peroxide, EDTA was used to remove smear layer and smear plugs [19].

Several studies described the dentinal wall adaptation of thermoplasticized gutta-percha in absence or in presence of smear layer [5, 7]. In vitro studies demonstrated that removing smear layer significantly reduces apical leakage, which improves the seal (although other variables need to be considered, e.g., the kind of sealer) [5].

In contrast, other studies demonstrated that removing smear layer had no effect on the apical seal [7, 32] and no obliteration of accessory channels.

Furthermore, with regard to irrigation regimes and their correlation with enhanced penetration into the accessory channels, a recent study demonstrated that the removal of organic (NaOCl) and inorganic (EDTA) substance did not produce statistically significant differences in the obturation material penetration rate [32].

As suggested by the SEM pictures obtained during this study, the thickness of endodontic smear layer is probably 1–5 μ, and this smear layer must be easily pushed with enough pressure by warm gutta-percha inserted inside root channels. In spite of the use of a 17% EDTA solution, it is also plausible that in the apical area, debris and smear layer were also still present in the smear layer-free group. Recent studies demonstrated that the apical thirds also have a considerable amount of smear layer after using chelating agents [19]. The inability of EDTA to completely remove the smear layer from apical third may suggest that morphology of dentin in this area is similar in both groups.

For several reasons, it may be difficult for AH Plus and warm gutta-percha to penetrate smaller lateral channels partially or completely closed by smear layer. However, in this study, they appeared able to penetrate (or partially infiltrate) even smaller lateral channels in both groups.

Finally, a recent investigation by Saleh et al. [24] demonstrated that AH Plus adhesion to dentin may be negatively influenced by EDTA smear layer removal. They suggest that smear layer removal may impair sealer adhesion to dentin. These findings are extremely interesting, as they provide further insight into the results obtained in the study presented in this paper. A previous study of AH Plus observed greater penetration of lateral channels compared to other sealers [31]. Lee et al. [14] indicate that compared to other endodontic sealers, AH Plus has the highest bond strength to dentin (2.06 MPa) and also to gutta-percha (2.93 MPa). However, it is difficult to evaluate whether or not adhesive properties of sealer may improve penetration inside lateral channels and the interaction with smear layer.

The presence of a large number of open dentinal tubules at coronal and medium thirds in the EDTA-treated group may suggest that during the insertion of warm gutta-percha, many small gutta-plugs may penetrate the tubules and may reduce the pressure at the apical third. Furthermore, the thixotropic behavior of α-phase gutta-percha may have influenced the filling of lateral channels at the apical third [15]. Future studies should evaluate the penetration of gutta-percha and endodontic sealer inside dentinal tubules.

Conclusion

The goal of this study was to evaluate if removal of smear layer at the bottom orifice by EDTA improved the quality of lateral channels filling. The null hypothesis was rejected.

This study confirms that the presence of smear layer does not prevent the injection of lateral channels. In other words, the findings presented here support the concept that smear layer does not represent an obstacle to the penetration of sealers such as AH Plus and warm gutta-percha inside the lateral channels.

Acknowledgments This study was supported by grant ex 60% and Progetti Pluriennali–Department of Dental Science of University of Bologna. The authors thank Dr. Silvia Marchionni for technical support and assistance in editing the figures.

References

1. Baumgartner JC, Cuenin PR (1992) Efficacy of several concentrations of sodium hypochlorite for root canal irrigation. J Endod 18:605–612
2. Becker TA, Donnelly JC (1997) Thermafil obturation: a literature review. Gen Dent 45:46–55
3. Bradshaw GB, Hall A, Edmunds DO (1989) The sealing ability of injection-molded thermoplasticized gutta-percha. Int Endod J 22:17–20
4. Chu CH, Lo CM, Cheung GSP (2005) Outcome of root canal treatment using Thermafil and cold lateral condensation filling techniques. Int Endod J 38:179–185
5. Clark-Holke D, Drake D, Walton R, Rivera E, Guthmiller JM (2003) Bacterial penetration trough canals of endodontically treated teeth in the presence or absence of smear layer. J Dent 31:275–281

