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Self Limiting Therapy in Deep Dentinal Lesions

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To my mother, husband and son with love, respect and admire…

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Introduction

Dental research over the last century has advanced our understanding of the aetiology and pathogenesis of caries lesions (Aoba, 2004). Evidence that caries is an initially reversible chronic disease with a known multi-factorial aetiology is being appreciated more widely (Pitts, 2004) in which numerous episodes of de- and remineralization rather than an unidirectional demineralization process (Kawasaki and Featherstone, 1997; Bjorndal and Mjör, 2001; Aoba, 2004) result in numerous minute pH fluctuations at the interface between the tooth surface and the microbial deposits (Baelum and Fejerskov, 2003). The presence of this microbial biofilm and the constant metabolic activity taking place within it, is believed to be the all-important driving force for caries to occur (Baelum and Fejerskov, 2003; Kidd 2004). Thus, better understanding the caries aetiology, pathogenesis and activity together with the development of a standard international system for caries detection and lesion assessment (Pitts, 2004) have to determine the quality and quantity of dentinal carious tissue to be removed before restoration to insure arrestment of carious process (Kidd, 2004), and to avoid bacterial reactivation and caries re-initiation (Foley and Blackwell, 2003).

Dentinal Lesions in Clinical Practice

Chapter 1

The aim of the following review is to discuss the scientific basis of preservative dentistry, and to relate these principles and basis to clinical practice. Both the caries process and caries lesions will be described. Clinical criteria for caries removal, various caries removal methods and the clinical studies which have been carried out in this area are reviewed, too.

Composition of Sound Dentin

70 wt% (50 vol%) of dentin is of an inorganic phase in which carbonated calcium phosphate micro-crystals form the major portion while part of this mineral phase may not be apatitic (Nikiforuk, 1985a). These crystals are of 50-60 nm long and 3-30 nm thick (ten Cate et al., 2003). The dentin crystallites are well known to be smaller (Nikiforuk, 1985a; ten Cate, 2001) and less systematically oriented (LeGeros, 1990) than enamel crystallites, resulting in an increased surface area and rapid dissolving rate under acid attacks (Ostrom, 1980). Dentin hydroxyapatites are located within an organic matrix that forms 20 wt% of dentin, the remainder 10 wt% is water.

Organic Dentin material contains 90% collagen and 10% non-collagenous compounds (NCC) (Beeley et al., 2000; Heinrich-Weltzien and Kneist, 2001). Collagen type I is the predominant collagen in dentin (89%), type I trimer is 11% and 1% is of types III, V, VI (Heinrich-Weltzien and Kneist, 2001). Dentin collagen forms a fibrous three-dimensional network which remineralizes to provide the fundamental building blocks of dentin (Balooch et al., 2004). Collagen molecules are composed of the amino acids proline, glycine, hydroxylysine and hydroxyproline (Kuboki et al.,

1977; Beeley et al., 2000) in which glycine has to be the third residue in the amino acid sequence (Butler and Richardson, 1980; Kleter, 1997), proline occupies almost 40% of the X positions and hydroxyproline occupies 30% of the Y positions of the repeating sequence in each chain (Butler and Richardson, 1980). These amino acids are bonded to each other through peptide bonds forming polypeptide α chains $[\alpha_1(I)]_2\alpha_2$. Every three polypeptide α chains are twisted about each other in a supercoiled form into the tropocollagen triple helix (Habelitz et al., 2002). The triple helix contains 1011 residue per α chain and is flanked by short non-helical ends which compose of 6-25 residues per α chain (Kleter, 1997). Each tropocollagen triple helix together with its non-helical ends constitutes a collagen molecule which is rod-like in shape, 300 nm long and 1.5 nm in diameter (Butler and Richardson, 1980). The tropocollagen subunits orientate parallel to each other to form the fibrils (Habelitz et al., 2002). Direct and water mediated hydrogen bonds between carbonyl groups and amide, hydroxyproline or another carbonyl groups in the same α chain (intramolecular) and between different α chains (intermolecular) form the collagen covalent cross-links (Brodsky and Ramshaw, 1997). However, there are four intermolecular cross-links materials in collagen fibres of sound dentin; they are dihydroxynorleucine, hydroxynorleucine, dihydroxylysinorleucine and hydroxylysinorleucine in which the first two are precursors of cross-links (Kuboki et al., 1977). These cross-links connect the non-helical extension of one molecule with the adjacent helical part of another molecule (Kuboki et al., 1993). The distinctive arrangement of the adjacent collagen molecules combined with gap zones between the ends of the successive molecules lead to the formation of alternating specific bands or what is the so called D-distance that ranges between 60 and 67nm depending at the hydration of the fibrils (Balooch et al., 2004). The fibrils' mechanical stability, insolubility, and acid and thermal

resistance are due to these covalent bonds forming the intra- and inter-molecular cross-links within the fibril (Beeley et al., 2000; Heinrich-Weltzien and Kneist, 2001). When collagen is irreversibly degenerated, these cross-links are broken, the banding pattern is disappeared and collagen molecules are destructed; this will result in the so called denaturated collagen (Kuboki et al., 1977) which is irreversibly damaged and degenerated.

Dentin cellular and extra-cellular proteins are synthesized, controlled and secreted by the odontoblasts (Goldberg and Smith, 2004). The unmineralized extra-cellular matrix (predentin) changes into dentin as the collagen mineralize (Butler, 1998). NCP mostly glycoproteins and proteoglycans cover the collagen fibrils (Habelitz et al., 2002). Phosphoproteins which are the most abundant NCP found to be critical for proper biomineralization of dentin in which they induce mineral nucleation (Fujsawa and Kuboki, 1998; Baolooch et al., 2004) but only when they are in low amounts (Lussi et al., 1988; Clarkson et al., 1998; Saito et al., 1998) and only when the negatively charged phosphate esters bound covalently to the positively charged collagen gap zones (Saito et al., 1997). This is followed by binding of calcium and phosphate to the resultant three dimensional protein conformations within these regions initiating plate-like apatite crystal formation (Butler, 1998; Ritchie et al., 1998; Saito et al., 2000). Therefore, collagen has been considered the structural backbone of dentin (ten Cate et al., 2003), which holds together the apatite crystals in a proper orientation on (extrafibrillar) and in between (intrafibrillar) its fibers (Klont and ten Cate, 1991; Kinney et al., 2003; Balooch et al., 2004). Moreover, it provides stable support for the NCPs and the proteoglycans (Lussi and Lindi, 1993; Saito et al., 2000). The different organizational levels of collagen are shown in fig 1.1 (Butler and Richardson, 1980).

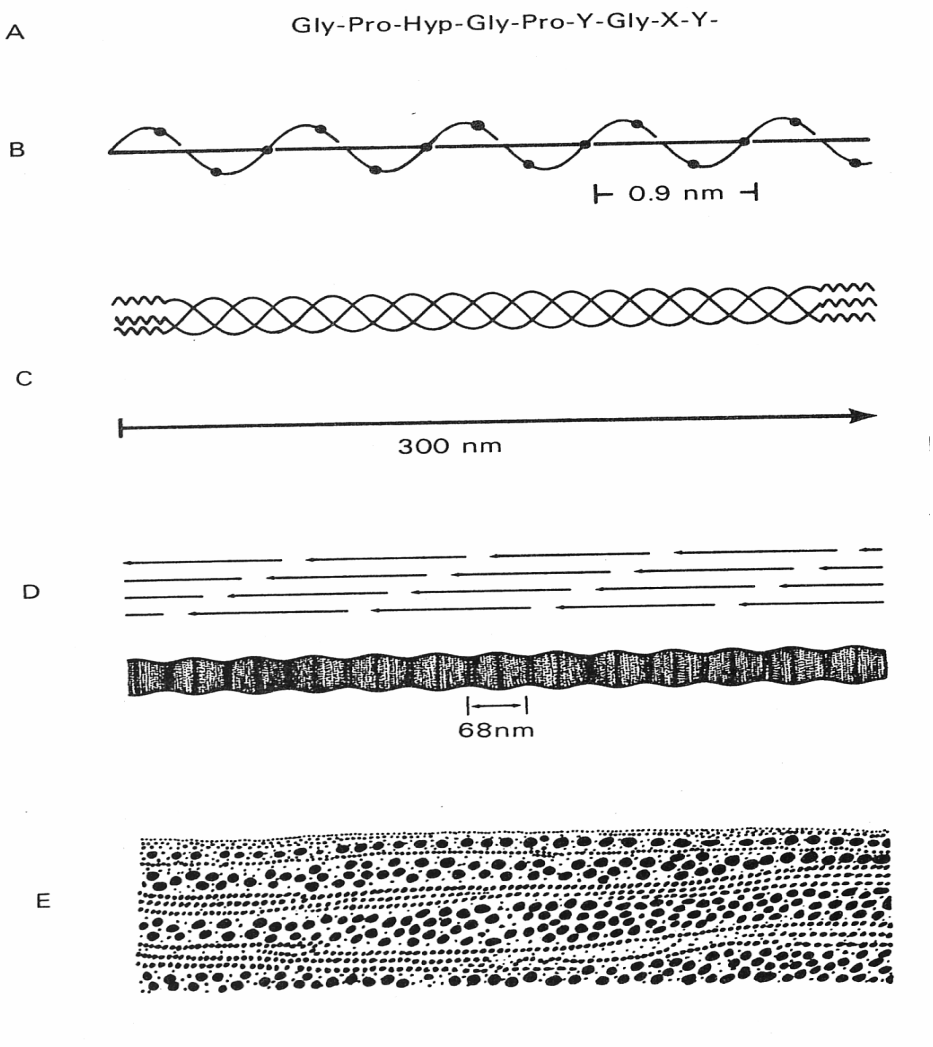


Fig 1.1. The different organizational levels of collagen (Butler and Richardson, 1980).

A. Amino acid sequence – the general form of the amino acid sequence found in a helical portion of an α chain is (Gly-X-Y)n.

B. Minor helix – this diagram illustrates the fact that an individual α chain twists into a helix with three amino acid residues (represented by balls) per repeat.

C. Major helix – three polypeptide α chains are coiled about each other to form the triple helix. Each end of all three chains is nonhelical. Single collagen molecules of this form are represented by elongated arrows about 300nm long.

D. Collagen fibrils – a large number of collagen molecules aggregate in a staggered way to form a fibril large enough to be seen by electron microscopy. The staining procedure reveals that bands in the fibril repeat about every 68nm.

E. Connective tissue – collagen fibrils are laid down into bundles to form networks of stable fibers in extracellular spaces. The fibers are large enough to be seen by light microscopy. The tissues also contain other interfibrillar materials such as proteoglycans.

The non-collagenous matrix consists of non-collagenous proteins (NCP) and non-proteinaceous components (NPC) (Heinrich-Weltzien and Kneist, 2001). Dentin phosphoprotein (DPP) and dentin sialoprotein (DSP) are thought to be unique dentin proteins (Butler, 1998), however, it seems that bone cells synthesize also these proteins but in a ratio of 1:400 to that of dentin (Quin et al., 2002; Goldberg and Smith, 2004). Phosphoproteins which are the dominant components of the NCC (Nikoforuk, 1985) are found in the dentin matrix in two forms; 90% are soluble (electrostatically bound to collagen) in a free form, and the other 10% are insoluble (bounded phosphophoryn) in inextricable form (Lussi et al., 1988; Saito et al., 2003). They are composed mainly of aspartic acid residues (40-50%) and serine and phosphoserine (35-40%) (Saito et al., 2003). They were found to be critical for proper biomineralization of dentin in which they induce mineral nucleation (Fujisawa and Kuboki, 1998; Baolooch et al., 2004) but only when they are in low amounts (Lussi et al., 1988; Clarkson et al., 1998; Saito et al., 1998) and only when the negatively charged phosphate esters bound covalently to the positively charged collagen gap zones which traverse the negatively charged type I collagen fibrils forming phosphodiester bonds (Saito et al., 1997). This is followed by binding of calcium and phosphate to the resultant three dimensional protein conformations within these regions initiating plate-like apatite crystal formation (Butler, 1998; Ritchie et al., 1998; Saito et al., 2000). However, controlling the size and the orientation of crystals' growth, which are positively charged due to high calcium concentrations, is influenced by the binding capacity of these phosphoproteins and their abundance in the matrix, mainly when collagen is degraded and they are extensively released in a soluble form (Lussi et al., 1988; Clarkson et al., 1991; Lussi and Linde, 1993; Saito et al., 1997, 1998; Butler, 1998; Clarkson et al., 1998; Ritchie et al., 1998; Saito et al., 2003). Moreover, the reconstituted collagen fibrils

alone and the DPP adsorption to these fibrils do not induce mineral formation; thus irreversible binding of cross-linked phosphophoryn to the insoluble collagen is essential for mineral induction (Ritchie et al., 1998). For more details concerning DPP, the reader is advised to review the mentioned references. The organic and inorganic components of dentin are shown in tables 1.1 and 1.2.

Table 1.1. The organic and inorganic components of sound Human dentin

(Adapted from Tissue Preservation in Caries Treatment, 2001)

<table>
<tr><td colspan="2">Inorganic Components</td><td colspan="4">Organic Components</td></tr>
<tr><td>Ions</td><td>Percentag
(dry weight)</td><td colspan="3">Non Collagenous Matrix</td><td>Collagen</td></tr>
<tr><td>Calcium</td><td>25.1%</td><td>Non collagenous Proteins</td><td colspan="2">Non Proteinaceous Components</td><td>Type I</td></tr>
<tr><td>Phosphate</td><td>13.9%</td><td rowspan="10">Components are in table 2.</td><td>Glucosaminog-lycn</td><td rowspan="10">Lipids
Phospholipi-ds</td><td>Type I trimer</td></tr>
<tr><td>Carbonate</td><td>4.5%</td><td rowspan="3">Chondroitin -4-sulphate</td><td rowspan="4">Type III</td></tr>
<tr><td>Magnesium</td><td>0.85%</td></tr>
<tr><td>Sodium</td><td>0.54%</td><td rowspan="3">Chondroitin -6-sulphate</td></tr>
<tr><td>Potassium</td><td>0.019%</td><td rowspan="4">Type V</td></tr>
<tr><td>Chloride</td><td>0.072%</td><td rowspan="3">Dermatan sulphate</td></tr>
<tr><td>Fluoride</td><td>It is higher than bulk enamel fluoride concentration and it increases with deeper layers (Fejerskov and Kidd, 2003).</td><td rowspan="3">Type VI</td></tr>
<tr><td></td><td></td><td>Hyaluranate</td></tr>
</table>

Table 1.2. The Non-Collagenous Proteins

(Adapted from Tissue Preservation in Caries Treatment, 2001)

<table>
<tr><td colspan="6">Non Collagenous Proteins</td></tr>
<tr><td>Specific Dentin Protein</td><td>Mineral Tissue Associated Matrix Components</td><td>Serum Derived Proteins</td><td>Growth Factors
(Goldberg
and Smith, 2004)</td><td>Matrix Metalloproteina-ses (MMPs)</td><td>Enamel Proteins</td></tr>
<tr><td rowspan="4">Phosphophor-yn (50% wt)
-It has an important role to play in remineralizati-on through crystal nucleation initiation and growth inhibition.
(Discussed in text)</td><td>Dentin Matrix Protein 1</td><td rowspan="4">Albumin</td><td rowspan="4">- TGF-ß1
-Insulin-like Growth Factor-1 (IGF-1)
-Bone Morphog-enic Protein-2
(BMP-2)</td><td rowspan="4">MMPs and other tissue inhibitors</td><td rowspan="4">7K Da chondro-genic protein</td></tr>
<tr><td>Osteopoitin</td></tr>
<tr><td rowspan="2">Osteocalcin</td></tr>
<tr><td rowspan="2">Dentin Sialoprotein
-(5-8%wt) (Ritchie et al., 1998)
- It is related to phosphoproteins and coded by the same gene (located on human chromosome 4), so it is believed that it has to have a role in remineralization (Butler, 1998).</td></tr>
<tr><td>Bone Sialoprotein</td></tr>
<tr><td></td><td>Biglycan
Decorin
Lumican</td></tr>
</table>

Dentinal Tubules and Fluid

Dentinal Tubules contained in dentin matrix are important structural features which have to be mentioned when talking about dentin composition. These tubular structures act as diffusion channels, permitting the flow of ions, molecules and fluids such as acids (hydrogen ions), fluoride and dentinal fluid toward the pulp or/and outward. The direction of the flow of the tubules' contents is influenced by the osmotic pressure exerted by tissue fluid within the pulp; so, hypotonicity at the dentin surface causes inward water flow while hypertonicity moves it out (Ostrom, 1980). Although no significant correlation was found between the flow rate of dentinal fluid and dentin demineralization, mineral contents of in vitro dentinal lesions were higher and the lesion's depth was less when they were perfused with simulated dentinal fluid (SDF) than others perfuse with water (Özok et al., 2004). It is thought to be due to interaction between the demineralizing buffer and dentinal fluid which resulted in precipitation of minerals from the tubular fluid and/or dissolved peritubular dentin (Özok et al., 2004). The density and orientation of these tubules together with the hardness of the intertubular dentin and tissue mineral state play an important role in determination the hardness of sound dentin, in which softer dentin can be found at the Dentino-enamel Junction (DEJ) and at the pulpal surface due to these tubules (Banerjee et al., 1999). Moreover, dentin permeability also increases toward the pulp due to the high tubule number (which increases from 15.000 tubule/mm^2 in the outer third of dentin up to 35.000-55.000 tubule/mm^2 near the pulp) (Heinrich-Weltzien and Kneist, 2001) and increased diameter from 3µm to 5µm in the same direction (Ostrom, 1980). This increase in number and diameter will decrease and even eliminate the protective effect of the dentinal flow against demineralization (Özok et al., 2004). However, with age (Ostrom, 1980) or/and chronic irritation (trauma, caries) they become smaller in

diameter due to progressive deposition of peritubular dentin (Ostrom, 1980; Fejerskov and Kidd, 2003) which is absent near the pulp (Murray et al., 2003; Hara et al., 2005). Moreover, dentinal tubules are occupied with odontoblastic processes, but they are limited to the inner dentin in humans and do not extend to the DEJ (Weber and Zaki, 1986). Although other studies reported that these processes are extended through the whole dentin up to DEJ (La Fleche et al., 1985).

Caries Process

The outlines of caries mechanism can be summarised as follows:

Metabolically active cariogenic bacteria in a biofilm (in particular Streptococcus mutans and Lactobacilli) + carbohydrates (mostly succrose) →→ anaerobic fermentation →→ acids (such as lactic, acetic, propionic, butyric, formic and succinic acids) →→ enamel minerals dissolve →→ if the process progresses →→ acid penetration through the dentinal tubules →→ subsequent bacterial invasion →→ reduction in pH in the dentin environment →→ acidogenic aciduric bacteria lead to further acid attack →→ demineralization continues in dentin →→ degradation of collagen and other matrix components →→ bacterial proteases and hydrolases →→ digestion of the organic matrix →→ further demineralization and mineral dissolution →→ more organic matrix is degraded to be digested →→ if the process progresses, it will end with pathological pulp involvement.

However, it is now assumed that the caries process can be arrested at any stage if the bacterial biofilm can be disturbed and remineralization is allowed to take place.

Arrested and Active Lesions

Sarnat and Massler (1965) showed that these two lesion types are closely related in respect to their layers, suggesting that as progression of the caries process slows or ceases, changes occur which modify its appearance (Sarnat and Massler, 1965).

Schüpbach et al. (1992) concluded from previous studies that the main differences between active and arrested lesions are:

1- The higher content of minerals in the surface layer of the arrested lesions.

2- Viable bacteria are absent in dentinal tubules of the arrested lesions.

3- The latter are impermeable to dyes and isotopes.

4- The great resistance of these arrested lesions to acid dissolution and proteolytic enzymes activity.

The first characteristic point for the arrested lesions has its implication in clinical practice since sometimes restoring an arrested lesion is required due to aesthetics reasons (Hara et al., 2004). Bonding to such lesions showed low tensile strength and lack of tag formation (Hara et al., 2004). Moreover, no hybrid layer was observed in the same mentioned paper. These results are not surprising since the bonding between this layer and the adhesive to be used through tag formation is prevented by intra-tubular occlusion (Franklin and Pashley, 2004; Hara et al., 2004; Nakajima et al., 2004) and the micromechanical interlocking between the monomer and the intertubular dentin will be minimized by the heavily mineralized collagen and bacteria in these areas (Franklin and Pashley, 2004; Hara et al., 2004). Although increasing the acid etching time to the highly acid resistant layer will increase the depth of the demineralized dentin, it will -at the same time- decrease the tensile bond strength and create an extensive

demineralized layer which could not be totally enveloped with monomer, resulting in a weak point and microleakge (Piemjai et al., 2004). However, extending the bonding to include peripheral sound dentin was another suggestion (Franklin and Pashley, 2004). Other alternatives and suitable treatments to improve adhesion between such substrates and resins should be investigated.

Clinically, an arrested lesion is diagnosed as dark-brown or even black discolored lesion (Schüpbach et al., 1992), with a smooth and shiny surface (Miller and Massler, 1962) that is hard on probing with moderate pressure (Fejerskov and Nyvad, 1986).

The dark brown discoloration in such lesions is thought to be due to the melanin from amino acids and carbohydrates derivatives, and degenerated bacteria or/and their proteins and nucleic acids degradation products (Sarnat and Massler, 1965). Millard reaction (sugar-protein reaction) which modifies amino acids in collagen making them more resistant to enzymatic degradation and proteolytic attacks (Kleter et al., 1998; Beeley et al., 2000) seems the most accountable possibility for lesion discoloration (Kleter, 1998). In the deep, acidic and anaerobic environment the Millard reaction occurs with small aldehydes (bacterial metabolism derivatives), at the outer surface oxidation can permit the melanin and lipofuscin participation and discoloration (Kleter, 1998). However, when the lesion is exposed to the oral environment external pigments can add to the biochemical reactions in collagenous dentin (Fejerskov and Kidd, 2003).

Histologically, an arrested dentinal lesion, whether it is a coronal or root dentin lesion, is sandwiched between two highly mineralized layers; the hypermineralized surface layer and the sclerosed white opaque sclerotic zone. The contents of the intertubular dentin in the hypermineralized

surface layer are calcified and heavily mineralized, while the lumina of the dentinal tubules in the sclerotic zone are filled with calcified contents (Sarnat and Massler, 1965; Schüpbach et al., 1992). Detailed description of such lesions is written in the mentioned references (Sarnat and Massler, 1965; Schüpbach et al., 1992).

Sclerotic Dentin

This hypermineralized zone consists of increased mineral content at the active front of advancing carious lesion; its thickness varies with the intensity and chronicity of the carious attack (Ostrom, 1980). It can be as narrow as a small band walling off the lesion in acute caries or considerably wide extending from the lesion up to the root canal in chronic and more slowly progressing ones (Ostrom, 1980; Nikiforuk, 1985; Schüpbach et al., 1992).

Tubular sclerosis is considered to be the most common defense reaction by the pulpo-dentinal organ, in which deposition of mineral along and within dentinal tubules results in their gradual occlusion (Schüpbach et al., 1992; Fejerskov and Kidd, 2003). These deposited minerals make the tissue more homogenous, reducing the scattering of light passing through the affected tissue under the transmitted light microscope, thus appears as an area of transparency; the transparent zone (Fejerskov and Kidd, 2003).

This layer is suggested to form due to precipitation of mineral salts (dissolved apatite) in apatitic or crystallites and non-apatitic forms at the site during each reversal of the demineralization-remineralization equilibrium (Sarnat and Massler, 1965; Ostrom, 1980; Heinrich-Weltzien and Kneist, 2001). Therefore, the formation mechanism apparently depends on the microbial metabolism within the infected body of the lesion and parallels the remineralization of the surface layer (Ostrom, 1980; Schüpbach et al., 1992; Fejerskov and Kidd, 2003). Intra-tubular

calcification is an alternative explanation, in which obliteration of deep layers underneath the lesion due to minerals mediated by odontoblastic processes with the source of the minerals is the pulp blood supply (Sarnat and Massler, 1965). But whether this sclerosis is initiated as intracytoblasmic calcification followed by a secondary periodontoblastic mineralization or at the peritubular space followed by calcification of the odontoblastic process is not yet solved (Frank and Voegel, 1980; Schüpbach et al., 1992; Fejerskov and Kidd, 2003). Occasionally, if a lesion develops rapidly, odontoblasts will degenerate leaving (partially) empty tubules forming the so called dead tracts (Fejerskov and Kidd, 2003).

Dentinal Lesion

It is now well documented that the dentinal carious lesion consists of two layers. These layers differ in their microscopic structure and biochemical and physiological characteristics (Shimizu et al., 1981):

1. ***The superficial layer, which is:***

1.1 Clinically soft, wet and yellow (Biological Basis, 1980).

1.2 The infected layer, which comprises the body of the carious infection (Ostrom, 1980).

1.3 Composed of outermost necrotic zone that consists of disintegrating dentinal tubules and structurless matrix degraded by the proteolytic bacterial enzymes of the mixed flora which penetrated the dentinal canals. (Johansen and Parks, 1961; Sarnat and Massler, 1965; Nikiforuk, 1985).

1.4 This layer is to be removed because it is the main body of the lesion that burries the cariogenic bacterial mass (Massler, 1967; Baelum and Fejerskov, 2003; Kidd, 2004).

1.5 Physiologically unremineralizable (Fusayama et al., 1966; Kuboki et al., 1977; Shimizu et al., 1981). This layer is irreversibly demineralized due to the following structural, biochemical and physiological characteristics:

1.5.1 It has been appreciated that the structure of the collagen fibrils is an important determinant for the phosphophoryn binding sites as well as apatite induction controlled by this phosphophoryn-collagen complex (Saito et al., 2003). Consequently, the presence of the aforementioned denaturated collagen fibres is unremineralizable. It is a dead layer (Kato and Fusayama, 1970) and the degradation of collagen had proceeded to an extent were the nucleating properties nearly completely diminished (Johansen and Parks, 1961; Levine and Rowles, 1973; Klont and Ten Cate, 1991; Ten Cate, 2001), so neither remineralization as a vital reaction will take place (Kato and Fusayama, 1970) nor remineralization from an external origin can occur due to the above mentioned characteristics.

1.5.2 The concentrations of calcium ions in this layer are very low and few minerals if any are available for crystal nucleation or/and growth (Kato and Fusayama, 1970).

1.5.3 Odontoblastic processes which are important remineralizing contributors are dead (Kuboki et al., 1977).

1.5.4 Other organic matrix components such as glucosaminoglycan GAG and proteoglycan PG, are thought to play a role in remineralization inhibition, because they were found only in the predentin and they were degraded during dentinogenesis and mineralization of dentin (Butler et al., 1979). Thus, organic material removal from dentinal lesions is recommended if remineralization is to take place (Inaba et al., 1996). Moreover, Moreno and Zahradnik (1979) concluded that the alteration of the organic matrix of enamel may be the limitation for reversibility of the incipient lesion (Butler et al., 1979). It is logic that this is also true in the case of dentin were the organic

material contributes to 20% of the dentin weight and not just 0.5-2% as in enamel.

Adhesion to the infected layer:

On the other hand, subsequent bonding of the restorative material to such a layer is dramatically affected. The microtensile bond strength tests showed lowered bond strength between resins and demineralized dentin (Fuentes et al., 2004; Hara et al., 2004; Nakajima et al., 2005). The bonding quality was severely altered, due to lack of tag formation, loss of the mineral support resulted in low mechanical properties and collagen matrix collapsing and improper resin infiltration (Hara et al., 2004; Nakajima et al., 2005). Consequently, there was no hybrid layer formation which is the basic mechanism of resin-dentin bonding through the resin and collagen fibril network molecular interlocking (Prati et al., 1999). Despite the extensive and abnormal interdiffusion at the resin-dentin interface (Hara et al., 2004) the collapsed collagen fibers in the inter- and intra-demineralized dentin prevented the monomers from penetrating and complete infiltrating within the dentinal layer (Fuentes et al., 2004; Piemjai et al., 2004). This has its vital clinical implication, in which the sealing quality is impaired and microleakage can take place due to the microscopic voids between the collagen fibrils which were left by the incomplete diffusion of the adhesive monomers (Fuentes et al., 2004; Hara et al., 2004; Piemjai et al., 2004). This leads to the so called nanoleakage (Prati et al., 1999; Hashimoto et al., 2004; Nakajima et al., 2005), which is exposed unprotected collagen beneath the resin-dentine zone which adds to the microleakage (Piemjai et al., 2004). This naked unsupported collagen is a weak link at the bonding interface because it is liable for hydrolytic or/and proteolytic degradation by bacterial enzymes or/and host-derived matrix metalloproteinase (Fuentes et al., 2004). On the other hand, this apatite depleted collagen

zone has a low modulus of elasticity; therefore, it undergoes more strain than the hybrid layer overlying it (Yang et al., 2005). This in return largely controls the strength, quality, durability and longevity of the bond (Prati et al., 1999; Fuentes et al., 2004; Hara et al., 2004; Piemjai et al., 2004; Nakajima et al., 2005; Yang et al., 2005).

2. *The inner layer:*

Clinical practice increasingly encourages leaving this layer during excavation. Sarnat defined caries as an infectious disease, with the infection localized in the superficial layer. Therefore, removing just the superficial layer means eliminating the causative agent which results in arresting the carious process (Sarnat and Massler, 1965). Massler considered the inner layer as a partially demineralized but otherwise morphologically intact dentin (Ostrom, 1980). Moreover, Fusayama's observations (1975) suggested similarity between the collagen fibers of this layer and sound dentin with characteristic cross-links and regularly arranged fibrils (Ohgushi and Fusayama, 1975). In addition, Kuboki et al., (1977) analysis of the amino acids and collagen biochemistry of this layer concluded the retrogression of the cross-links from the mature to premature form. That was explained by normal shifting of the equilibrium state in collagen to precursor at acidic pH and to cross-link at neutral pH (Kuboki et al., 1977). Menaken (1980) described it as a partially demineralized dentin which retains much of its tubular structure although it is distorted (Ostrom, 1980).

2.1 Clinically this layer is dry, leathery or hard and brown (light/dark) (Ostrom, 1980).

2.2 It is further subdivided into zones, the discolored layer, the transparent zone and the subtransparent zone (Marshall et al., 2001). Two

points have to be mentioned in this area; the importance of discrimination between the transparent zone and the sclerotic layer which should not be used interchangeably except in very limited and atypical cases (fig. 1.2). The transparent layer is a part of the softened and demineralized carious lesion while the hardest layer of dentin is at the top of the normal dentin, beneath the transparent zone (Ogawa et al., 1983; Banerjee et al., 1999). This is very much different from the sclerotic dentin (discussed later) in chronic and arrested caries as a defensive mechanism of dentin through reactionary/secondary or reparative/tertiary dentin (Sarnat and Massler, 1965; Nikiforuk, 1985; Schüpbach et al., 1992; Kidd and Fejerskov, 2003; Goldberg and smith, 2004; Kidd, 2004). The Second point to be considered is the insignificance of the mineral deposits and loosely packed crystals within the tubules which do not contribute to the microhardness of dentin, where the overall mechanical properties of it are governed by the properties and mineralization state of the intertubular dentin (not the peritubular one), which is soft and partially demineralized in this transparent layer (Marshall et al., 2001).

2.3 The numbers of viable microbial cells in this affected layer are less than 0.1% of those in the infected one, in a gradual transition from the surface of the layer to the front of the lesion (Ostrom, 1980). Hence the challenge of the impossibility of exact discrimination between different layers with a definite border line, in which a residual remaining bacteria can be left in the cavity to be restored.

2.4 This layer is physiologically recalcifyable (Fusayama et al., 1966; Kuboki et al., 1977; Shimizu et al., 1981).

2.5 Again this layer is remineralizable due to the following structural, biochemical and physiological characteristics:

2.5.1 Collagen fibers in this layer are demineralized but not denaturated (Johansen and Parks, 1961; Kuboki et al., 1977), in which the exrafibrillar minerals that lie within the interstitial spaces separating the fibrils (Landis et al., 1996) were dissolved. These minerals are suggested to form the major portion of the mineral phase (Kinney et al., 2003). On the other hand the intrafibrillar minerals which are confined within or immediately adjacent to the gap junctions of the collagen (Klont and ten Cate, 1991; Landis et al., 1996) were not affected. Consequently, the internal structure of the fibers were not destructed or degenerated.

2.5.2 Moreover, the phosphophoryns are in the phosphorylated insoluble bounded form and covalently cross-linked to the collagen, so they are able to bind calcium and initiate crystal nucleation and hydroxyapatite formation (Saito et al., 2000; Milan et al., 2006).

2.5.3 Residual crystals and calcium ions are found in this layer (Kato and Fusayama, 1970).

2.5.4 Odontoblastic processes which contribute to recalcification (Heinrich-Weltzien and Kneist, 2001) are living (Kuboki et al., 1977).

Last point to be considered in this area is that the recalcification of this layer if not external due to saliva (Almqvist and Lagerlöf, 1993; ten Cate, 2001; Mukai et al., 2001) or remineralizing capping or/and restorative material (Dijkman et al., 1993), it occurs internally as a vital reaction by pulp function (Kato and Fusayama, 1970).

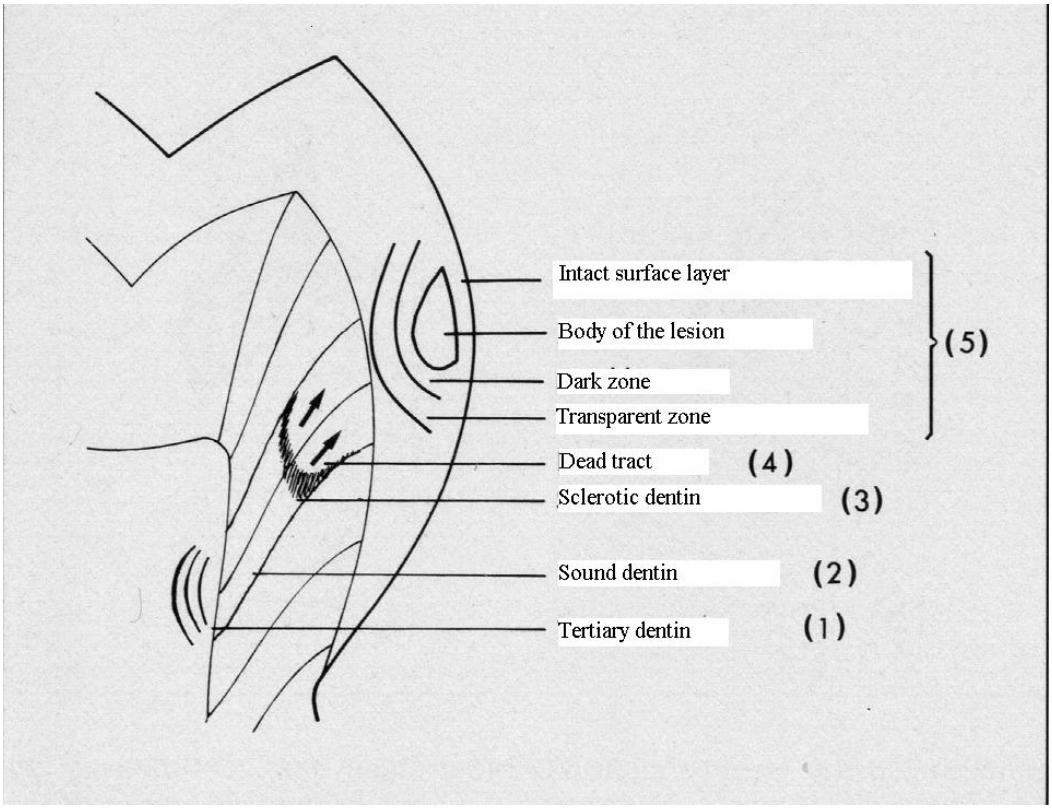


Fig 1.2. The different histological zones of dental caries. The transparent zone (zone 5) and the sclerotic dentin (zone 3) are two different zones.

The Current Criteria for caries removal

Several studies were made to evaluate the clinical outcome of leaving the affected dentinal tissue behind after excavation before restoring the tooth (Bjorndal et al., 1997; Weerheijm et al., 1999; Maltz et al., 2002; Lager et al., 2003; Foley and Blackwell, 2003). These authors and others believe that when they eliminate the harmful microbial mass at the lesion surface, they permit the underlying layer to heal gradually through the biological properties of the tissue after sealing it with a restorative material. At the same time they preserve the remineralizable tissue, maintain pulp vitality by avoiding its exposure and arrest caries progression (Ostrom, 1980; Nikiforuk, 1985; McComb, 2000; Heinrich-Weltzien and Kneist, 2001; Fejerskov and Kidd, 2003). Accordingly step wise excavation described by Bodecker (1939) (Kidd, 2004) and indirect pulp capping adopted by Nygaard-Östby (1972) (Fisher, 1981) using different cements and dental materials, mainly $Ca(OH)_2$ and ZOE (Fisher, 1981; Fejerskov and Kidd, 2003), were and are still up to date widely used.