6. Dummer PMH, Lyle L, Rawle J, Kennedy JK (1994) A laboratory study of fillings in teeth obturated by lateral condensation of gutta-percha or Thermafil obturator. Int Endod J 27:32–38
7. Evans JT, Simon JH (1986) Evaluation of the apical seal produced by injected thermoplasticized gutta-percha in the absence of smear layer and root canal sealer. J Endod 12:100–107
8. Foschi F, Nucci C, Montebugnoli L, Marchionni S, Breschi L, Malagnino VA, Prati C. (2004) SEM evaluation of canal wall dentine following use of Mtwo and ProTaper NiTi rotary instruments. Int Endod J 37:832–839
9. Gencoglu N, Garip Y, Samani S (2002) Comparison of different gutta-percha root filling techniques: Thermafil, Quick-Fill, System B and lateral condensation. Oral Surg Oral Med Oral Pathol 93:333–336
10. Grandini S, Balleri P, Ferrari M (2002) Evaluation of Glyde File Prep in combination with sodium hypochlorite as a root canal irrigant. J Endod 28:300–303
11. Haikel Y, Wittenmeyer W, Bateman G, Bentaleb A, Allemann C (1999) A new method for the quantitative analysis of endodontic microleakage. J Endod 25:172–177
12. Jarret IS, Marx D, Covey D, Kamazin M, Laviv M, Gound T (2004) Percentage of canals filled in apical cross-section an in vitro study of seven obturation techniques. Int Endod J 37:392–398
13. Kopper PMP, Figueiredo JAP, Della Bona A, Vanni JR, Bier CA, Bopp S (2003) Comparative in vivo analysis of the sealing ability of three endodontic sealers in post-prepared root channels. Int Endod J 36:857–863
14. Lee K, Williams M, Camps J, Pashley D (2002) Adhesion of endodontic sealers to dentin and gutta-percha. J Endod 28:684–688
15. Levitan ME, Himel VT, Luckey JB (2003) The effect of insertion rates on fill length and adaptation of a thermoplasticized gutta-percha technique. J Endod 29:505–508
16. Lim TS, Wee TY, Choi, WC Koh, Sae-Lim V (2003) Light and scanning electron microscopic evaluation of Glyde File Prep in smear layer removal. Int Endod J 36:336–343
17. Love RM, Jenkinson HF (2002) Invasion of dentinal tubules by oral bacteria. Crit Rev Oral Biol Med 13:171–183
18. McGurkin-Smith R, Trope M, Caplan D, Sigurdsson A (2005) Reduction of intracanal bacteria using GT rotary instrumentation, 5.25% NaOCl, EDTA, and Ca(OH)2. J Endod 31:359–363
19. O' Connel MS, Morgan LA, Beeler WJ, Baumgartner JC (2000) A comparative study of smear-layer removal using different salts of EDTA. J Endod 26:739–733
20. Prati C, Chersoni S, Pashley DH (1999) Effect of removal of surface collagen fibrils on resin dentin-bonding. Dent Mater 15:323–331
21. Prati C, Foschi F, Nucci C, Montebugnoli L, Marchionni S (2004) Appearance of the root canal walls after preparation with NiTi rotary instruments: a comparative SEM investigation. Clin Oral Investig 8:102–110
22. Reader CM, Himel VT, Germain LP, Hoen MM (1993) Effect of three obturation techniques on the filling of lateral canals and the main canal. J Endod 19:404–408
23. Rud J, Andreasen JO (1972) Operative procedures in periapical surgery with contemporaneous root filling. Int J Oral Surg 1:297–310
24. Saleh IM, Ruyter IE, Haapasalo M, Orstavik D (2002) The effects of dentine pretreatment on the adhesion of root-canal sealers. Int Endod J 35:859–866
25. Saleh IM, Ruyter IE, Haapasalo M, Ørstavik D (2004) Survival of *Enterococcus faecalis* in infected dentinal tubules after root canal filling with different root canal sealer. Int Endod J 37:193–198
26. Schafer E, Olthoff G (2002) Effect of three different sealers on the sealing ability of both Thermafil obturators and cold laterally compacted gutta-percha. J Endod 28:638–642
27. Sen BH, Wesselink PR, Turkun M (1995) The smear layer: a phenomenon in root canal therapy. Int Endod J 28:141–148
28. Siqueira JF (2001) Aetiology of root canal treatment failure: why well-treated teeth can fail. Int Endod J 34:1–10
29. Torabinejad M, Handysides R, Ali Khademi A, Bakland LK (2002) Clinical implications of the smear layer in endodontics: a review. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 94:658–666
30. Venturi M, Di Lenarda R, Prati C, Breschi L (2005) An in vitro model to investigate filling of lateral canals. J Endod 31:877–881
31. Venturi M, Prati C, Capelli G, Falconi M, Breschi L (2003) A preliminary analysis of the morphology of lateral channels after root canal filling using a tooth-clearing technique. Int Endod J 36:54–63
32. Villegas JC, Yoshioka T, Kobayashi C, Suda H (2002) Obturation of accessory canals after four different final irrigation regimes. J Endod 28:534–536
33. Weis MV, Parashos P, Messer HH (2004) Effect of obturation technique on sealer cement thickness and dentinal tubule penetration. Int Endod J 37:653–663
34. Wu M-K, Özok AR, Wesselink PR (2000) Sealer distribution in root canals obturated by three techniques. Int Endod J 33:340–345

New Microbiologica, 31, 235-240, 2008

Recovery of *Enterococcus faecalis* in root canal lumen of patients with primary and secondary endodontic lesions

Chiara Pirani[1], Angelica Bertacci[1], Francesca Cavrini[3], Federico Foschi[1], Giovanni Luca Acquaviva[1], Carlo Prati[1], Vittorio Sambri[2]

[1]Endodontics Unit, Department of Dental Science, Master Clinical Endodontology, University of Bologna, Alma Mater Studiorum, Italy;
[2]Section of Microbiology, DMCSS, Ospedale S. Orsola, University of Bologna, Italy;
[3]Centro di Riferimento Regionale Emilia Romagna per le Emergenze Microbiologiche (CRREM), Bologna, Italy