These methods showed high success rates, but the effect and interaction of the restorative material on and with the dental tissue is still questioned and undetermined (Fisher, 1981). Moreover, current clinical criteria for complete removal of the cariogenic flora are dependent on individual judgments and different schools, which make them more or less reliable criteria (Ostrom, 1980; Lager et al., 2003; Fejerskov and Kidd, 2003).

However, in the absence of safe clinical parameters guiding total caries removal, it is well accepted that excavation never attempts to sterilize a cavity. Today it is well recognized that the clinically judged caries-free cavity is never steril what ever approach is adopted (Kidd, 1996) and micro-organisms will never be totally eliminated even if the soft dentin is completely removed (Lager et al., 2003). Even when dentin is

hard on probing without sticking or tug-back sensation, the cavity is never caries-free according to the investigations of Seltzer (1940), Shovelton (1968, 1970) and Lichtenberg-Crone (1968) due to the irregular and diffuse nature of the front of the lesion, which permit ingression of micro-organisms through dentinal tubules and their retention beneath the hard surface (Fisher, 1981; Bjorndal et al., 1997).

The typical dark brown discoloration together with hard (judged clinically by the probe) and dry texture are the conventional criteria we rely on in our clinical examination, although recent studies had claimed that there are no associations between bacterial growth quantities in a lesion and its coloration (Kidd et al., 1996; Maltz et al., 2002; Foley and Blackwell, 2003). Moreover, when both stained and stainless dentin harbors bacteria whether at the pulpal floor or at the Dentin Enamel Junction (DEJ), why stained dentin is to be excavated (Kidd et al., 1996)? Although wet soft caries is a heavily infected one (Kidd et al., 1993, 1994), dry old caries can be found under restorations (Kidd, 2004) and a universal definition of a reasonable hard firm floor is much dependent on the examiner (Fejerskov and Kidd, 2003) and it is not easy to find it in acute cases (Fusayama et al., 1966; Ostrom, 1980). However, it seems that these conventional clinical criteria, although blunt, they are sufficient and satisfactory assessments (McComb, 2000; Lager et al., 2003).

Caries Detector (1.0% acid red in propylene glycol) developed by Fusayama (1979) claiming to discriminate between infected and affected layers (Fusayama, 1988) seems to fail in differentiation between infected and soften dentin (McComb, 2000) because it depends on the demineralized organic matrix not on the bacterial presence (McComb, 2000). Thus, routine usage of this dye may lead to over preparing the cavity and unnecessarily sound dentin removal from the circumpulpal

dentin and at the DEJ since both are stained -due to lower mineral density- although they are sound (McComb, 2000).

Dentin caries autofluorescence (AF) was subjected to different investigations to relate its signaling to a particular component or process in carious lesion. No direct correlation or exact matching was found between AF distribution and mineral content within the lesion using Confocal Laser Scanning Optical Microscope (CLSM) and Backscattered Electron Microscope (BSE-SEM) (Banerjee and Boyde, 1998). Nevertheless, Banerjee et al. (1999) reported a correlation between carious dentin AF and its microhardness and discoloration suggesting it as a reproducible, objective and histological marker for the excavatable carious dentin (Banerjee et al., 1999). Moreover the nature of carious dentin natural fluorescence is not known although it is suggested to be of organic origin (Banerjee and Boyde, 1998). Currently, the careful visual inspection combined with radiographs would appear to best fulfill the diagnostic requirements (McComb, 2000).

Alternative Methods of Carious Dentine Excavation

It is not surprising that many researchers, companies and studies are trying to find an objective parameter that can be specific, reliable and valid as a clinical caries detector. Therefore, alternatives to the mechanical excavation using a spoon excavator or a bur, aimed to be more selective in caries removal avoiding pulp irritation, cavity over preparation and pulp exposure by removing only the infected denaturated and cariogenic layer leaving behind the affected demineralized but remineralizable one (Ericson et al., 1999; Banerjee et al., 2000; Beeley et al., 2000; Fure et al., 2000; Nadanovsky et al., 2001; Lager et al., 2003; Rafique et al., 2003; Flückiger et al., 2005). Patient's comfort beside his dental health sake was also considered and tested (Ericson et al., 1999; Banerjee et al., 2000; Beeley et al., 2000; Fure et al., 2000; Nadanovsky et al., 2001; Rafique et al., 2003).

Various caries removal methods together with their advantages and disadvantages are shown in table No. 1.3 and table No. 1.4 (adapted from Banerjee et al., 2000; Rafique et al., 2003).

Table 1.3. Various caries removal methods

<table>
<tr><td colspan="5">Methods of excavation</td></tr>
<tr><td colspan="2">Mechanical</td><td>Chemomechanical</td><td colspan="2">Non-Mechanical</td></tr>
<tr><td>Rotary</td><td>Non-Rotary</td><td>-</td><td>Chemical</td><td>Photo-Ablation</td></tr>
<tr><td rowspan="5">Hand-pieces and Burs</td><td>Hand excavation</td><td rowspan="5">Carisolv</td><td rowspan="5">Enzymes</td><td rowspan="5">Laser</td></tr>
<tr><td>Air polishing</td></tr>
<tr><td>Air abrasion</td></tr>
<tr><td>Ultra sonic</td></tr>
<tr><td>Sono-abrasion</td></tr>
</table>

Table 1.4. Comparison between various caries removal methods

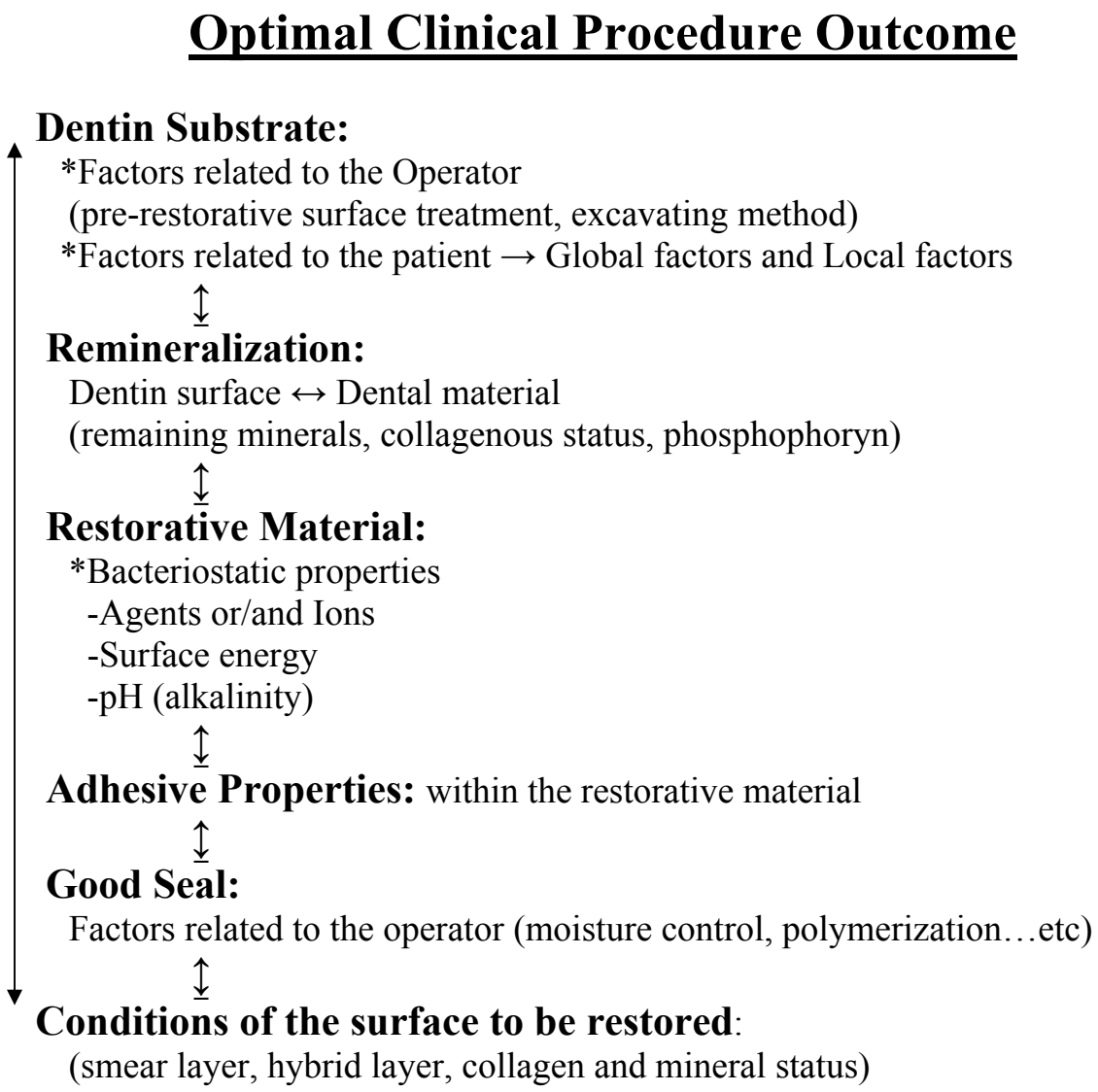
The Method	Advantages	Disadvantages
Burs	1-Effective in caries removal 2-Efficient (fastest method) 3-Almost always needed for initial access and cavity outlining	1-Anesthesia (time, anxiety, pain, numbness, bad taste) 2-Discomfort (noise, water cooling system, vibrating) 3-Pain (sensitivity) 4-Pulp irritation (thermal, mechanical) 5-Smear layer 6-Overpreparation
Hand Excavation	1-Effective 2-More self-limiting than bur (sensitive tactile feed back)	1-Time consuming 2-Painful 3-Smearing 4-Overpreparation
Carisolv ➢ Carisolv System by Medi Team (1997-1998) Composition: 2 Carboxymethyl-cellulose (CMC) based gels : - 0.5% NaOCl (Clear liquid) - 0.1M amino acids, NaCl, NaOH (PH 11), erythrosine dye, CMC 200-800 cps, purified water.	1- Effective (Ericson et al., 1999; Beeley et al. 2000; Fure et al., 2000; Lager et al., 2003; Flükiger et al., 2005). 2- Efficient (extra time is compensated with the time needed for anaesthesia). Fure et al., 2000). 3- Eliminates over prepared cavities. 4- Decreases the risk of pulp exposure. 5- Antibacterial effects of chloramines (Lager et al., 2003). 6- Removes the smear layer improving bond strength and quality (Beeley et al., 2000). 7- Less discomfort and pain: a - Advantageous for the dental phobic, medically compromised patients, children and patients with high dental fear and anxiety. b- Thermal insulator. c- No vibrations, noise and pressure. d- No need for anesthesia and	1-Limited accessible lesions (Nadanovsky et al., 2001) 2-Efficiency is questionable (Yip et al., 1999) 3-Taste and Smell (Maragakis et al., 2001) 4-Time consuming (Maragakis et al., 2001; Flückiger et al., 2005) 5-Render dentin substrate weak (Fuentes et al., 2004) and effect the bonding quality (Parti et al., 1999).

	associated dislikes. e- Eliminates the thermal and pressure effects on the pulp.	
Enzymes (bacterial acromobacter collagenase, pronase enzyme)	-	Promising
Laser: U.V. Emission (Excimer Laser 377nm)	1-Decreased post operative sensitivity (sealed tubules)	1-Thermal irritation to the pulp. 2-Distruction or alteration in adjacent tissue. 3-Expense and size of equipment. 4-Control of procedure.
Air Abrasion (Disadvantages are when abrasive material is only Aluminum oxide and advantages when it is mixture with hydroxyapatite)	1-Effective 2-Efficient 3-Painless 4-No pulpal irritation	1-Non-Selective 2-Loss of tactile sensation 3-Overpreparation 4- Inadequate carious dentin removal
Air Polishing	-	Non-Selective
Ultra sonic	-	Overpreparation
Sono Abrasion	-	1-Underpreparation 2-Smearing

The Role of the Restorative Dental Material

The dental material to restore a tooth has a significant role in the success of the operative procedure and on the long term outcome. Thus, the practitioner's work with a particular tooth will be ended by the end of the session, while the effect of the material on the surface it restores begins. Therefore, it is very much logic to focus at the requirements we need in a material to restore a deep dentinal lesion. It is obvious from the following figure (fig 1.3) that the restorative material interacts with the tooth tissue in many different ways. For example, sealing properties of a dental material are considered to be the most important properties in preventing caries progression or/and recurrent caries (Ostrom, 1980; Mertz-Fairhurst et al., 1998; Heinrich-Weltzien and Kneist, 2001; Maltz et al., 2002; Fejerskov and Kidd, 2003; Kidd, 2004). At the same time, the bacteriostatic properties would be beneficial for eliminating or at least reducing the number of residual bacteria left in the cavity (Fisher, 1981; Mjör, 1996; Weerheijm et al., 1999; Foley and Blackwell, 2003; Splieth et al., 2003). However, it is claimed that reduction can be achieved by just sealing the cavity (Bjorndal et al., 1997; Mertz-Fairhurst et al., 1998; Maltz et al., 2002; Foley and Blackwell, 2003; Lager et al., 2003). Another way of interaction is the remineralizing effect that the material can exert on the dentin surface (Saito et al., 2003; Deng et al., 2005). The figure illustrates clearly that there is no independent factor responsible for the success or failure of a restoration. Instead, it is the vital, sensitive, essential and precise interaction between the operator knowledge, skills, facilities and sense with an improved adhesive material that is qualified to the roles (sealing, antibacterial, remineralizing) it will play in the cavity. These together with the available tooth substrate bearing in mind the individual patient related factors will influence the end results of the dental operative procedure.

Fig 1.3. Illustration of the integration of the determinants of the "Perfect" restoration.



The "Perfect" Restoration

It is worthwhile to mention here that although secondary caries is the most common cause of restorative failure (Splieth et al., 2003), the imperfect seal is claimed to be the most important etiological factor behind recurrent caries (Bjorndal et al., 1997; Heinrich-Weltzien and Kneist, 2001; Fejerskov and Kidd, 2003; Kidd, 2004).

Therefore, it is reasonable to double check the accusing of the remaining residual bacteria for caries re-initiation (Bjorndal et al., 1997; Foley and Blackwell, 2003; Kidd, 2004). Although these remained bacteria could stay alive up to two years (Ostrom, 1980; Bjorndal et al., 1997; Kidd, 2004), receiving nutrients -most probably- from tissue fluid via the pulp or/and degradation products of remaining dead bacterial cells (Ostrom, 1980) or possibly through the porous enamel, it is not determined until when these supplies are sufficient? And until when these bacteria can survive (Ostrom, 1980; Foley and Blackwell, 2003; Kidd. 2004)?

In addition, the association of these residual bacteria with dentin demineralization, pulpal pathology and secondary caries is not yet proved (Ostrom, 1980; Bjorndal et al., 1997; Foley and Blackwell, 2003; Kidd, 2004)!

Are these retained hidden bacteria powerful in the term of causing recurrent caries after not known years (Ostrom, 1980; Foley and Blackwell, 2003; Kidd, 2004)? How many can we leave behind? Although Kidd et al. (1993) suggested that mutans streptococci (recognized as initiators for carious process and their presence with lactobacilli that is associated with lesion development is indicative for lesion activity) numbers can reach 100 CFU/sample (burful) without being clinically significant (Kidd et al., 1993). Foley and Blackwell (2003) found arrested lesions under black

copper cement with even more than this number (Foley and Blackwell, 2003).

Maybe by the traditional cultivation methods there are other undetected bacteria associated with lesion progression, since dentin caries is a complicated environment which involves a complex multi-species flora (Lager et al., 2003). However, the new environment of these viable cells after sealing the cavity and cutting of the sufficient amounts of nutrient sources needed for surviving, multiplication and cariogenesis seems to influence not only the quantity of the existing flora, but also the quality of the dominant species (Ostrom, 1980; Mertz-Fairhurst et al., 1998; Love and Jenkinson, 2002; Splieth et al., 2003; Kidd, 2004), but the exact role of the new conditions (nutrients depletion, pH raising, anaerobic environment) in changing the cariogenicity and species of the dominant flora under restoration is not clarified yet.

The answer may be so much dependent on the conditions left behind (Murray et al., 2002) such as the quality of the prepared cavity, the efficacy of the cavity sealing and the restorative material to be used together with its pH and remineralizing properties, antibacterial and adhesive characteristics.

On the other hand, despite caries arrestment reported in many studies upon re-entering the cavity based on clinical criteria and dentin appearance, radiography and microbiological examination (Bjorndal et al., 1997; Mertz-Fairhurst et al., 1998; Weerheijm et al., 1999; Maltz et al., 2002; Foley and Blackwell, 2003; Lager et al., 2003), slower destruction activities due to fewer nutrient supplies can not be precluded (Ostrom, 1980; Foley and Blackwell, 2003; Kidd, 2004) and long clinical trials are essential if these questions are to be definitely answered (Kidd, 2004). Moreover, systemic review with direct comparison between these studies can not be made due to the variable clinical conditions (Kidd, 2004). However, all of these

studies except for Weerheijm et al. (1992) reported dark, dry, firm and leathery dentin upon re-opening the cavity, with decreased radiolucency and marked reduction in viable cell counts (Fejerskov and Kidd, 2003; Kidd, 2004) which is indicative for an arrested lesion (Miller and Massler, 1962).

Nevertheless, the remaining question which was the main reason behind this review is not yet answered. Instead it evoked even more branching related questions. If G.V. Black was too radical in his operative approach (Fisher, 1981), and we accepted the more conservative attitudes which are facilitated by the daily improvement of the dental materials. What is the reliable guide to our excavation, if we have to excavate something? I mean if we stand in the middle of the way and removed just the outermost layer which contains the biofilm due to the previously explained reasons, and we intend to be so carefully conservative not to unnecessarily remove any healthy remineralizable dentin, what is our universal, standard, sharp criterion?

Figure 1.3 (pp 34) is a trial to collect, understand and connect these elements in order to achieve an optimal clinical outcome.

The aim of this study:

(1) Based on the literature we know that in the presence of fluoride even dentinal caries lesions have the potential to remineralize. However, it is unknown, which fluoride concentration will be optimal. In addition it is not really clear whether the remineralization is a general increase of the hydroxyapatite concentration throughout the lesion or whether the depth of the lesion is reduced.

The first experiment studied the influence of various concentrations of fluoride on the remineralization of dentinal lesions. In addition the mineral distribution throughout the remineralized lesions was evaluated.

(2) As we figured out throughout the literature review, only a part of the carious dentin has to be removed and another part has the potential to remineralize. Based on the reviewed studies the presence of hydroxyapatite is mandatory in order to remineralize collagen fibers after a carious attack. To evaluate new selective, self-limiting carious therapies we need a model which has both types of collagen fibers, those which should be removed because they are denatured and those which can be left because they are only demineralized but have an intact structure enabling their remineralization.