SUMMARY

The presence of *Enterococcus faecalis* in root canal teeth affected by primary and secondary periapical lesions was studied using polymerase chain reaction (PCR) assays. The association between presence of *E. faecalis* with clinical signs of apical lesions was assessed to evaluate a possible relationship between clinical findings.
Microbial samples were obtained from healthy patients affected by different periapical lesions, 79 teeth with primary periapical lesion and 23 with secondary periapical lesion. For each tooth, clinical symptoms and X-ray appearance were examined.
E. faecalis was detected in 6 of 79 samples with primary lesion (7.6%), and in 9 of 23 with secondary lesion (39.1%). Suggested association was found between *E. faecalis* and secondary apical lesions. As regard specific signs and symptoms *E. faecalis* was more associated with asymptomatic lesions (all $p<0.05$) than with symptomatic apical lesions.
The study confirms the high presence of *E. faecalis* in secondary apical lesions. However, its effective role in endodontic pathogenesis such as bone periapical lesions needs to be clarified.

KEY WORDS: Clinical signs, *Enterococcus faecalis*, PCR, Endodontic lesion

Received October 02, 2007 *Accepted* October 30, 2007

INTRODUCTION

In dentistry, Enterococci have long been implicated in secondary or persistent root canal infection (Sedgley *et al.*, 2005). *Enterococcus faecalis* is a persistent micro-organism that is probably able to survive in the root canal as a single organism or as a major component of the flora (Evans *et al.*, 2002; Portenier *et al.*, 2003). It has been suggested that this species is involved in the pathogenesis of secondary endodontic apical lesions (Tronstad and Sunde, 2003). Nevertheless, there are some reports in the literature that have demonstrated that *Enterococci* can also be found in root-filled teeth with no apical (periapical) lesions (Zoletti *et al.*, 2006) and also in primary endodontic lesions (Ferrari *et al.* 2005, Siqueira, 2002; Sakamoto *et al.*, 2006). Finally, the association of *E. faecalis* with the specific signs and symptoms of periapical lesions is not well defined. The typical symptoms associated with apical lesions are pain to percussion, swelling and tenderness to percussion. Apical radiolucency detected by intra-oral Rx is more frequent in chronic apical lesion or in re-exacerbated apical lesions and are caused by a localized bone defect in the

Corresponding author
Chiara Pirani
Department of Dental Sciences
University of Bologna
Via San Vitale 59,
40125 Bologna, Italy
E-mail: chiara.pirani4@unibo.it

root apical region (Nair *et al.*, 2005). Apical bone defects are more common in chronic lesions than in acute symptomatic lesions (Nair *et al.*, 2005 and 2006).
Only few clinical studies have been performed in an Italian population detecting the presence of this pathogen in primary and in secondary endodontic lesions (D'Arcangelo *et al.*, 1999).
The aim of the present study was to use PCR techniques to investigate the correlation between *E faecalis*, identified within root canals in primary and secondary endodontic lesions, and the presence of signs and symptoms. The role of this micro-organism in patients with primary and secondary apical endodontic lesions with and without bone lesions has to be clarified.

MATERIAL AND METHODS

Patients

The study population consisted of 102 patients presenting at the Endodontic Clinical Section of the Department of Dental Science-University of Bologna, Italy for endodontic treatments. Medical histories revealed that all patients were in good general health and had no important systemic diseases such as diabetes. Patients that had received antibiotic therapy during the last two months before root canal therapy were excluded from the study. The patient ages ranged from 16 to 73 years, mean ± SD: 36.7 ± 15.6 years. During the first visit, written informed consent was obtained from each patient before inclusion in the study.
Only third molars were excluded from the study for anatomical reasons, but all the other types of teeth (i.e. molars, canines etc.) were included. Lesions with periodontal pocket probing greater than 4.0 mm were excluded due to possible endodontic-periodontal infection. Another exclusion criterion was teeth in which proper rubber dam isolation could not be achieved during the sampling procedures and followed endodontic retreatment. We collected 79 primary endodontic (peri)apical lesions and 23 secondary (peri)apical endodontic lesions.

Clinical signs and symptoms

Clinical features were recorded for each tooth. The following clinical data were collected: presence of previous root canal filling, pain, tenderness to percussion or palpation, swelling, and periapical radiolucency.
For all teeth the presence of a periapical radiolucency was assessed using the periapical index (PAI), determined with a paralleling X-ray technique, according to Orstavik *et al.* (1986). Teeth with a PAI score equal to or greater than '3' (signs of structural changes of bone periapical structure with mineral loss and anatomical lesion) were considered to be affected by (peri)apical bone lesions.