The literature is not too detailed about these different types of collagen. Neither the way to produce a reliable model nor the proof of its validity is mentioned.

In the second part of the thesis a model was developed to obtain a reproducible artificial dentin lesion which has denaturated and in addition demineralized dentin.

(3) In the third part the artificial carious lesions were treated with different solutions of known working mechanism, because we wanted to learn how much materials can be removed with these different mechanisms. In addition the morphology of the remaining substrate was documented as a reference for future studies because this information is currently not available in the literature.

(4) Finally, it is necessary to know whether the principles of remineralization, as evaluated in part 1, can be applied to the dentinal tissue which is left after treating the lesion with the solutions that we used in part 3. This step was necessary to prove that it is possible

a) to be less invasive than it is current practice with the bur which is what we call “self-limiting”,

b) to leave softer, but not denatured collagen in a lesion and

c) that this dentin quality has the potential to remineralize.

Influence of Fluoride Concentration on the Distribution of Minerals in Dentinal Lesion

Chapter 2

Introduction

In the last few decades enamel remineralization has been a major subject (Silverstone et al., 1969, 1981; Moreno and Zahradnik, 1979; Strang et al., 1987; Featherstone et al., 1990; LeGeros, 1990; Heskströter et al., 1991; ten Cate et al., 2003). The role of different fluoride concentrations in calcium-phosphate containing remineralizing solutions (ten Cate and Duijsters, 1983a, 1983b), toothpastes, varnishes, gels and dentifrices (Schmit et al., 2002; Demito et al., 2004), glass ionomer cements (Thornton et al., 1986), bonding agents (Corry et al., 2003), composites (Arends et al., 1990; Dijkman et al., 1993), chewing gums (Lamb et al., 1993) and slow release devices (Greene et al., 1986) in remineralizing incipient and advanced natural as well as artificial enamel lesions (Silverstone et al., 1981; ten Cate and Duijsters, 1982; ten Cate, 2001) is well-documented in the literature.

The thermodynamic driving forces and kinetic factors involved in enamel lesion formation have been intensively investigated and analysed in situ and in vitro (Silverstone, 1968; Higuchi, 1974; Moreno and Zahradnik, 1974, 1979; Featherstone et al., 1983, 1985; ten Cate and Duijsters, 1983a, 1983b; Arends and Christoffersen, 1986; Robinson et al., 2000; Zhang et al., 2000). Moreover, remineralizing such lesions with various concentrations and forms of fluoride (sodium fluoride, stannous fluoride, monofluorophosphate sodium, acidulated phosphate fluoride, amine fluoride, silver fluoride and silicate fluoride) to enhance remineralization

has also been extensively studied (Mukai et al., 2001; ten Cate et al., 2003; Tanoue et al., 2004).

Relatively few studies have tested the remineralization of dentin together with the effect of fluoride on remineralizing dentinal lesions. Although remineralizing dentin with fluoride containing remineralizing solutions follows the same general physicochemical principles of enamel biomineralization, such a process is more complicated in dentin than in enamel due to the compositional and ultrastructural differences between both tissues. Dentin is composed of 20 wt% organic matrix while in enamel it is about only 1 wt%. 90% of the organic phase in dentin is made up of collagen (mainly type I) while the remaining 10% are of non-collagenous components. In enamel proteins form the major portion of the small inorganic phase. Moreover, the presence of dentinal tubules, their orientation, numbers and diameters influence dentin permeability and affect the diffusion process. Not only the volume but also the composition of the inorganic phase is different in dentin and enamel; 70 wt% in dentin and 96 wt% in enamel. The small dimensions of dentin crystallites, the proportions of carbonate and magnesium ions incorporated in the hydroxyapatite lattice, their crystallinity and composition with dentin porosity complicate the remineralizing process even more. In addition to these differences, dentin which is of mesenchymal origin is a biologically active tissue that forms one complex with the pulp through their histological, structural and chemical interactions, unlike the ectodermal acellular enamel which is a biologically inert tissue.

The role of fluoride in enamel remineralization is of a particular interest for preventive measures. In addition these preventive purposes were also the driving forces behind remineralizing dentinal lesions for the sake of arresting dentin root caries (Almqvist and Lagerlöf, 1993; Kawasaki et al., 1999; Mukai et al., 2001, 2002) while remineralizing

coronal dentinal lesions is of a curative importance and has its clinical implications in repairing deep dentinal carious lesions under dental fillings. Such deep dentinal lesions are prone to remineralization under certain conditions which favour crystal growth on partially demineralized dentin (Levine and Roweles, 1973; Kuboki et al., 1977; Lussi et al., 1988; Arends et al., 1989; Klont and ten Cate, 1991; Lussi and Linde, 1993; Inaba et al., 1996; Saito et al., 1997, 2003; Clarkson et al., 1998; Ritchie et al., 1998; Butler, 1998; Damen et al., 1998; Kawasaki et al., 1999, 2000; ten Cate, 2001).

Dentin is capable of remineralization but the distribution of mineral ions in the presence of fluoride within the lesion body and the depth at which the lesion can still be remineralized are not well clarified at present.

The purpose of this study was to determine the level of fluoride which could enhance the remineralization of a dentinal lesion. The role of fluoride in remineralizing the lesion surface, body and front is to be studied together with the possible influence of the dense surface mineralized layer on remineralizing the lesion body and/or front. In this part we aimed to test the hypothesis that fluoride is capable of remineralizing the dentinal lesion front and thus it is efficient in decreasing the lesion depth.

Materials and Methods

Sample Preparation:

Seventy-five extracted human third molars were obtained from an oral surgeon’s private clinic and used within 5 months of extraction. After extraction teeth were immediately stored at room temperature in distilled water to which sodium-azide was added to prevent bacterial growth. All teeth were clinically sound and they were carefully observed for caries, abrasions or any mechanical traumas. Teeth were cleaned with a tooth brush and sometimes with a scalpel to remove the periodontal ligament and

intercrestal bone remnants and rinsed under running tap water. They were embedded individually in transparent cold-curing methylmethacrylate (Technovit 4004, Kulzer GmbH, Wehrheim, Germany).To expose deep-coronal dentin the occlusal half of each tooth was cut using a slow speed water-cooled diamond saw (Isomet, Beuhler, Illinois, USA). Dentin exposed surfaces were then polished flat with water proof silicon carbide abrasive paper (P500-grit) with Leco VP 100 (GmbH, Neuss, Germany). Subsequently they were polished using wet polishing paper with a silicone paste of polycrystalline diamonds of size 9 μm (DAP-7, Struers, Copenhagen, Denmark).

Lesion Formation:

Dentin surfaces and the surrounding Technovit were coated with two coats of nail varnish (Keyte GmbH, Munich, Germany) to avoid the penetration of the solution into any marginal gaps that could exist between the tooth and the acrylate, whilst leaving two windows of exposed deep-coronal dentin per tooth. Adhesive paper was cut into 2x5 mm² pieces and attached to the dentin surfaces before applying the nail varnish to standardize the windows' sizes. The samples were then kept in air for about half an hour to dry the nail varnish and after the removal of the adhesive paper they were immersed in the demineralizing solution (40 ml per sample). The demineralization solution contained 50 mM acetic acid, 2.2 mM $CaCl_2.2H_2O$, 2.2 mM $KHPO_4$, 1 mM $NaNa_3$, 2 M KOH. No fluoride was added to the demineralizing solution. The pH was adjusted to 5.0 with drops of KOH and was measured through out the demineralizing period (two weeks with gentle shaking (Müller Schüttler, Munich, Germany) at 37°C). The demineralizing solution was refreshed weekly to avoid changes of the solution's pH of more than half a pH unit.

Remineralization:

After artificial lesion formation the samples were washed with distilled water and divided into six groups (n = 12 per group). Each group was transferred to a flask containing 1 l of remineralizing solution composed of 20 mM HEPES, 1.5 mM $CaCl_2.2H_2O$, 0.9 mM $KHPO_4$, 130 mM KCl, and 3.08 mM sodium-azide with the pH adjusted to 7 with KOH. Different fluoride concentrations were used for each group 0, 0.1, 0.5, 1.0, 5.0 and 10.0 ppm as NaF. Again remineralization was performed with shaking at 37.0°C. After the first week the pH of the solutions was measured to be 7.2 for all groups, three samples were taken from each group and kept in Ringer solution until and during the processing period which always began on the same day, the solutions were refreshed, and the flasks were returned to the shaker once again. The same procedure was repeated every week for four weeks. The experimental groups are shown in table 2.1.

Samples for Lesion Assessment:

The teeth were cut perpendicularly to the two windows at the dentin surface with a thin diamond blade on a saw microtome (Leica SP 1600, GmbH, Nußloch, Germany) under tap water into thin (120 µm) and thick (280 µm) sections. Each section was then polished flat with wet silicon carbide abrasive paper (800-grit) to obtain a plano-parallel slice of 110 µm and 250 µm thickness. Thin slices from each tooth were kept in air for 10 min to dry before imbibition in quinoline (Quinoline 22650, Fluka Chemie GmbH, Hamburg, Germany). They were then mounted for microscopic examination. Lesion depth was measured along a vertical line perpendicular to the tooth surface extending from a point at the lesion surface to a point at the non-demineralized surface through out the lesion body to the inner most border of the lesion. The thick samples were divided into two groups. The first was taken for microhardness testing and the

second was prepared for the morphological evaluation in a Field Emission Scanning Electron Microscope (FE-SEM).

Analytical Tools:

Microscopy:

Quinoline with polarized light (Axioskope 2, MAT, Carl Zeiss Jena GmbH, Göttingen, Germany) was used for the visual qualitative analysis of the lesions before and after remineralization. Digital images were taken with the image analysis software Axiovision (Rel. 4.4, SP2, Carl Zeiss Jena GmbH, Göttingen, Germany).

Microhardness:

Testing the microhardness of the remineralized dentin was performed with a Vickers pyramid diamond indenter at 500 mN/mm² and an automatic microhardness tester Fischerscope H100C (Helmut Fischer GmbH, Sindelfingen, Germany). Two lines were made per lesion in which each line was composed of 4-6 points which were spaced by 50-70 µm. Each line extended vertically through the lesion from a point just beneath the lesion bottom up to the surface to determine cross surface microhardness (CSMH) through out the lesion.

FE-SEM:

To obtain information on the morphology of the mineral depositions a FE-SEM was used. Samples were immersed in 50% alcohol for 20 min, then in 70%, 80% and 90% alcohol each for 20 min. Finally they were kept overnight in 96% alcohol. Samples were immersed in Hexamethyldisilazane for 10 min and air dried at room temperature according to Perdigao et al. (1995). Then liquid nitrogen (-70°) was used for each sample for few seconds to facilitate the fracture before using a scalpel to initiate a crack from the pulpal side. Each sample was then fixed on the SEM sample holder with carbon paste. Gold sputtering was done for

1 min, with 1.0 Kv, 0.3 mbar and 40 mA (Edwards Sputter Coater S15OB, Sussex, UK). Pictures where then made with a Leo FE-SEM (Leo DSM 982, Carl-Zeiss NTS GmbH, Oberkochen, Germany).

Through out the whole experimental procedure care was taken to avoid sample drying and dentin desiccation particularly after lesion was formed with the exception of the SEM samples were drying was mandatory.

Results

Lesion depth before and after remineralization with various fluoride concentrations after 1, 2, 3 and 4 weeks was measured by polarized light microscope (PLM) and is shown in table 2.2.

Lesions which were remineralized without any fluoride additions to the remineralizing solution (group A) showed no decrease in lesion depth either microscopically determined or with the microhardness profiles. Moreover, no changes were observed in the dentin hardness throughout the lesion even after 4 weeks (A4) of remineralization except for the surface layer where the Vickers indentations showed higher values. The surface values were equal to and sometimes even exceeded the values of sound dentin in the third (A3) and fourth week (A4) (diagram 2.1). Table 2.3 shows the mean values of the CSMH per group.

FE-SEM pictures of the surface of the control group (A) showed well-mineralized intertubular dentine with some mineral precipitates at the surface. Peritubular dentin was also seen with tubules' diameters within the normal range (1.5-2.5 μm), decreased or even occluded. Intertubular dentin at the fracture surface was more mineralized in group A3 than in A2 (fig. 2.1, 2.2).

With the presence of fluoride ions in the remineralizing solution (0.1, 0.5 and 1.0 ppm) the distribution of minerals and the pattern of

remineralization changed. After one week there were no differences between the groups B1, C1 and D1 and the control group A1 in any of the used analytical tools except that the lesion depth was decreased by 40µm in the first three groups although the decrease was not constant in all samples and was not affected by differences in the fluoride concentrations. However, the SEM pictures of groups B2, C2 and D2 showed much more surface mineralization with well-remineralized intertubular dentin and prominent thick peritubular dentin, and many of the dentinal tubules were occluded (fig. 2.3). Crystalline precipitates were also observed at the surface. The upper most surface layer of the fractured side appeared morphologically to be more mineralized than the remainder of the lesion body although the lesion body was also mineralized to the extent that borders of the dentinal tubules within the lesion were not very distinguishable (fig. 2.4). Although the hardness tests did not demonstrate an increased hardness of the surface of these lesions, they showed an improved hardness in the lesion body in comparison with the control group. After the fourth week the hardness values were also increased at the surface but did not exceed the normal values.

PLM showed banding near the surface and within lesion body in groups B, C and D (fig. 2.5). The banding seen with the PLM and the improved hardness measured by the Vickers indenter were strongly related to the fluoride concentration (D > C > B). Increasing the remineralization time also enhanced these effects (3^{rd} week > 2^{nd} week). After the first week there was no further decrease in lesion depth throughout the experimental period for all groups. Groups E and F had better hardness values from the first week (E1 and F1). Moreover, an apparent surface layer appeared in the PLM, especially during the last two weeks (E3, E4, F3 and F4). Although the hardness measurement with the Vickers indenter did not show higher values at the surface in groups E and F, the SEM pictures were full of

precipitates that occluded the dentinal tubules which were clearly surrounded with peritubular dentin and hyper-mineralized areas (Fig. 2.6, 2.7). The hyper-mineralized areas were not continuous in group E while they formed a dense hyper-mineralized layer in group F. Mineral precipitates were also found at the fracture side within the lesion body in both groups (Fig. 2.8).

Discussion

In the 90 s, caries researchers such as ten Cate and Arends had focused on the effects of fluoride on dentin de- and remineralization in addition to their known studies of its effects on both phenomena in enamel. Ten Cate demonstrated that dentin has a much higher uptake capacity for fluoride than enamel (ten Cate, 1999), while Arends showed the ability of both bovine and human dentin to 'over-remineralize' (Arends et al., 1989, 1990). However, in our study the remineralizing solution without fluoride addition did not contribute at all in remineralizing the lesion body together with its front. Moreover, a well-remineralized surface layer can still be formed even without fluoride and without observing a significant remineralization within the lesion body or decrease in lesion depth. These results were partially in agreement with Kawasaki et al., (2000) who found that a surface layer was formed even without fluoride addition although this case showed a better overall remineralization than lesions which were remineralized in the presence of high fluoride levels (10 ppm). The difference in mineral distribution within the lesion in both studies can be due to the differences in the study's design and materials and methods so that a direct comparison between both studies can not be made. First because his results were relative to other types of lesions in that study and second because the methods of evaluation used in the two experiments are not comparable because mineral deposition can occur within the lesion without contributing to its hardness (Marshall et al., 2001). In addition,

differences in the structure and behaviour between crown and root dentin due to differences in their development have been suggested by Goldberg (2004). Furthermore, the volume of the remineralizing solution per sample in the mentioned study was much smaller than ours which in return affects the remineralization rate.

In our experiment increasing the volume of demineralizing solution, demineralization duration, solution stirring and refreshment probably resulted in an increased demineralization rate (Theuns et al., 1985), and increased lesion depth with increased baseline mineral loss (Arends et al., 1997).

The increase in mineral loss increases the concentration gradient after putting the sample into a remineralization solution which in turn increases the initial remineralization rate (Strang et al., 1987).

According to Fick's first and second laws the flux of a material across a membrane is a function of both the concentration gradient (thermodynamic factor) as well as the diffusion coefficient (kinetic parameter). The rate of transport is faster when the concentration gradient is steeper (Hopfenberg, 1974). The diffusion of mineral ions into and through the lesion is the rate limiting step for remineralization (Exterkate et al., 1993; Mukai et al., 2001). Rapid precipitation of ions at the first reactive mineral surfaces leads to fast removal of the ions from the solution which retards any mineral deposition deep in the lesion (Exterkate et al., 1993). In this case fast precipitation at the surface of the lesion will prevent ions from reaching the innermost part of the lesion because of the sharp reduction of the thermodynamic force at the beginning and the blockage of pores at lesion surface later in the process (ten Cate, 2001). Therefore ion precipitation can be also considered to be a rate limiting factor in the remineralization process where faster diffusion means faster precipitation

at the surface which in return forms a dense mineralized surface layer which inhibits further diffusion.

In the presence of fluoride the overall remineralization pattern showed by FE-SEM, PLM and through the microhardness measurements changed in terms of mineral distribution within the lesion. Low levels of fluoride (0.1, 0.5 and 1.0 ppm) resulted in significant remineralization although this was not apparent in the first week. Remineralization occurred at the lesion front as detected by decreased lesion depth under PLM. The higher hardness values which were evident throughout the lesion correlated well with the banding of the same lesions under PLM. No attempts were made to analyse the mineral bands shown in the microscope although some references suggest that fluoride is responsible for this lamination phenomenon in dentin (Wefel et al., 1995; Nyvad et al., 1997) and the total double refraction in water for enamel was correlated well with its mineral content (Featherstone et al., 1983). Mineralized inter- and peritubular dentin with the decreased in diameter or partially or totally occluded dentinal tubules were clearly visible in FE-SEM pictures. According to the SEM and PLM pictures the remineralization in the three groups (B, C and D) was enhanced with increasing the remineralization time (4^{th} week > 3^{rd} week > 2^{nd} week). In the present study, no attempts were made to compare directly between the three used methodologies since the information obtained from each quantifies a different physical property related to the tissue (Featherstone et al., 1983). When fluoride was added to the remineralizing solution at higher concentrations (5.0 and 10.0 ppm) the SEM pictures revealed an obvious well-mineralized dentinal surface with dense precipitates accumulated in and on the inter- and intra-tubular dentin as well as partially or totally occluded dentinal tubules. We tried to measure the thickness of the hyper-mineralized surface layer depending on its morphology for both groups (E and F) from the fractured side. There

were always differences in the measurements so that we could not estimate its thickness but we concluded that the hyper-mineralized surface layer in groups E2 and E3 was not continuous because there were differences in its thickness within the same sample. Based on the SEM and PLM pictures and the microhardness values of groups E2, E3 and E4 we hypothesize that the non-continuous surface layer in these groups could not inhibit the diffusion process into the lesion or prevent lesion body remineralization. Our results are in agreement with Arends et al., (1990) who found that the lesion front could be remineralized even after the formation of a hypermineralized surface layer using 5.0 ppm fluoride. A mineralized surface layer does not always prevent the deposition of minerals elsewhere in the lesion (Damen et al., 1998). In comparison remineralization behaviour in groups F2, F3 and F4 were similar to the control groups in which a hyper-mineralized surface layer was formed without evident remineralization in the lesion body. This possible inhibitory effect of a hypermineralized fluoridated surface layer on the remineralization of the lesion front was stated by Kawasaki et al., (2000).