Specimen sampling

Endodontic samplings from teeth of different patients were obtained during the first visit for root canal therapy. After anaesthesia, a rubber dam was placed and surface disinfection of intact enamel was carried out using a small cotton pellet immersed in NaOCl 5.25% (Niclor 5, Ogna, Muggiò, Italy) as described by Ng *et al.* (2003). The antimicrobial solution was soaked up with a second dry sterile cotton pellet. No rubber dam leakage was observed during the access cavity procedure. Access cavity preparations were made using sterile burs with sterile water spray supplied by Logos Junior and Duo dental units (Castellini S.p.A., Castel Maggiore, Italy), equipped with an Autosteril system (Montebugnoli & Dolci, 2002). The patency of each canal was assessed by inserting a sterile #10 or 15 K-file (Dentsply-Maillefer, Ballaigues, CH) so that the tip was approximately 2-4 mm short from the apex, previously measured on the pre-operative radiograph. In cases of previously filled root canals (secondary apical lesions group), gutta-percha was preliminary removed without chemical solvents with the use of # 4, 3 and 2 Gates Glidden burs (Dentsply-Maillefer, Ballaigues, CH) and # 10-15 K-files. To obtain microbial samples, two or more paper points (ADA products-Mynol, Milwaukee, WI, USA) were placed into the root canal and retained inside for 40 seconds. The paper points were then immediately transferred to sterile 1.5 ml tubes (Eppendorf AG, Hamburg, Germany) containing 500 µl of sterile phosphate buffered saline (PBS) solution. Samples were frozen immediately at -20°C and stored up to one-two months until assayed by PCR.

PCR assays

DNA extraction of samples was performed using the QIAamp DNA Blood Mini Kit (Qiagen GmbH,

Hilden, Germany) according to the manufacturer's instructions. To control for the efficiency of DNA extraction and the absence of PCR inhibitors, a partial region of the human *Hfe* gene (390 bp) was amplified for each sample using a specific pair of primers (Hfe1 5'-TGGCAAGGGTAAACAAGATCC-3', Hfe2 5'-CTCAGGCACTCCTCTCAACC-3').

In addition, the presence of different *Enterococcus* species within the root canal samples was first investigated by amplifying the *Enterococcus* spp. *tuf* gene with genus-specific primers (Table 1). The samples yielding a positive result for the presence of *Enterococcus* spp. were further investigated for *E. faecalis* using specific primers targeting the *ddl* gene (Table 1). The DNA extracted from two clinical isolates of *E. faecium* and *E. faecalis* respectively was amplified as a positive control. The specificity of each primer-pair was confirmed using the BLAST software available on-line at http://www.ncbi.nlm.nih.gov/blast. Primers were custom synthesized by PRIMM (Milan, Italy). The amplifications were performed in 30 µl total final volume, containing 10 mM Tris-HCl, 50 mM KCl, 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 1.5 U Taq polymerase (Takara, Shiga, Japan) and a specific primer pair.

The concentration of primer was 0.4 µM for *Enterococcus* spp. and the human *Hfe* gene. For each sample 10 µl of extracted DNA was added to the reaction mixture, and PCRs were performed in a Mastercycler thermalcycler (Eppendorf, Hamburg, Germany) under optimized conditions as reported in Table 1. For detection of the *Hfe* gene, 35 amplification cycles were used (1 min at 95°C, 1 min at 61°C and 1 min at 72°C). An initial denaturation step of 3 min at 95°C preceded the amplification cycles, followed by a final extension step of 3 min at 72°C in each PCR reaction. The amplification products were analyzed by 2% agarose gel electrophoresis in TBE buffer (Tris-borate EDTA) at 100V for 2h. The gels were stained with ethidium bromide (0.5 µg/ml) and the PCR products were visualized under UV light with a TFX-20M Gibco BRL (Gaithersburg, MD, USA) UV Transilluminator. The identity of each band was inferred by comparison with a molecular weight ladder (DNA Marker IV, Roche, Penzberg, Germany) using the 1D image analysis software (Kodak Digital Science, Rochester, NY, USA).

Data analysis

Data collected for each sample were recorded on an electronic data spreadsheet and analyzed with SPSS 12.0 (SPSS Inc., Chicago, IL, USA). Descriptive statistical analysis was performed using the Pearson Chi-square test or the one-sided Fisher's Exact test, as appropriate. The null hypothesis was that there was no correlation between different clinical signs of apical lesions and the detection of specific bacteria strains in sampled root canals.

RESULTS

Table 2 shows the incidence of cases in the study groups, according to their different clinical categories. Specifically, 79/102 teeth presented primary endodontic infection, while 23/102 pre-

TABLE 1 - *PCR primers, with expected amplicon size and thermocycling parameters, for endodontic pathogens investigated in the present study.*

Bacterial species	*Primers sequence*	*Amplicon size*	*Amplification*	*Reference*
	(from 5' to 3')	*(bp)*	*cycles*	
Enterococcus species	TACTGACAAACCATTCATGATG AACTTCGTCACCAACGCGAAC	112 bp	35 cycles 95°C 30 s 58°C 45 s 72°C 20 s	Ke et al. (1999)
E. faecalis	ATCAAGTACAGTTAGTCT ACGATTCAAAGCTAACTG	941 bp	36 cycles: 95°C 30 s 47°C 45 s 72°C 40 s	Dutka-Malen et al. (1995)

TABLE 2 - *Distribution and percentage of e.faecalis in primary and secondary apical lesion groups according to different clinical signs and symptoms.*