No attempts were made to qualify the precipitated crystallites in and on the lesion surface. According to the literature, under conditions where fluoride levels are low and the pH is higher than 4.5, fluorohydroxyapatite (ten Cate et al., 2003) or even fluoroapatite (Iijima et al., 1993) have the highest probability to form.

Our results were very much similar to those found in literature regarding remineralization of the lesion front. In the absence of fluoride, remineralization did not appear to take place at the lesion front and the lesion depth did not decrease (Inaba et al., 1996; Kawasaki et al., 1999, 2000). Limited decrease in lesion depth after fluoride addition to the remineralizing solution was also previously documented (Arends et al., 1989, 1990; Exterkate et al., 1993: Kawasaki et al., 1999; Mukai et al.,

2001; ten Cate, 2001).Various levels of fluoride (0.1- 10.0 ppm) dramatically effected the surface mineralization. The surface remineralization was proportional to both fluoride concentration and duration of remineralization (Arends et al., 1989, 1990).

We concluded from our results that incorporation of relatively small amounts of fluoride in the remineralizing process (0.5, 1.0 and 5.0 ppm) has the highest beneficial effect on dentinal lesion remineralization because such concentrations seem to be high enough to maintain a gradient at the lesion front, thus activating the thermodynamic driving force through out the whole lesion. On the other hand they are low enough to keep a constant diffusion rate to the innermost part of the lesion, thus controlling the kinetic of the precipitation process at least until the appearance of other inhibitory factors which spontaneously stop the process.

Such inhibitory factors could be:

1- The concentration gradient is not strong enough to maintain effective thermodynamics.

2- The rapid precipitation of ions at the first reactive surface areas of the dentinal crystallites which in turn blocks the lesion pores at the surface (Exterkate et al., 1993; Kawasaki et al., 2000; ten Cate, 2001).

3- The limited capacity of the dentinal front to remineralize, which is -most probably- due to the physical presence and chemical composition of the remaining organic phase where both properties can strongly restrict crystal growth (Inaba et al., 1996; Saito et al., 1997;Fujisawa and Kuboki, 1998). Hence, remineralization in this deepest area of the lesion is always limited and independent of fluoride concentrations.

Therefore, we suggest that neither number of available sites for remineralization alone (Mukai and ten Cate, 2002) nor diffusion of ions

solely (Mukai et al., 2001) is completely responsible for controlling the remineralization phenomenon at dentinal lesion front.

In conclusion, the present study indicates:

1- The influence of fluoride concentration in determining the rate as well as the pattern of mineral deposition in dentinal lesion.

2- The independence of lesion front remineralization from fluoride concentration which could be due to its limited capacity for remineralization.

Table 2.1. After lesion formation samples were divided into groups to be remineralized with various fluoride concentrations for different periods of time.

Time / Group	Week 1	Week 2	Week 3	Week 4
A(F=0.0ppm)	A1	A2	A3	A4
B(F=0.1ppm)	B1	B2	B3	B4
C(F=0.5ppm)	C1	C2	C3	C4
D(F=1.0ppm)	D1	D2	D3	D4
E(F=5.0ppm)	E1	E2	E3	E4
F(F=10ppm)	F1	F2	F3	F4

Table 2.2. Lesion depth before remineralization (BR) and after remineralization (AR) in each group (Mean ± SD) as observed with the polarized light microscope.

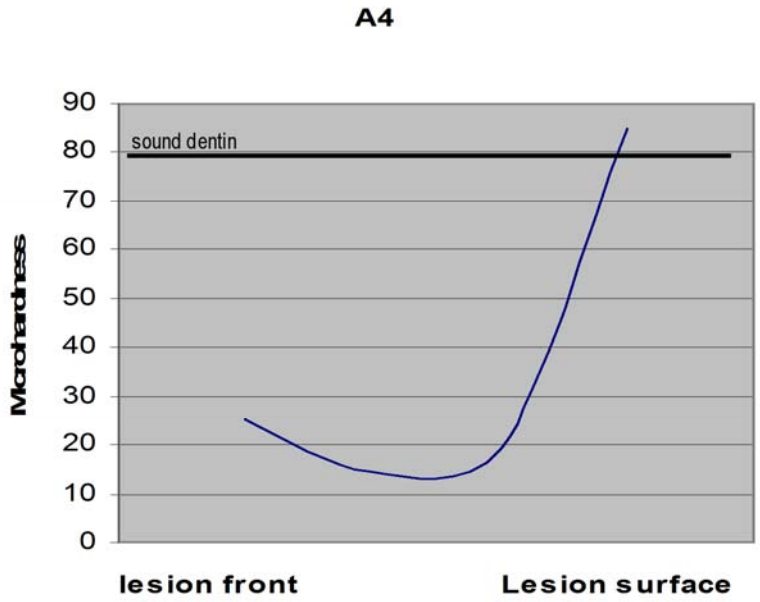
	F level (ppm)	Time (days)	Lesion depth (μm)
BR	≈0.0	14	210 ± 10
AR	0.0	7	195 ± 10
		14	200 ± 15
	0.1	7	165 ± 20
		14	168 ± 20
	0.5	7	170 ± 10
		14	165 ± 15
	1.0	7	169 ± 15
		14	167 ± 15
	5.0	7	165 ± 10
		14	163 ± 20

	10.0	7 14	170 ± 30 169 ± 20

Table 2.3. The mean microhardness values measured with the Vickers indenter through out the lesion per group. The average of the surface layer at week 4 for each group is given.

Time / Group	Week 1	Week 2	Week 3	Week 4	CSMH values at the lesion surface at week4
A	6.023	15.012	10.110	9.192	88.079
B	5.533	16.091	19.073	18.212	16.714
C	5.045	23.784	22.998	23.719	21.926
D	7.013	29.109	28.534	28.113	27.152
E	9.942	28.075	36.159	36.991	56.166
F	10.554	10.962	15.382	11.987	79.688

Diagram 2.1. Microhardness representative profiles for groups A4 (0.0 ppm fluoride, week 4) and E4 (5.0 ppm fluoride, week 4). Note the low microhardness values measured within the lesion body without fluoride in comparison with the high values when fluoride is added to the remineralzing solution (5.0 ppm).



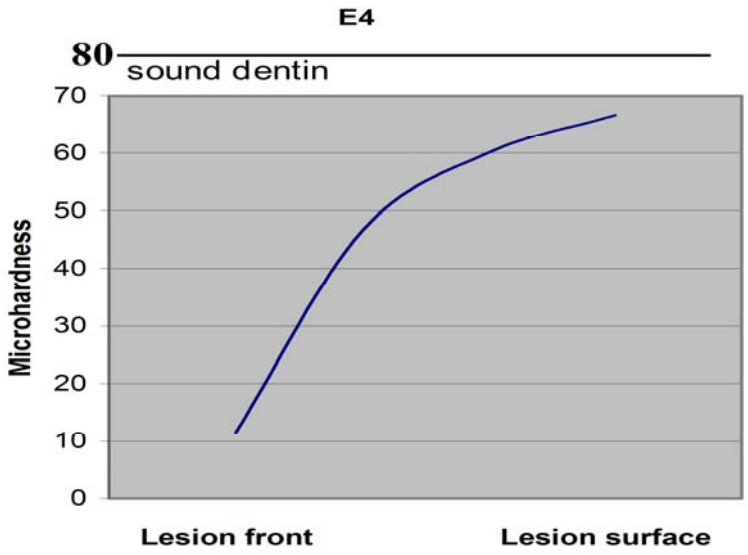


Fig. 2.1 Fractured side from the upper most surface of the lesion from the control group at the second week (A2) (x10000). Note the remineralized inter- and peri-tubular dentin.

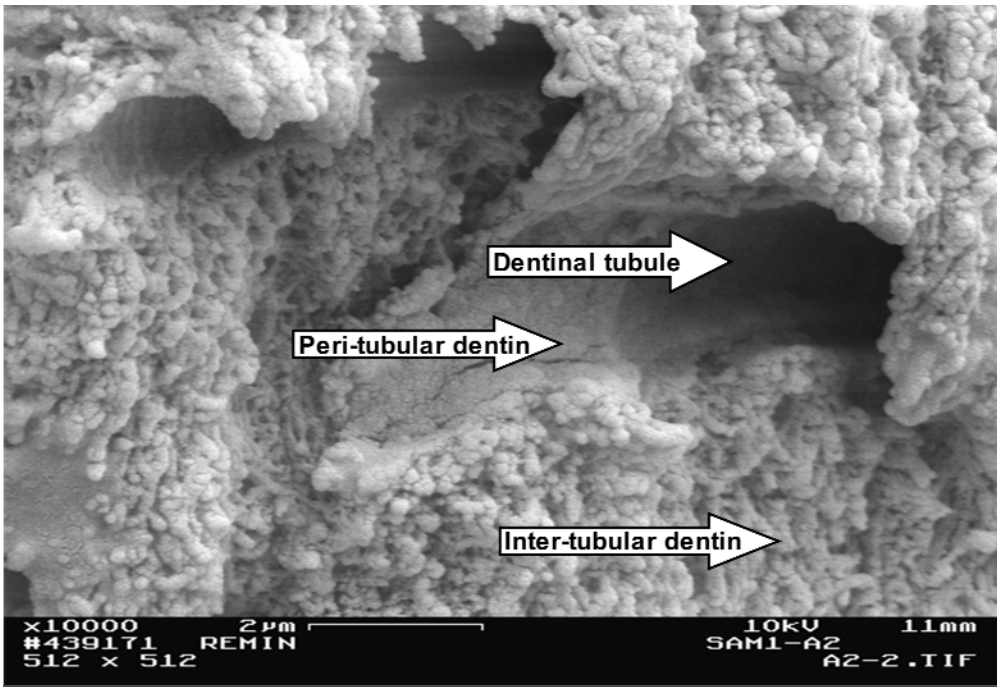


Fig. 2.2 Fractured side from the upper most surface of the lesion from the control group at the third week (A3) (x10000). Note the hyper-mineralized inter- and peri-tubular dentin.

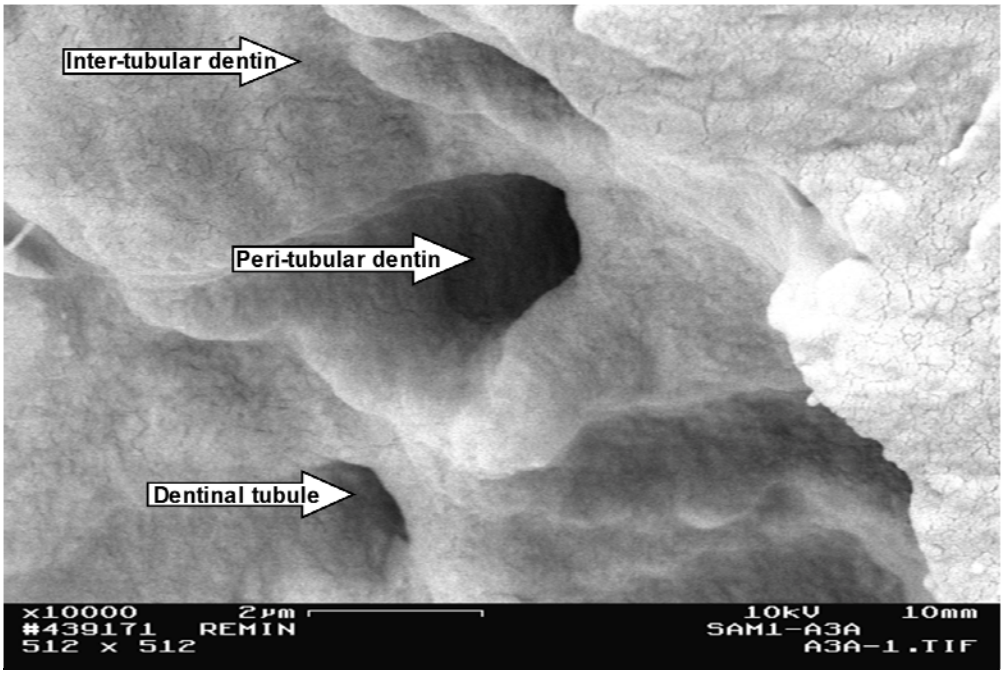


Fig.2.3 The surface of a remineralized lesion from group B2 (0.1 ppm fluoride, 2 weeks). Note the well-remineralized inter- and peri-tubular dentin (x5000).

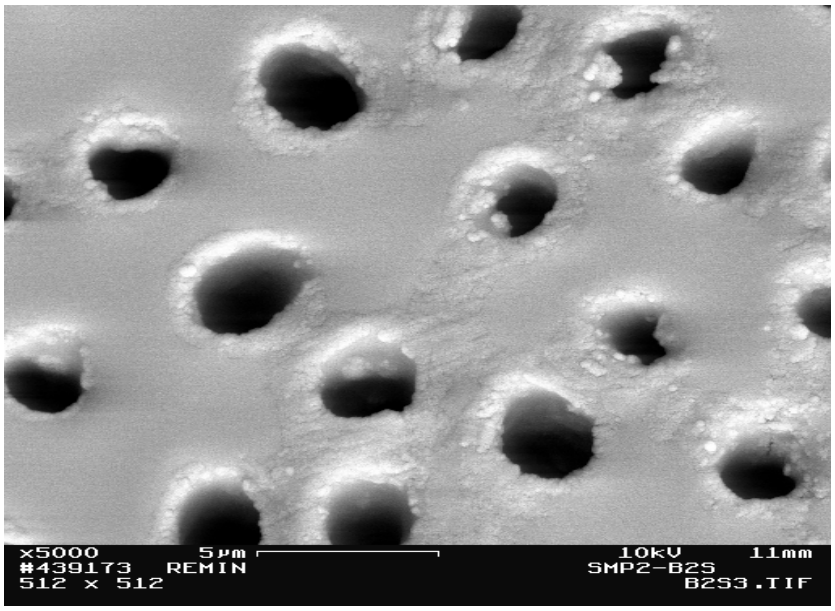


Fig. 2.4 Fractured side of a lesion from group B3 shows clearly the remineralized intertubular and peritubular dentin as well as remineralization within the dentinal tubules (x3000).

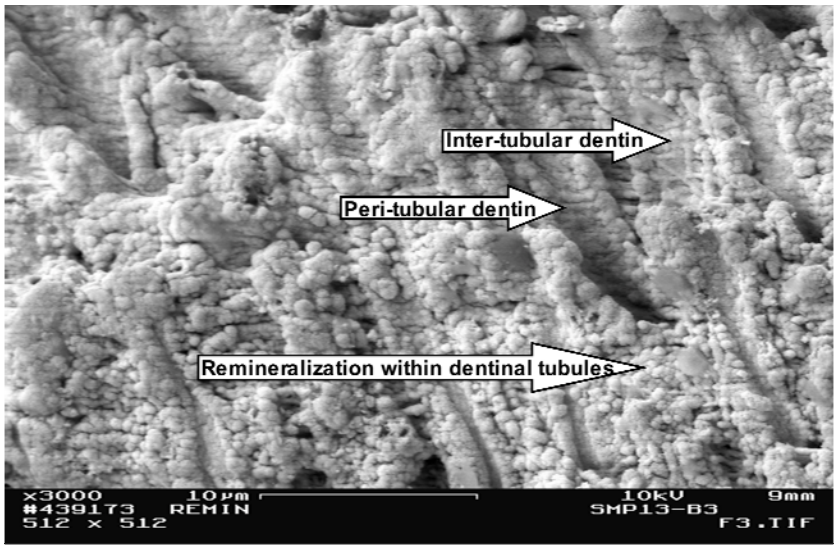


Fig. 2.5 Remineralized dentinal lesion from group C3 (0.5 ppm fluoride, 3 weeks) with Polarized light microscope (x10). Note the remineralization band within the lesion (arrows). The method of lesion depth measuring is shown.

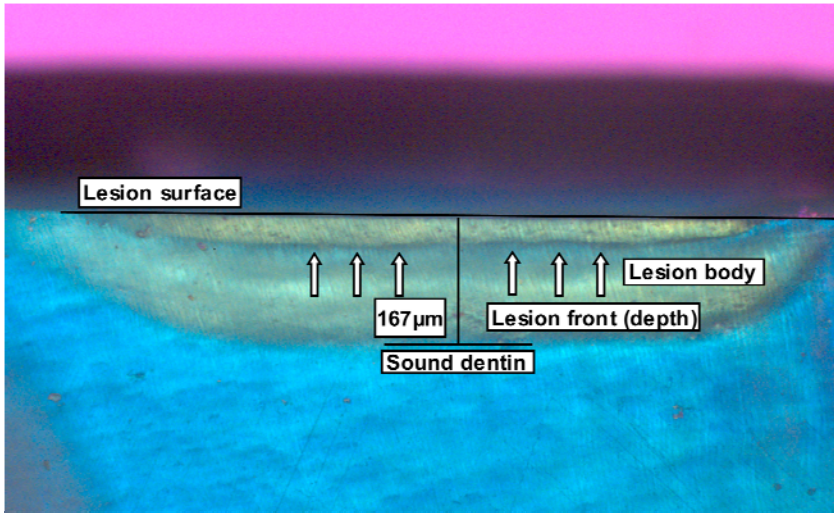


Fig. 2.6 The occluded tubules at the surface of a sample from group E3 (5.0 ppm fluoride, 3 weeks) (x5000).

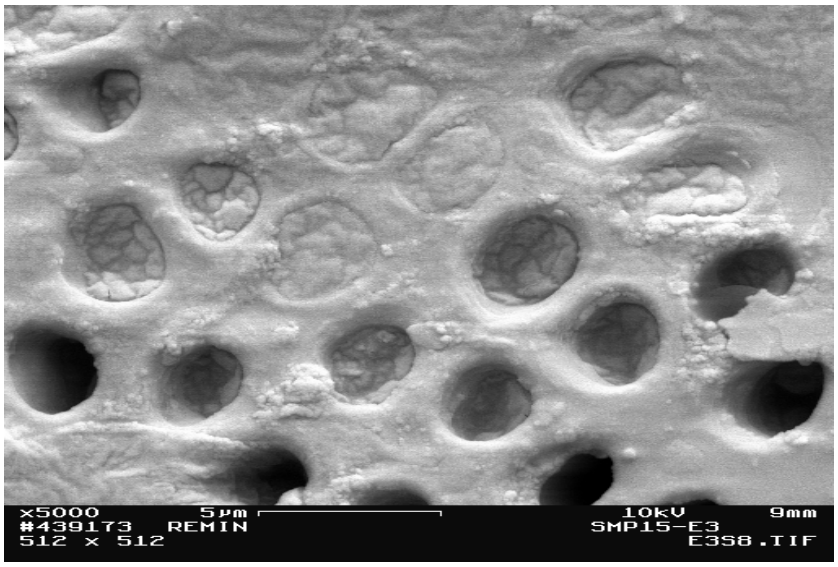


Fig. 2.7 Hyper-mineralized inter-tubular dentin and thick peri-tubular dentin at the surface of a sample from group F2 (10.0 ppm fluoride, 2 weeks) (x10000).

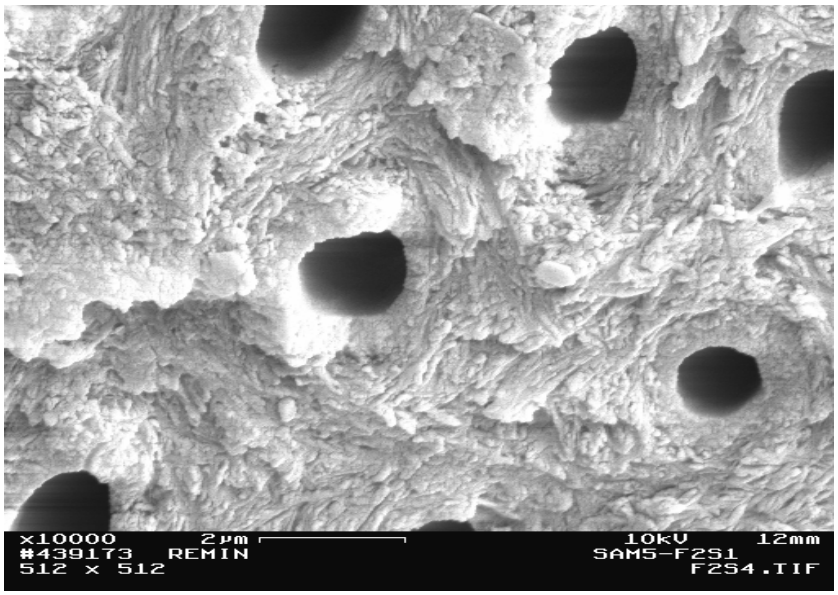
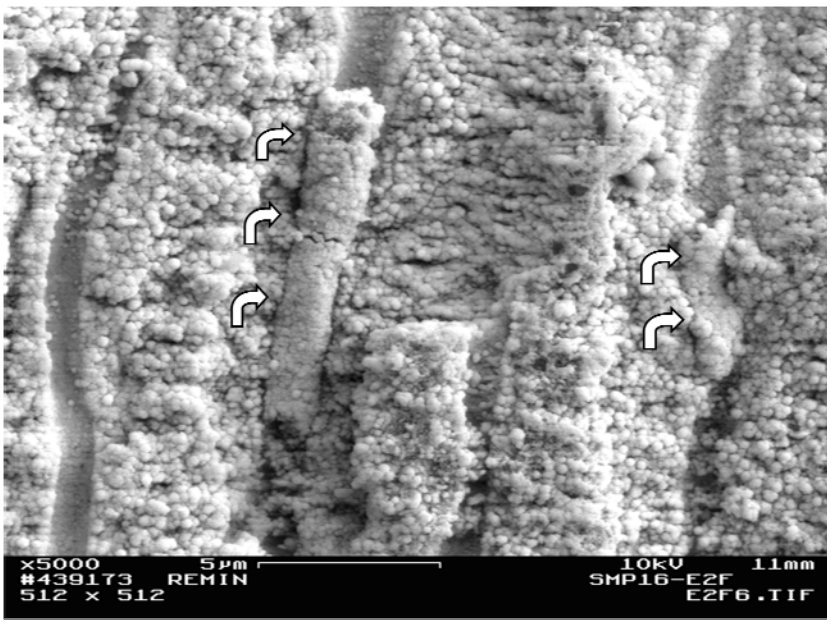


Fig. 2.8 Remineralized precipitates (arrows) within the dentinal tubules of the lesion body, the sample is from group E2 (5.0 ppm fluoride, 2 weeks) (x5000).



An Artificial Caries Model for Better Understanding Dentin Caries in vitro

Chapter 3

Introduction

Although dental research has very much improved our understanding of the etiology and pathology of dental caries over the last century (Aoba, 2004), caries research community is still in need for better scientific understanding of the caries process to enhance more effective methods of prevention, control and treatment (Fejerskov, 2004; Pitts, 2004). An appropriate knowledge of such a process can be facilitated by tracing and understanding all the associated key factors and their role in dental caries.

Three basic facts which constitute the basics of dental caries:

1- Dental caries is a dynamic process (Featherstone, 2004) which is initiated by the microbial deposits (biofilm) on a tooth surface (Fejerskov, 2004; Kidd, 2004; Pitts, 2004). The metabolically active cariogenic bacteria in this biofilm cause numerous minute pH fluctuations at the interface between tooth surface and the microbial deposits (Baelum and Fejerskov, 2003). These fluctuations will cause loss or gain of minerals from the tooth depending on the pH drop or increase respectively, cumulatively resulting in a net loss of minerals, dissolution of dental hard tissues and the formation of a carious lesion (Kidd and Fejerskov, 2004). Thus, the all important driving force in the caries process is the metabolic activity in the biofilm in which demineralization of enamel or dentin beneath may be considered as a reflection of these dynamic events (Kidd, 2004; Bjorndal and Mjör, 2001).

2- Carbohydrates and in particular the low molecular weight disaccharide succrose which is the most consumed dietary sugar and most related to caries is utilized by the cariogenic plaque bacteria where streptococcus mutans emerges as the predominant organism initially and lactobacilli growth is associated with lesion progression in deep dentinal lesions (Nikiforuk, 1985a). These acidogenic aciduric bacteria are able to hydrolyze succrose through the enzyme invertase to glucose and fructose which are in return converted to glucan (dextran and mutan) and fructan by glucosyltransferase and fructosyltransferase respectively (Loesche, 1996).

Under these acidic conditions pH drops in the vicinity of 5.0-5.5 (Loesche, 1996). The critical pH for enamel is 5.5 where the saliva or any other solution that surrounds enamel in vitro is just saturated with respect to hydroxyapatite (HAP) and any further fall leads to undersaturation, in which the ion activity product (IAP_{HA}) with respect to HAP is decreased and is much less than the constant of solubility product of the HAP (KSP_{HA}) and thus dissolving the HAP crystals is initiated (ten Cate et al., 2003).

Whether or not a solution is saturated with respect to HAP can be determined from the solubility product theory which is derived from the law of mass action, which states that the velocity of a reaction is proportional to the product of the masses of the reacting substances, each raised to a power equal to the number of molecules taking part.

For example, when 1 unit mass of solid HAP dissolves, 5 calcium ions, 3 trivalent phosphate ions and 1 hydroxyl ion are released into solution:

$$Ca_5(PO_4)_3OH \leftrightarrow 5Ca^{2+} + 3PO_4^{3-} + OH^-$$

On the other hand, when a solution containing hydroxyapatite is saturated and the mineral is in equilibrium with the ions in solution, the

IAP_{HA} equals the KSP_{HA}, a constant which has a value of 7.41×10^{60} mol^9/L^9 at 37°C. Thus, at equilibrium: (ten Cate et al., 2003)

$$KSP_{HA} = IAP_{HA} = (Ca^{2+})^5 \times (PO_4^{3-})^3 \times OH^- = 7.41 \times 10^{60}\ mol^9/L^9$$

The rate of enamel dissolution and carious lesion formation is a function of both the degree and undersaturation of plaque fluid (Theuns et al., 1985; ten Cate et al., 2003) together with the rate of diffusion of ions into and out of enamel (Featherstone and Rodgers, 1981) in which the latter depends on the total demineralization time, acid strength, concentration and pH (Featherstone and Rodgers, 1981; Theuns et al., 1985; Herksröter et al., 1991).The critical pH for dentin is higher than that of enamel (Hoppenbrouwers et al., 1986), hence even milder acidic attacks cause dissolution and lesion formation in dentin (Wefel, 1994)

As we have mentioned before such a process is not a one-way demineralizing process (Bjorndal and Mjör, 2001). Saliva and plaque fluid have a high potency to neutralize the acidic environment within 30-60 min (Loesche, 1996). Their buffering effect is known as the Stephan curve (Nikiforuk, 1985b). The partially demineralized apatite crystals can be remineralized and equilibrium may be re-established by the dissolving and reforming effects (ten Cate et al., 2003). However, it is only when the pH value remains under 5.5 tooth mineral is solubilized and plaque fluid tends then to loose its potential for counteracting the pH change and this is why frequency of sugar intake is considered to be more cariogenic and harmful than the total sugar ingested (Nikiforuk, 1985b; Loesche, 1996).

3- Although the physicochemical rationale to explain de- and remineralization process applies to both enamel and dentin (Wefel, 1994; ten Cate et al., 2003), enamel caries and dentin caries are two independent entities due to the remarkable differences between both tissues (Arends et al., 1989; Fejerskov et al., 2003). Dentin which is of a mesenchymal origin

is a biologically active tissue while the ectodermal acellular enamel is a biologically inert tissue (Arends et al., 1989). Therefore, dentin possesses the potential to respond to different external stimuli through several reactions which vary according to the severity and rate of progression of the stimulus.

Moreover, 20 wt% of dentin is composed of organic matrix while the organic matter in enamel is not more than about 1 wt% in which proteins form the major portion of it. The organic phase in dentin constitutes of 90% collagen (mainly type I) and 10% non-collagenous proteins (NCP) and non-protinacious components (NPC). Dentin porosity (Arends et al., 1992) together with the presence of dentinal tubules, their orientation, contents, numbers and diameters influence dentin permeability and affect the diffusion process (Murray et al., 2003; Hara et al., 2005). The inorganic phase differs between enamel and dentin in volume (96 wt% in enamel and 70 wt% in dentin) and in composition. Dentin crystallites are randomly oriented in comparison with enamel crystals; they are with smaller dimensions and larger surface area per unit volume and with higher carbonate and magnesium ions incorporating in their HAP lattice (Ostrom, 1980; LeGeros, 1990).

It is quite clear from the aforementioned differences between the two tissues that the chemical events associated with the caries process limited to de- and remineralization phenomena can be applied to enamel but are deficient in the case of dentin. Strictly speaking, the presence of the organic matrix plays a significant role in lesion formation during the demineralizing phase in which it retards the acid dissolution rate and hampers lesion progression (Klont and ten Cate, 1990, 1991a; Kleter et al., 1994; Kleter, 1997; Hara et al., 2005). Thus, dentinal caries involves both demineralization of the hard tissue followed by degradation of the organic material (Klont and ten Cate, 1991a). Therefore, the caries process in

dentin is an intermittent course of demineralization interspersed with remineralization (Bjorndal and Mjör, 2001) in which the demineralization phase is alternating with organic degradation. Detailed description of dentin collagen and other dentin organic matrix components is already mentioned in the introduction in chapter 1 (pp 7-12).

However, although it is well established that lactic and acetic acids are the major bacterial anaerobic fermentation products causing demineralization in dental caries, the distinct role of each in the demineralization and degradation of the dentin substrate is not established. To gain more information and better understanding the effect of these acids on demineralizing and/or denaturating dentin collagen a model for artificial dentinal caries was developed to study the morphological appearance of coronal dentin surface after demineralizing with acetic or lactic acids. Chemical analysis of the demineralizing solutions was done to identify degraded collagen type I in order to differentiate between demineralized and denaturated collagen.

The aim in this part of the study was to present this artificial dentinal caries model to clarify the possible variable effects of both acetic and lactic acids on collagen degradation and organic matrix breakdown.

Materials and Methods

Sample Preparation:

Twelve extracted human third molars were obtained from an oral surgeons' private clinic and used within 4 months of extraction. After extraction teeth were immediately stored at room temperature in Ringer solution to which sodium-azide was added to prevent bacterial growth. All teeth were clinically sound and they were carefully observed for caries, abrasions or any mechanical traumas. Teeth were cleaned with a tooth brush aided sometimes with a scalpel to remove the periodontal ligament

and intercrestal bone remnants and rinsed under running tap water. The roots were cut 1.5-2.0 mm below the cementoenamel junction. The teeth were then embedded individually in transparent cold-curing methylmethacrylate (Technovit 4004, Kulzer GmbH, Wehrheim, Germany). To expose mid-coronal dentin each tooth was sectioned parallel to its long axis into two halves using a slow speed water-cooled diamond saw (Isomet, Beuhler, Illinois, USA), the occlusal part of each half was then removed by a cut perpendicular to the long axis of the tooth and 1.5-3.0 mm away from the pulp. Each half was divided into four slices parallel to the long axis of the tooth and extends from the middle of the tooth to the outer enamel with a thin saw microtome (Leica SP 1600, GmbH, Nußloch, Germany) under tap water. Dentin exposed surfaces were then polished flat with water proof silicon carbide abrasive paper (P500-grit) with a Leco VP 100 (GmbH, Neuss, Germany) device. Subsequently they were polished using wet polishing paper with silicone paste of polycrystalline diamonds of size 9 μm (DAP-7, Struers, Copenhagen, Denmark).

Dentin surfaces together with the surrounding Technovit were covered with two coats of nail varnish (Keyte, GmbH, Munich, Germany) to avoid the penetration of the solutions' molecules in any marginal gaps that could exist between the tooth and the acrylate, leaving one window of exposed mid-coronal dentin per slice. Adhesive paper was cut into 1x1.5 mm^2 pieces and attached to the dentin surfaces before applying the nail varnish to standardize the window. Two of the four exposed surfaces were perpendicular to the dentinal tubules and the other two were parallel to the tubules. The samples were then kept in air for about fifteen minutes to allow the nail varnish to dry. After the removal of the adhesive strips each window was etched for 20 s with 37% phosphoric acid gel (Total etch, Ivoclar Vivadent GmbH, Schaan, FL) to remove the smear layer. The

successive steps of the slices preparation are schematically shown in figure 3.1.

Lesion Formation:

Each four slices from each half were immersed in 80ml of 0.1 M lactic acid (pH 4), while the other four slices from the opposite half of the same tooth were immersed in 80 ml of 0.5 M acetic acid (pH 5) for 3, 7 and 14 days. The demineralization process was always with agitation of the solution with 150 rpm at 37°C (Forma Orbital Shaker, Thermo Electron Corporation, Ohio, USA) with 20 ml per slice. KOH and stock lactic acid solution (90%) were used sometimes to adjust the acetic acid and lactic acid pH respectively. After the first week the demineralizing solutions were refreshed to avoid changes of the solution's pH of more than half a pH unit.

FE-SEM:

To obtain information on the morphology of the demineralised dentin surfaces a high resolution FE-SEM was used. Samples were fixed in 0.25 M Glutaraldehyde in 0.1 M Cacodylatebuffer pH 7.4 for 1 h, washed with 0.1 M Cacodylateuffer pH 7.4, then immersed in 50% alcohol for 20 min, subsequently in 70%, 80% and 90% alcohol, each for 20 min, and they were kept finally overnight in 96% alcohol. According to Perdigao et al. (1995) samples were put in Hexamethyldisilazane for 10 min and air dried at room temperature. Each sample was then fixed with carbon paste on the SEM sample holder. Gold sputtering was done for 1 min, with 1.0 kV, 0.3 mbar and 40 mA (Edwards Sputter Coater S15OB, Sussex, UK) and the pictures were then made with a Leo FE-SEM (Leo DSM 982, Carl-Zeiss NTS GmbH, Oberkochen, Germany).

Through out the whole experimental procedure care was taken to avoid dentin desiccation particularly after the lesion was formed to avoid collapsing of the demineralised and denaturated collagen fibrils. Exposing

the demineralised fibrils to air drying before critical point drying would lead to collapsed collagen fibrils and protinacious precipitates which would mask the accurate morphological appearance of the surface.

Spectrophotometer:

After the removal of the samples from the demineralizing solutions, the solutions were kept in 4°C until they were used for the hydroxyproline analysis measured with a double beam spectrophotometer (UVIKON 933, Double beam UV\VIS, Kontron Instruments, Zurich, Switzerland).

Lyophilizing and Hydrolyzes:

Samples were taken from each demineralizing solution after 3, 7 and 14 days and neutralized with $Na(OH)_2$, frozen at -20°C , then they were left for 1 h in -80°C immediately before lyophilizing (Freeze Dryer ALPHA 2-4 LSC, Christ GmbH, Osterode am Harz, Germany). Lyophilized samples were hydrolyzed with 6 M HCl (1 ml/1 mg) for 24 h in sealed borosilicate test tubes at 110°C in 78% glycerin bath. To remove HCl the samples were neutralized again with $Na(OH)_2$ before lyophilizing once again.

Preparations of the reagents according to Jamall et al., (1981) and Kleter et al., (1998):

-Chloramine T reagent: Acetate/citrate buffer was composed of 57 g sodium acetate trihydrate, 37.5 g trisodium citrate, 5.50 g citric acid monohydrate and 395 ml of isopropanol. They were brought to 1 l with distilled de-ionized water and the pH was adjusted to 6.0 by dropwise addition of 12 M hydrochloric acid. 5.6 mg of chloramine T reagent (Chloramine T puriss, Tolud-4-Sulforsäurechloramid Natriumsalz Trihydrat, Sigma-Aldrich GmbH, Seelze, Germany) was added to 1 ml of the acetate/citrate buffer.

-Ehrlich’s reagent: 10 g of dimethylaminobenzaldehyde (4-Dimethylamino benzaldehyde, Sigma-Aldrich Chemie GmbH, Steinheim,

Germany) was added to 11 ml of 60% perchloric acid. 3 ml of the mixture were mixed with 8 ml of 50% isopropanol to form the working Ehrlich reagent.

Sample preparation:

-Each dried sample was dissolved in 1.2 ml of 50% isopropanol for 1 h before any further addition. 0.2 ml of chloramine T buffer was added to each sample for 25 min before the addition of 1.0 ml of Ehrlich reagent. The samples were then incubated for 20 min at 65°C to develop the red color chromophore. Each reagent solution was freshly prepared at the same day to be used.