Signs and symptoms	*Detected (yes/no)*	*Primary apical lesion (n=79)*		*Secondary apical lesion (n=23)*	
		E. faecalis positive (7.6%)	*E. faecalis negative (92.4%)*	*E. faecalis positive (39.1%)*	*E. faecalis negative (60.9%)*
Pain	Yes	0%	49.4%	8.7%	30.4%
	No	7.6%	43.0%	30.4%	30.5%
Periapical radiolucency	Yes	6.3%	49.4%	30.4%	47.8%
	No	1.3%	43.0%	8.7%	13.1%
Swelling	Yes	8.7%	24.1%	8.7%	8.7%
	No	5.1%	68.3%	30.4%	43.5%
Tenderness to percussion	Yes	2.5%	53.2%	8.7%	34.8%
	No	5.1%	39.2%	30.4%	26.1%

sented secondary endodontic lesions.
Suggested association were found between *E. faecalis* and secondary apical lesions ($p<0.05$). *E. faecalis* resulted associated with a large number of asymptomatic apical periodontitis ($p<0.05$) in primary apical lesions.

DISCUSSION

The purpose of this study was to evaluate the presence of *E faecalis* in the root canals of teeth with endodontic apical lesions and to associate its presence with clinical symptoms.
Bone defect is the principal condition determining a diagnosis of primary or secondary apical lesions (Nair 2006). In many cases the bone defect is radiographically detectable as an apical radiolucency. Bacteria, toxins, foreign bodies have been considered responsible for apical lesions such as apical granulomas and apical cysts (Nair 2006). The presence of radiographically detected apical bone resorption is indicative of a complex pathogenic mechanism which involves large numbers of bacteria at root apical level and in the proximity of root apical bone (Fabricious *et al.*, 1982) for a sufficient period of time to stimulate bone destruction and resorption and other complex immunological activities (Siqueira *et al.*, 2004; Siqueira & Rocas 2004; Nair *et al.*, 2005). In an innovative study, Sunde *et al.* (2003) revealed microorganisms directly in the apical region using fluorescent *in situ* hybridization techniques. These microorganisms were assumed to play a major role in the development of clinical symptoms (Jacinto *et al.*, 2003) and in tissue alterations and resorption (Siqueira *et al.*, 2004; Siqueira & Rocas 2004). Hancock *et al.* (2001) examined root filled teeth with persistent apical radiolucencies (considered secondary apical lesions) and found that as well as Enterococcus other genera, viz. Peptostreptococcus, Actinomyces and Streptococcus predominated.
Our results confirm that *E. faecalis* is associated with secondary apical lesions (i.e. previous treatment failures). No relationship was suggested with the symptoms studied both in primary and in secondary endodontic infections.
Using the DNA-DNA checkerboard technique, an 8.0% prevalence of *E. faecalis* was also reported in primary endodontic infections (Siqueira *et al.*, 2002), which agrees well with our study (7.6%). Another molecular-based study revealed the concurrent presence of *E. faecalis* and other bacteria (*Pseudoramibacter, Proprionibacterium, Dialister*, and *Filifactor*) (Siqueira *et al.*, 2004) in these types of lesions in asymptomatic patients.
It is not currently possible to consider *E. faecalis* responsible for the bone lesions, but it is evident that they may be only partially involved in the formation of the bone damage. It may be a sort of "in vivo index" and may be present in the api-

cal biofilm with other bacteria and play a critical support role (Johnson *et al.*, 2006). It has been demonstrated that dentinal tubules may represent a long-term *nidus* for secondary subsequent root canal infection and subsequent apical bone infection. Hence, these bacteria *may* reside not only in the canal lumen but also may invade the dentinal tubules for more than 200 microns. Hence, these structures may act as a reservoir for future dental and systemic infections (Oguntebi 1994; Peters *et al.*, 2001; Matsuo *et al.*, 2003).

To explain the reason for a high percentage of positive samples only in secondary lesions, *E. faecalis* survival is favoured during therapy, and can also persist for a long time inside dentinal tubules before initiating secondary disease (Pinheiro *et al.*, 2003). Adhesion to the dentin surface is an essential step determining the pathogenic potential of *E. faecalis* in the medicated root canal: serine protease and Ace aid *E. faecalis* binding to dentin (Hubble *et al.*, 2003). Therefore dentinal tubules may work as a great reservoir of bacteria completely outside immunological control.

Clearly, more effective clinical methodologies for disinfection of root canals must be established to eradicate this pathogen in the course of endodontic treatment. *E. faecalis*, may survive in the smear layer and in debris inside the root canal (and inside the lateral canals and dentinal tubules) and may be extremely difficult to remove by irrigation and instrumentation (Yang *et al.*, 2006, Estrela *et al.*, 2007). For these reasons, it is important to consider that when an *E faecalis* infection is suspected a different type of irrigation must be used in the root canal. Chlorexidine has a broad-spectrum antimicrobial effect and kills *E faecalis* in the dentinal tubules more effectively than other irrigations and disinfectants (Schafer and Bossman, 2005). Alternatively, ultrasound mechanical preparation and other sonic procedures must be used the remove and kill pathogen bacteria (Gulabivara *et al.*, 2004).

Lastly, the presence of these pathogens inside the root canal may increase the risk for iatrogenic exacerbations (flare ups) when infected dentin debris is transported into the apical region (Siqueira, 2001).