-Standard hydroxyproline solutions were prepared from hydroxyproline stock solution in which 1 mg of hydroxyproline (L-4-Hydroxyproline, Fluka 56250, Sigma-Aldrich GmbH, Steinheim, Germany) was dissolved in 1 ml of 50% isopropanol. Dilutions 750, 500, 250, 100, 75, 50, 25 µg were done with isopropanol. Chloramine T reagent and Ehrlish's reagent were added to the hydroxyproline standard solutions and incubated just the same as with the dentin collagen samples. The absorbance at 555 nm was measured on a double beam UV/VIS spectrophotometer against 50% isopropanol as a refernce. The machine was found to be insensitive to concentrations less than 25 µg/ml hydroxyproline. Therefore dilutions starting from 1000 µg/ml down to 25 µg/ml hydroxyproline were used to draw a working curve against absorbance. For calculation of collagen content, the hydroxyproline concentration (µg) was multiplied by a factor of 7.1, as calculated from the composition of human dentin collagen.

Results

3 days demineralization:

The surface of the demineralized human dentin samples after 3 days of demineralization in acetic acid as shown in the SEM pictures were similar to those were demineralized for 3 days in lactic acid in which the peri-tubular dentin was dissolved and the inter-tubular collagen fibres were partially demineralized (fig 3.2). When acetic acid was the demineralizing regime surface precipitates were scattered and partially covering the demineralized surface. The demineralization with lactic acid led to large amounts of precipitates that covered large areas of the demineralized surface and occluded many of the dentinal tubules (fig 3.3).

1 week demineralization:

The surface precipitates were almost similar in quantity and morphological appearance for both acetic and lactic acids (fig 3.4a, 3.4b). They were scattered at the surfaces in which they did not totally cover the inter-tubular dentin. Therefore the partially demineralized collagen fibres were clearly visible. However, with lactic acid there were always areas within the same surface that were covered with a thick layer of surface precipitates (fig 3.4c).

2 weeks demineralization:

The samples which were demineralized in acetic acid showed scattered patches of precipitates. With 2 weeks of demineralization in lactic acid the surface precipitates were much more than those at the acetic acid demineralized surfaces and they totally covered the underlying demineralized inter-tubular dentin surface and occluded mostly all the dentinal tubules (fig 3.5).

Although the precipitates covered the demineralized collagen fibres, partially demineralized fibres within the inter-tubular dentin and totally demineralized collagen fibres within the dentinal tubules were sometimes observed in the layer under the precipitates. However, they were always clearly visible at the margins of the artificial dentinal lesions where no precipitates covered them (fig 3.6).

The demineralized collagen fibers at the surfaces of the artificial lesions under the precipitates observed in the SEM pictures were always decreased in thickness when they were demineralized with lactic acid (fig 3.7).

There was no difference in the morphological appearance of the lesions which were formed perpendicular to the dentinal tubules and those which were parallel to the dentinal tubules. However, the intermolecular cross-links banding with periodicity of 62 nm were clearly visible in the slices where windows were made parallel to the dentinal tubules and only when demineralization was with acetic acid solutions (fig 3.8) because the exposed deep inter-tubular collagen between the cut tubules lacked the heavy precipitates that covered the superficial inter-tubular collagen at the edge of the cut.

Although no attempts were made to qualify these precipitates in the present study, fig. 3.9a, 3.9b, 3.9c, 3.9d demonstrate the differences of these precipitates in comparison to enamel hydroxyapatite at a higher magnification.

The biochemical analysis revealed that no hydroxyproline could be detected in the acetic acid after 3 and 7 days of demineralization, which indicated that, during these incubation periods no solubilization of collagen occurred. However, after 2 weeks of demineralization amounts of solubilized collagen were detected through the hydroxyproline analysis.

Hydroxyproline was found in lactic acid after 3, 7 and 14 days. The amount of extracted collagen in the lactic acid was found to be increasing with time in a linear constant rate until two weeks (fig 3.10).

Discussion

The results of the present study indicated a different effect of acetic and lactic acid on the collagen degradation. While lactic acid could denaturate collagen within three days acetic acid did not show to solubilize collagen to any significant level for one week.

In the course of our study, we needed to evaluate the effect of several proteolytic agents on the demineralized dentinal organic matrix. Natural caries is too variable for reproducible evaluations. Therefore, we developed an artificial dentinal caries model to be used for evaluating self-limited enzyme-based experiments.

Lactic acid is the dominant organic acid among the glycolysis end products, but other organic acids such as acetic, propionic, butyric, formic and succinic acids are also produced in variable amounts (Featherstone and Rodgers, 1981; Nikiforuk, 1985a; Hojo et al., 1991; Loesche, 1996; Featherstone, 2004). Moreover, lactic acid has potency to denaturate the dentin collagen (Kuboki et al., 1983). On the other hand, acetic acid at pH 5 lacks the potential to breakdown the collagen fibrils, destroy the cross-links bands and release hydroxyproline as an indicator for collagen denaturation (Kuboki et al., 1983; Van Strijp et al., 1992). To gain more information and better understanding the effect of these acids on demineralizing and/or denaturating dentin collagen we studied the morphological appearance of the lactic and acetic acids demineralized coronal dentin surfaces. To identify the denaturated collagen and differentiate it from the demineralized collagen fibers, we analysed the demineralizing solutions to quantify the released hydroxyproline.

Although bacterial enzymes and endogenous collagenases are considered to be responsible for collagen and NCPs degradation (Dung et al., 1995; Larmas, 2001) we proved through our model the ability of lactic acid at pH 4 to denaturate dentin collagen within three days. On the other hand, only after 14 days of dentin demineralization with acetic acid at pH 5, samples from the acetic acid solutions showed to contain hydroxyproline.

The degraded organic matrix together with the partially demineralized hydroxyapatite formed a surface layer that increased in quantity with increasing the demineralizing time. By the next week these precipitates had almost covered the inter-tubular dentin and occluded the dentinal tubules. Denaturated dentin collagen did not appear as it was expected as loose three polypeptide chains, instead the tropocollagen strands separated into globular and random coils (Wikipedia, 2006). Dung et al., (1994) had reported that human dentin collagen was insoluble in weak organic acids such as lactic and acetic acids. However, these different documented results can be due to differences in the demineralization duration. A primary results in our lab indicated that 24 h of lactic acid demineralization is not enough to solubilize collagen fibrils.

Although the partially demineralized collagen fibres were expected to be visible at the surface of the demineralized inter-tubular dentin, the degraded matrix masked their appearance partially in case of the acetic acid and totally with the lactic acid. Therefore our demineralized collagen SEM pictures were different than those which were documented in the literature after phosphoric acid, citric acid, nitric acid or other acidic conditioners etching (Willey and Steinberg, 1984; Gwinnett, 1994a, 1994b; Gwinnett et al., 1996; Breschi et al., 2002; Hara et al., 2005). However, we suggest as a hypothesis that the formation of this surface layer is due to both, first the nature of the demineralization process in which the acid diffuses in an un-

uniform pathways leaving remnants of hydroxyapatite at the surface together with calcium and phosphate reprecipitations (Moreno and Zahradnik, 1974; Featherstone et al., 1983; Fejerskov et al., 2003) and second the degraded collagen fibres together with the released NCPs which remained in the demineralized tissue (Klont and ten Cate, 1990). Moreover, it was argued that mineral precipitates from the demineralizing solutions could participate in the formation of the surface layer.

The acid diffusion process during artificial carious lesion formation is a three steps process in which the unionized form of the organic acid is the predominant diffusing molecules at the beginning followed by the dissolution of the hydroxyapatite crystals as a function of the dissociated hydrogen ion concentration and finally is the diffusion of the calcium and phosphate ions out of the lesion (Featherstone and Rodgers, 1981, Featherstone, 2004). Based on the mentioned hypothesis the lactic acid which is stronger than acetic acid has more potency to dissolve hydroxyapatite crystals under the same temperature. Moreover, the apatite solubility increases with factor 10 with each single drop of pH (ten Cate et al., 2003). Thus, lowering the pH of the demineralizing solution increases the dissolution rate, which in turn, increases the diffusion coefficient (Theuns et al., 1985). Our observations regarding the more demineralized (thinner) collagen fibres when they were demineralized with lactic acid than those which were demineralized with acetic acid can be explained accordingly.

In spite of these nice and helpful results, common critics of all in vitro simulations of dentin caries are that utilizing chemical or bacterial systems to produce dentinal artificial caries in vitro is of limited abilities in simulating natural dentin caries in vivo mainly due to the differences between vital and extracted teeth. Sclerosis, reactionary dentine, tertiary dentine, polymorphous crystallites formation in dentinal tubules, dentinal

tubules diameter and the outward flow of the dentinal fluid are examples of biological vital dentino-pulpal reactions to caries (Johansen and Parks, 1961; Sarnat and Massler, 1965; Fusayama et al., 1966; Frank and Voegel, 1980; Frank et al., 1989; Schüpbach et al., 1990; Larmas, 2001; Smith, 2002) that could reduce the rate of demineralization and lesion progression (Nyvad et al., 1997; ten Cate et al., 2003; Özok et al., 2004) which are absent in case of the extracted teeth. On the other hand comparing artificial dentinal caries produced in vitro with the partially saturated buffer systems acidified with 0.5 M acetic acid to natural dentinal caries using variable analytical tools demonstrated a great similarity between artificial and natural dentin caries in terms of lesion morphology, basic histological features and mineral distribution which concluded that experimentally induced caries-like lesions mimic natural lesions (Featherstone and Rodgers, 1981; Wefel et al., 1995; McIntyre and Featherstone, 2000).

Our model minimizes the biological variations between the dentin samples (Almqvist et al., 1993) through the very close location between the compared treated slices and thus, enabled us to compare effectively between different variables.

We concluded from the present study that our model is suitable for studying artificial dentinal caries in which only lactic acid is capable of producing both denaturated and demineralized dentin. Moreover, the ratio of denaturated/demineralized dentin is controlled through the type of acid used and the demineralization time.

Future work can correlate these morphological results of the in vitro artificial dentin caries with SEM images of natural dentin caries.

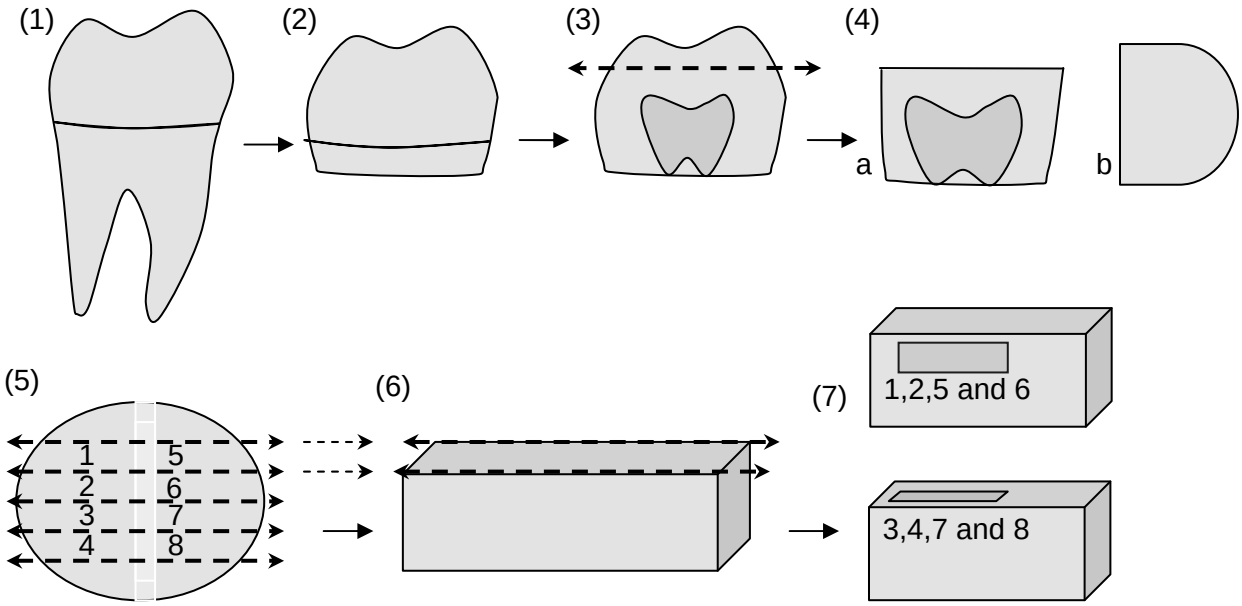


Fig 3.1. A schematic drawing illustrating the slice preparation. (1) Caries free molar. (2) The root was abraded short below the cemento-enamel junction and the tooth was cut longitudinally from the middle. (3) The occlusal part of each half was removed to expose deep dentin. (4) a. longitudinal section of one half of the crown after the occlusal part was removed, b. cross section. (5) Each half was sliced into four slices (1, 2, 3, 4) and (5, 6, 7, 8). (6) Each slice of dentin was prepared separately in which the close related slices had comparable windows. (7) One window per slice was prepared parallel to the dentinal tubules (slices 1, 2, 5 and 6) and slices 3, 4, 7 and 8 were prepared with the windows perpendicular to the dentinal tubules.

Fig 3.2. At high magnification the SEM pictures of the demineralized dentin for both acetic and lactic acid has the same morphological appearance (x20000).

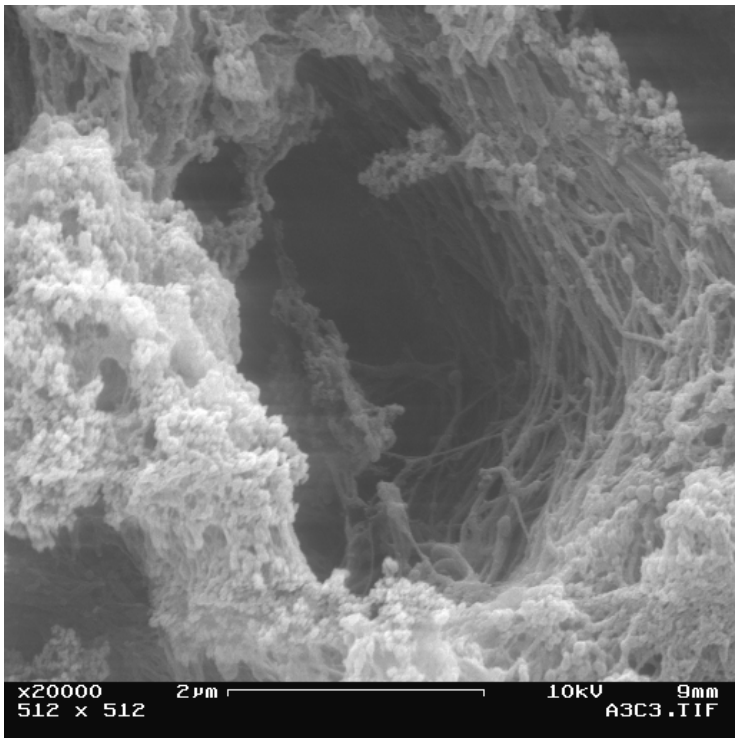
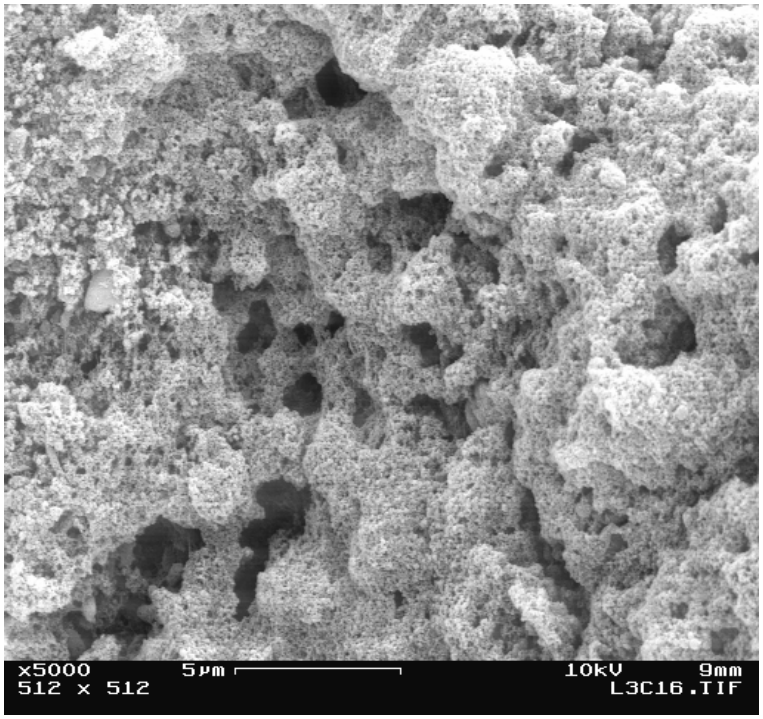


Fig 3.3. (a) The surface of the demineralized dentin was totally covered with surface precipitates after 3 days demineralization with lactic acid pH 4 (x5000). (b) Less surface precipitates were observed at dentin surface after 3 days when demineralization was with acetic acid pH 5 (x5000).