Based on the ubiquitous occurrence of enterococci in many food products, such as cheeses and milk derivates, it can be speculated that niches such as root canal lumens and dentinal tubules may favour their survival and long-standing local infection (Razavi *et al.*, 2007). The bacteria inside the root canal could be the consequence of a coronal colonization after contaminated food ingestion.

In conclusion, the present study confirms that *E. faecalis* inside root canal may be detected in teeth with secondary apical lesions (treatment failures). Surprisingly, signs and symptoms are not correlated to bacteria presence. We could speculate that coaggregation interactions between this and other bacterial species could play a major role in endodontic infection.

ACKNOWLEDGMENTS

This study was supported by RFO ex60% research grant 2004 and 2005 from Alma Mater Studiorum University of Bologna (Funds for selected research topics).

REFERENCES

D'Arcangelo C., Varvara G., De Fazio P. (1999). An evaluation of the action of different root canal irrigants on facultative aerobic-anaerobic, obligate anaerobic, and microaerophilic bacteria. *J Endod.* **25**, 351-353.

Estrela C., Estrela C.R.A., Decurcio D.A., Hollanda A.C.B., Silva J.A. (2007). Antimicrobial efficacy of ozonated water, gaseous ozone, sodium hypochlorite and chlorhexidine in infected human root canals. *Int End J.* **40**, 85-93.

Evans M., Davies J.K., Sundqvist G., Figdor D. (2002) Mechanisms involved in the resistance (1982). Predominant indigenous oral bacteria isolated from infected root canals after varied times of closure. *Scand J Dent Res.* **90**, 134-144.

Ferrari P.H.P, Cai S., Bombana A.C. (2005). Effect of endodontic procedures on *Enterococci*, enteric bacteria and yeasts in primary endodontic infections. *Int Endod J.* **38**, 372-380.

Gulabivala K., Stock C.J., Lewsey J.D., Ghori S., Ng Y.L., Spratt D.A. (2004). Effectiveness of electrochemically activated water as an irrigant in an infected tooth model. *Int Endod J.* **37**, 624-631.

Hancock H.H., Sigurdsson A., Trope M., Moiseiwitsch J. (2001). Bacteria isolated after unsuccessful endodontic treatment in a North American population. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* **91**, 579-586.

Hubble T.S., Hatton J.F., Nallapareddy S.R., Murray B.E., Gillespie M.J. (2003). Influence of Enterococcus faecalis proteases and the collagen-

binding protein, Ace, on adhesion to dentin. *Oral Microbiol Immunol.* **18**, 21-26.

Jacinto R.C., Gomes B.P., Ferraz C.C., Zaia A.A., Filho F.J. (2003). Microbiological analysis of infected root canals from symptomatic and asymptomatic teeth with periapical periodontitis and the antimicrobial susceptibility of some isolated anaerobic bacteria. *Oral Microbiol Immunol*. **18**, 285-292.

Johnson E.M., Flannagan S.E., Sedgley C.M. (2006). Coaggregation interactions between oral and endodontic Enterococcus faecalis and bacterial species isolated from persistent apical periodontitis. *J Endod*. **32**, 946-950.

Matsuo T., Shirakami T., Ozaki K., Nakanishi T., Yumoto H., Ebisu S. (2003) An immunohistological study of the localization of bacteria invading root pulpal walls of teeth with periapical lesions. *J Endod.* **29**, 94-200.

Montebugnoli L., Dolci G. (2002). A new chemical formulation for control of dental unit water line contamination: An 'in vitro' and clinical 'study'. *BMC Oral Health*. **2**, 1.

Nair P.N.R., Henry S., Cano V., Vera J. (2005). Microbial status of apical root canal system of human mandibular first molars with primary apical periodontitis after "one visit" endodontic treatment. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod.* **99**, 231-252.

Nair P.N.R. (2006). On the causes of persistent apical periodontitis: a review. *Int Endod J.* **39**, 249-281.

Ng Y.L., Spratt D., Sriskantharajah S., Gulabivala K. (2003). Evaluation of protocols for field decontamination before bacterial sampling of root canals for contemporary microbiology techniques. *J Endod*. **29**, 317-320.

Oguntebi B.R. (1994). Dentine tubule infection and endodontic therapy implications. *Int Endod J.* **27**, 218-222.

Orstavik D., Kerekes K., Eriksen H.M. (1986). The periapical index: a scoring system for radiographic assessment of apical periodontitis. *Endod Dent Traumatol*. **2**, 20-34.

Peters L.B., Wesselink P.R., Buijs J.F., Van Winkelhoff. (2001). Viable bacteria in root dentinal tubules of teeth with apical periodontitis. *J Endod*. **27**, 76-81.

Pinheiro E.T., Gomes B.P., Ferraz C.C., Sousa E.L., Teixeira F.B., Souza-Filho F.J. (2003). Microorganisms from canals of root-filled teeth with periapical lesions. *Int Endod J*. **36**, 1-11.

Pinheiro E.T., Gomes B.P., Ferraz C.C., Teixeira F.B., Zaia A.A., Souza Filho F.J. (2003). Evaluation of root canal microorganisms isolated from teeth with endodontic failure and their antimicrobial susceptibility. *Oral Microbiol Immunol*. **18**, 100-103.

Portenier I., Waltimo T.M.T., Haapasalo M. (2003). *Enterococcus faecalis*- the root canal survivor and "star" in post-treatment disease. *Endodontic Topics*. **6**, 135-159.

Razavi A., Gmur R., Imfeld T., Zehnder M. (2007). Recovery of Enterococcus faecalis from cheese in the oral cavity of healthy subjects. *Oral Microbiol Immunol.* **22**; 248-251.

Sakamoto M., Rocas I.N., Siqueira J.F., Benno Y. (2006). Molecular analysis of bacteria in asymptomatic and symptomatic endodontic infections. *Oral Microbiol Immunol*. **21**, 112-122.

Schafer E., Bossman K. (2005). Antimicrobial efficacy of chlorexidine and two calcium-hydroxide formulation against *Enterococcus faecalis*. *J Endod*. **31**, 53-56.

Sedgley C.M., Nagel A.C., Shelburne C.E., Clewell D.B., Appelbe O, Molander A. (2005). Quantitative real-time PCR detection of oral *Enterococcus faecalis* in humans. *Arch Oral Biol*. **50**, 575-583.

Siqueira J.F. Jr, Rocas I.N., Rosado A.S. (2004). Investigation of bacterial communities associated with asymptomatic and symptomatic endodontic infections by denaturing gradient gel electrophoresis fingerprinting approach. *Oral Microbiol Immunol*. **19**, 363-370.

Siqueira J.F. Jr, Rocas I.N. (2004). Polymerase chain reaction-based analysis of microorganisms associated with failed endodontic treatment. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. **97**, 85-94.

Siqueira J.F. Jr. (2002). Endodontic infections: concepts, paradigms, and perspectives. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*. **94**, 281-293.

Siqueira J.F. Jr, Rocas I.N., Souto R., De Uzeda M., Colombo A.P. (2002). *Actinomyces Species, Streptococci* and *Enterococcus faecalis* in primary root canal infection. *J Endod*. **28**, 168-172.

Siqueira J.F. (2001). Aetiology of root canal treatment failure: why well-treated teeth can fail. *Int Endod J*. **34**, 1-10.

Sunde P.T., Olsen I., Gobel U.B., Theegarten D., Winter S., Debelian G.J., Tronstad L., Moter A. (2003). Fluorescence in situ hybridization (FISH) for direct visualization of bacteria in periapical lesions of asymptomatic root-filled teeth. *Microbiology*. **149**, 1095-1102.

Tronstad L., Sunde P.T. (2003). The evolving new understanding of endodontic infections. *Endodontics Topics*. **6**, 57-77.

Yang SE, Cha J.H, Kim E.S, Kum K.Y, Lee C.Y, Jung I.Y. (2006). Effect of smear layer and chlorexidine treatment on the adhesion of *Enterococcus faecalis* to bovine dentin. *J Endod*. **32**, 663-667.

Zoletti G.O., Siqueira J.F., Santos K.R.N. (2006). Identification of *Enterococcus faecalis* in root-filled teeth with or without periradicular lesions by culture-dependent and-indipendent approaches. *J Endod*. **32**, 722-726.

Curriculum vitae

Dott.ssa Angelica Bertacci.
Nata a Prato il 05/10/1977.
Reparto di Endodonzia, Dipartimento di Scienze Odontostomatologiche
Alma Mater Studiorum- Università degli Studi di Bologna
Tel.: +39 051 2088124
fax: +39 051 225208
E-mail address: angelica.bertacci2@unibo.it

Studi e formazione

1996
Maturità classica conseguita presso il Liceo Classico Statale "F. Cicognini" di Prato, votazione **60/60**.
Iscrizione al corso di laurea in *Biologia* presso l'Università degli Studi di Firenze. Esami sostenuti: Istituzioni di Matematiche con votazione di 28/30, Citologia ed Istologia con votazione di 30/30.

1997
Vincitrice del concorso per l'iscrizione al corso di laurea in Odontoiatria e Protesi Dentaria a posti limitati (19) presso l'Università degli Studi di Pisa.

A.A. 2001/2002
Laurea in Odontoiatria e Protesi Dentaria conseguita presso l'Università degli Studi di Pisa, votazione **110/110 e Lode**.
Titolo della Tesi: "Disordini temporo-mandibolari extracapsulari: attualità sul ruolo delle indagini strumentali nel processo diagnostico". Relatore: Prof. M. Bosco.

Abilitazione all'esercizio della professione di odontoiatra.

A.A. 2003/2004 Conseguimento del *Master Universitario di II livello in Endodonzia Clinica* presso il Dipartimento di Scienze Odontostomatologiche dell'Alma Mater Studiorum, Università di Bologna.

A.A. 2004/2005 Professore a contratto ***Tutorato di assistenza agli studenti per l'insegnamento di Clinica Odontostomatologica II***, presso il Dipartimento di Scienze Odontostomatologiche, Alma Mater Studiorum-Università degli Studi di Bologna.

Vincitrice del concorso per il **Dottorato in Biotecnologie Mediche XXI ciclo** (Scuola di Dottorato**:** Scienze Mediche e Chirurgiche Cliniche. Coordinatore: prof. Marialuisa Zerbini), con assegnazione di **Borsa di Studio Triennale** finanziata dal MIUR.

2006 – oggi *Dottorando* Università degli Studi di Bologna Dottorato in Scienze biomediche progetto1:BIOTECNOLOGIE MEDICHE.

Pubblicazioni *in extenso*

Articoli su riviste nazionali

Biondi K, Bertacci A, Manfredini D, Tognini F, Bosco M. Ruolo dell'ultrasonografia nell'analisi morfo-strutturale del muscolo massetere. Il Dentista Moderno 2004;22:59-68.

Bertacci A, Cotti E, Marchionni S, Baroni C, Foschi F, Prati C. Ultramorphology of dentin after use of different irrigants with antibacterial activity. Giornale italiano di Conservativa 2006;1:40-47.

Bertacci A, Pirani C, Pacini G, Marchionni S, Prati C. Utilizzo degli ultrasuoni per la rimozione dello smear layer endodontico. Dental Cadmos 2006;10:29-33.

Migliau G, Giovannone T, Milia E, Bertacci A, Prati C, Gallottini G. Classificazione degli strumenti rotanti in nichel-titanio. Giornale italiano di Conservativa 2007;5:144-162.

Bertacci A, Breschi M, Acquaviva GL, Prati C. Nuove punte ultrasoniche Ni-Ti per la preparazione endodontica: valutazione morfologica al SEM Il Dentista Moderno 2008;5:58-74.

Baroni C, Bertacci A, Marchionni S, Gandolfi MG, Montanari M, Prati C. Gestione clinica e valutazione morfologica al SEM di elementi dentari ipomineralizzati (MIH). Giornale italiano di Conservativa 2008;6:185-192.

Zanarini M, Ruggeri O, Pazzi E, Bertacci A, Alessandri Bonetti G, Prati C. Demineralizzazione dello smalto dopo esposizione ad una soluzione di acido lattico. Valutazione al SEM/EDX ed al microscopio ottico metallografico di due sistemi adesivi ortodontici. Giornale Italiano di Conservativa (in press).

Articoli su riviste internazionali

Pirani C, Bertacci A, Cavrini F, Foschi F, Acquaviva GL, Prati C, SambriV. Recovery of Enterococcus faecalis in root canal lumen of patients with primary and secondary endodontic lesions. The New Microbiologica 2008;31:235-240.

Bertacci A, Chersoni S, Davidson CL, Prati C. The double origin of enamel fluid. Eur J Oral Sci 2007;115:523-4.

Bertacci A, Chersoni S, Davidson CL, Prati C. In vivo enamel fluid movement. Eur J Oral Sci. 2007;115:169-73.

Bertacci A, Baroni C, Breschi L, Venturi M, Prati C. The influence of smear layer in lateral channels filling. Clin Oral Investig. 2007;11:353-9.

Bertacci A, Landi N, Manfredini D, Ferronato G, Bosco M Coronoid hyperplasia. A case report. Minerva Stomatol 2005;54:461-70.

Manfredini D, Segù M, Bertacci A, Binotti G, Bosco M Diagnosis of temporomandibular disorders according to RDC/TMD axis I findings, a multicenter Italian study. Minerva Stomatol 2004;53:429-38.

Abstract e Atti di Congressi

Bertacci A, Biondi K, Tognini F, Manfredini D, Bosco M. "Risonanza Magnetica nella valutazione del muscolo massetere". Doctor Os 2004;15(1) suppl 1:124-127.

Bertacci A, Cantini E, Manfredini D, Statizzi G, Bosco M. "Prevalenza delle diverse forme di disordini temporomandibolari secondo i criteri RDC/TMD: dati preliminari". Doctor Os 2004;15(4) suppl 1:12-15.

Salvetti G, Manfredini D, Bertacci A, Landi N, Bosco M. Segni esintomi di disfunzione stomatognatica in corso di sindroma fibromialgica: studio descrittivo su 93 pazienti. In

Atti XII Congresso Nazionale Collegio dei Docenti di Odontoiatria, Roma 16-19 marzo 2005. Doctor Os (suppl) 2005;2 p.160.

Bertacci A, Pirani C, Pacini G, Clementi V, Gullifa A, Prati C. Utilizzo degli ultrasuoni per la rimozione dello smear layer endodontic. Abstarcr Congresso SIDOC, Roma, 10-12 febbraio 2005. In: Giornale Italiano di Conservativa (suppl) 2004;2:25-26.

Chersoni S, Sauro S, Bertacci A, Breschi L, Piana G, Prati C. "In vivo evaluation of fluid flow through enamel ". Giornale Italiano di Conservativa (Suppl) 2006:1:206.

Chersoni S, Bertacci A, Gandolfi MG, Iacono F, Tay FR, Pashley DH, Prati C. Fluoride and enemel permeability in vivo. In: IADR 86th General Session & Exhibition. Toronto, July 2-5, 2008.

Bertacci A, Taddei P, Prati C, Pashley DH, Tay FR, Gandolfi MG, Chersoni S. Acid treatments modify enamel permeability. In: IADR 87th General Session & Exhibition. Miami, April 1-4, 2009.