(a)



(b)

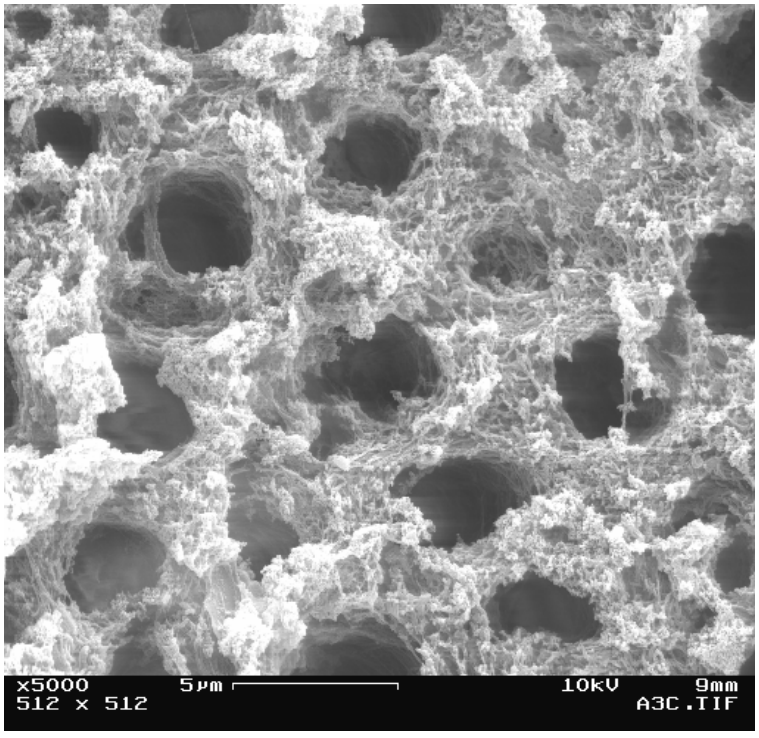
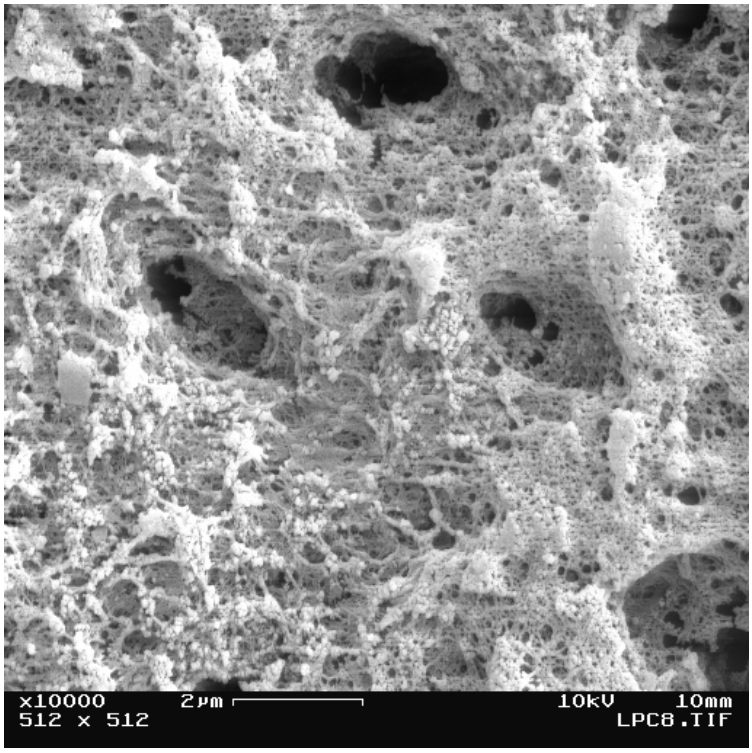
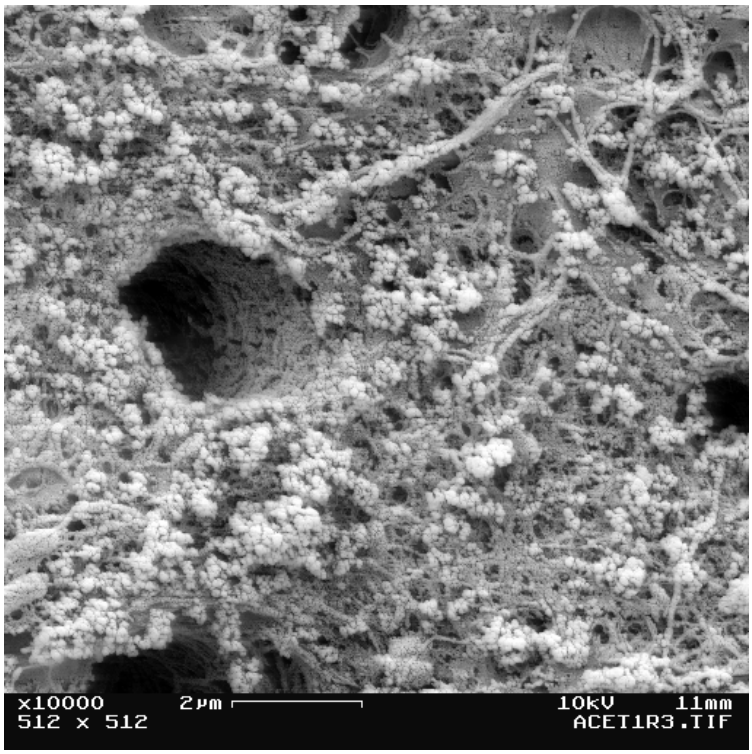


Fig 3.4. (a) The surface of lactic acid demineralized dentin after 1 week (x10000). (b) The surface of acetic acid demineralized dentin after 1 week (x10000). (c) In some samples after 1 week of lactic acid demineralization a surface layer was formed at the surface (x10000).

(a)



(b)



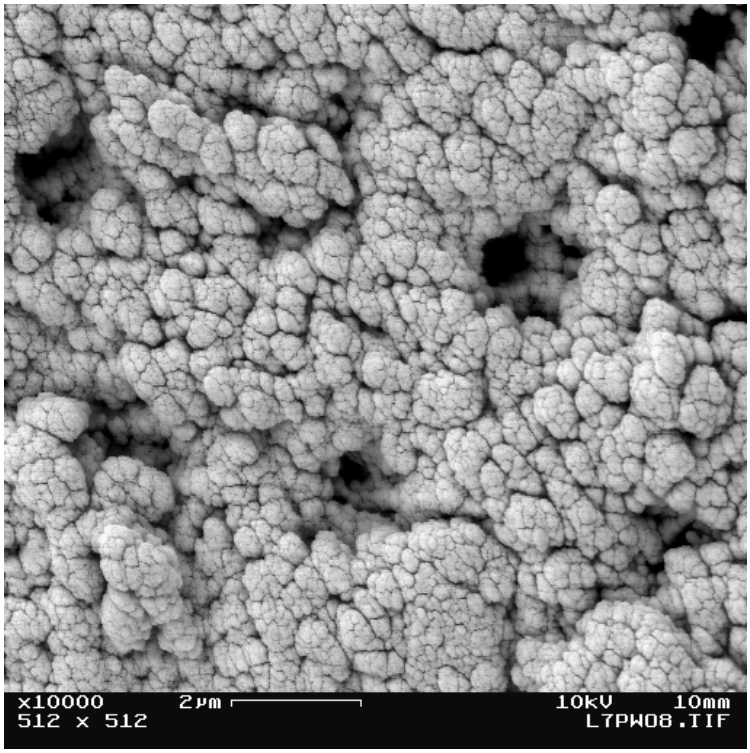
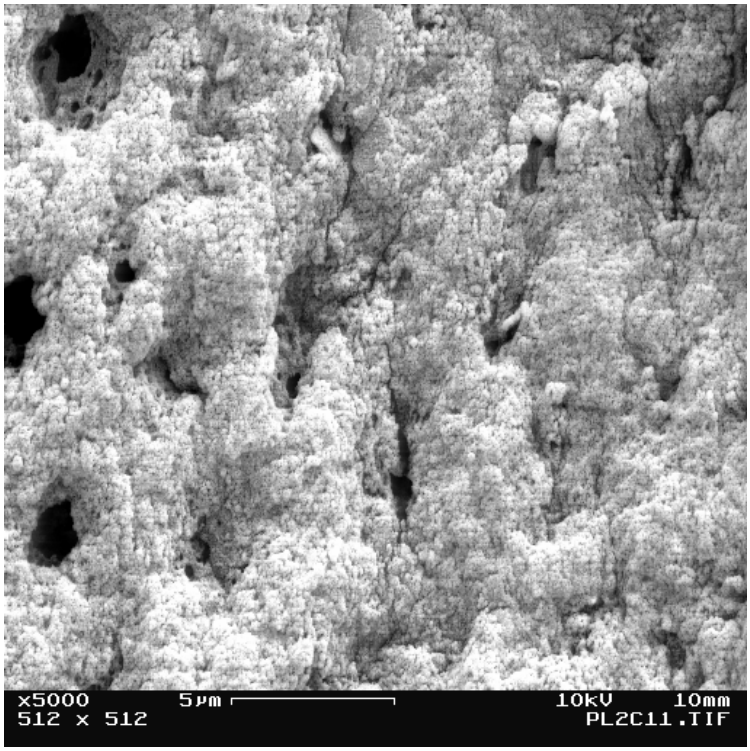
(c) 

Fig 3.5. (a) The surface of lactic acid demineralized dentin after 2 weeks was totally covered with a thick surface layer (x5000). (b) While the surface of acetic acid demineralized dentin after 2 weeks lacked these surface precipitates (x5000).

(a)



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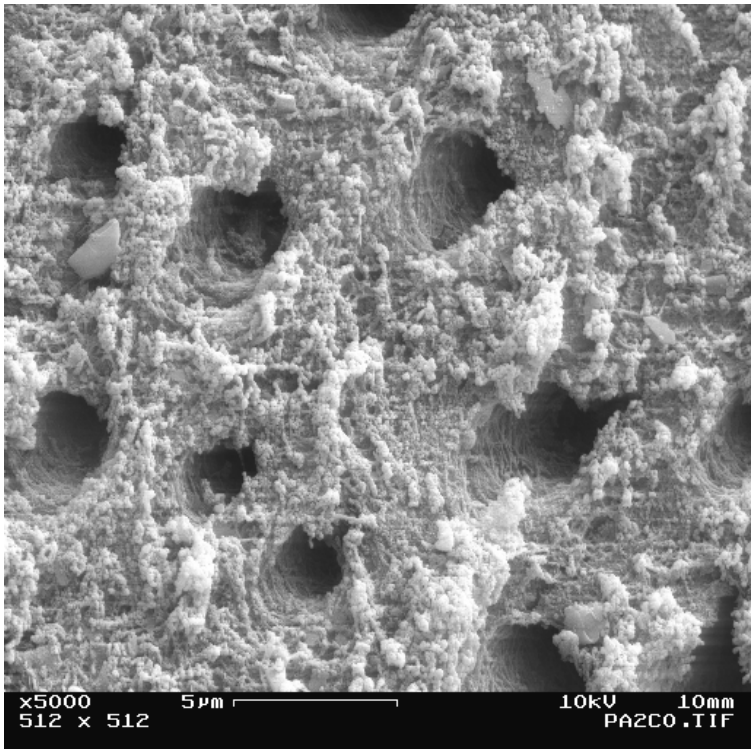
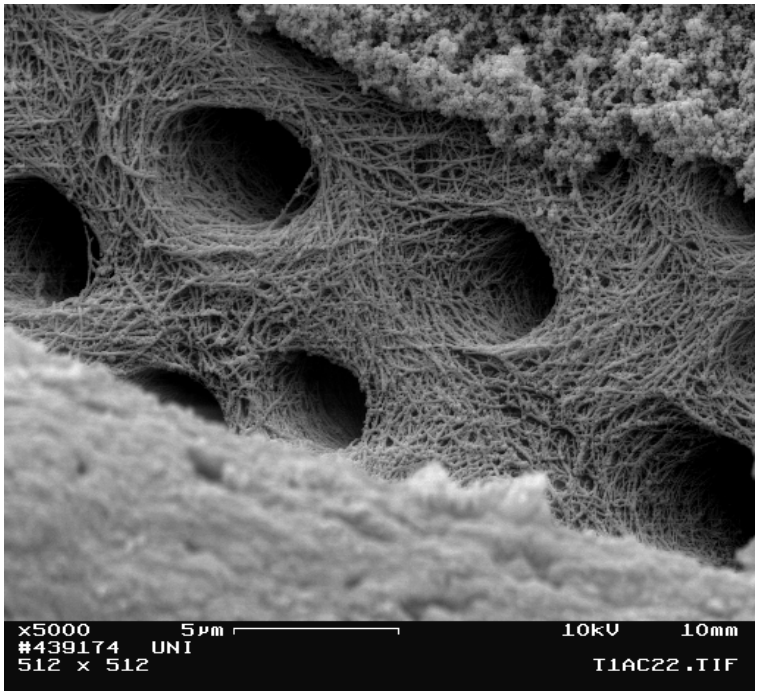


Fig 3.6. (a) At the edge of the lesion and under the surface precipitate the demineralized collagen fibers were clearly visible (x5000). (b) A higher magnification of the demineralized peri- and inter-tubular dentin (x1000).

(a)



(b)

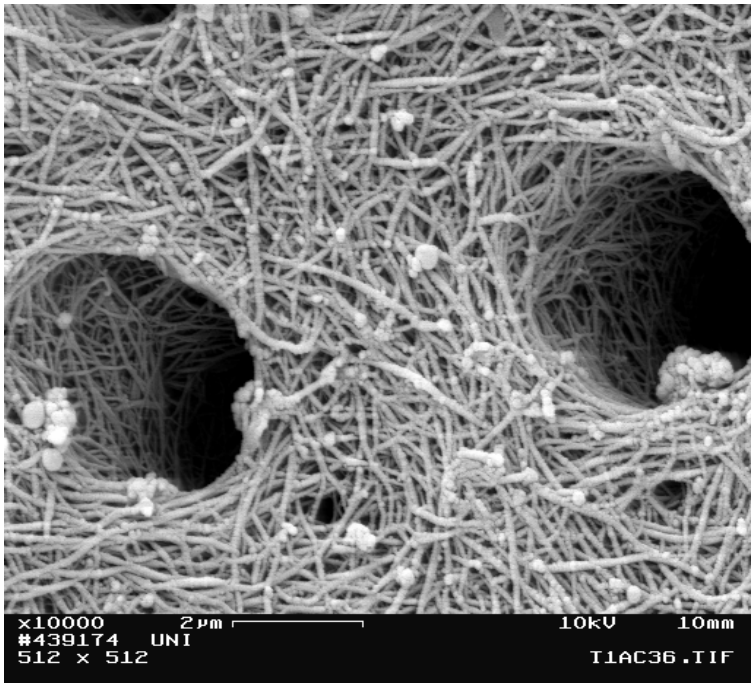
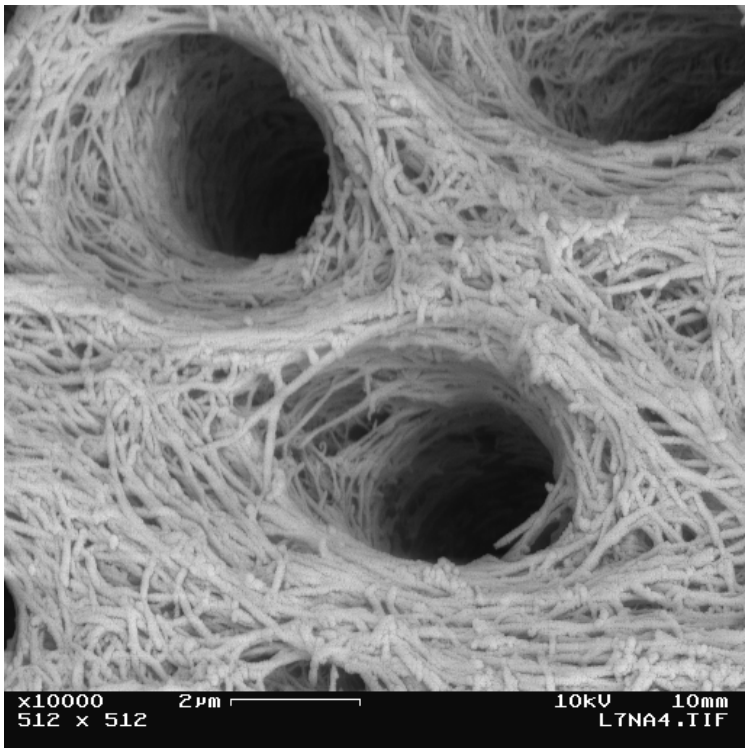
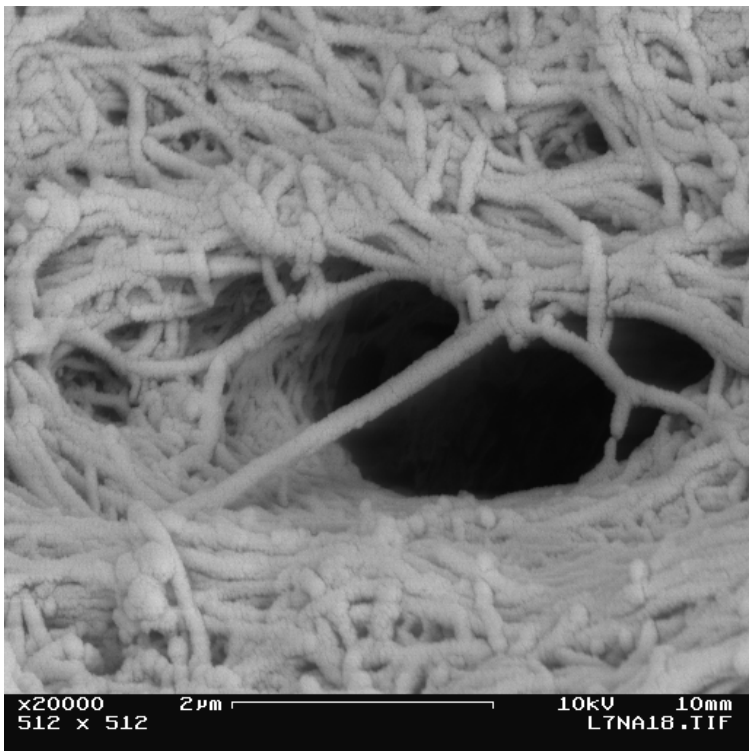


Fig 3.7. (a,b)The demineralized collagen fibers after demineralization with lactic acid were thinner than (c,d) those were demineralized with acetic acid (x10000) (x20000) (30000).

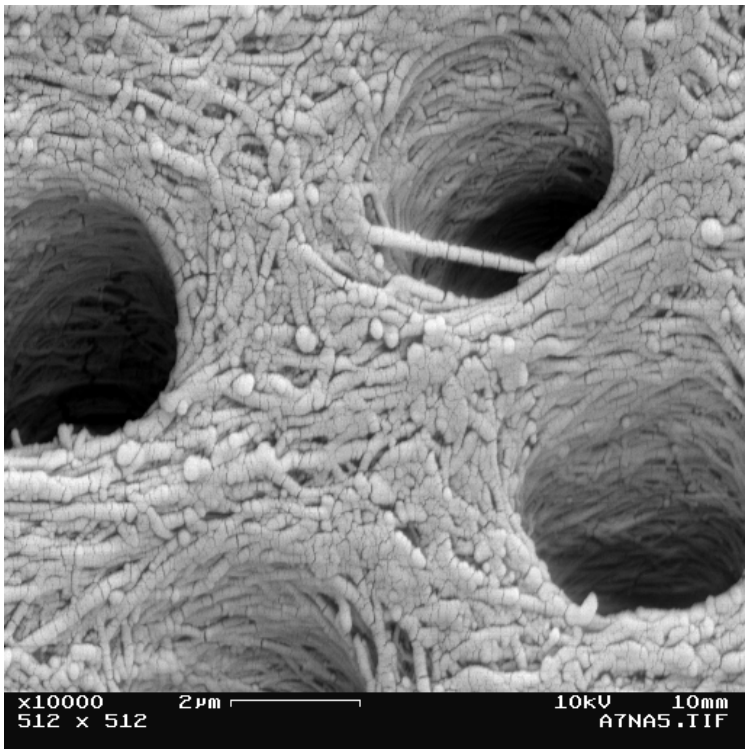
(a)



(b)



(c)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(d)

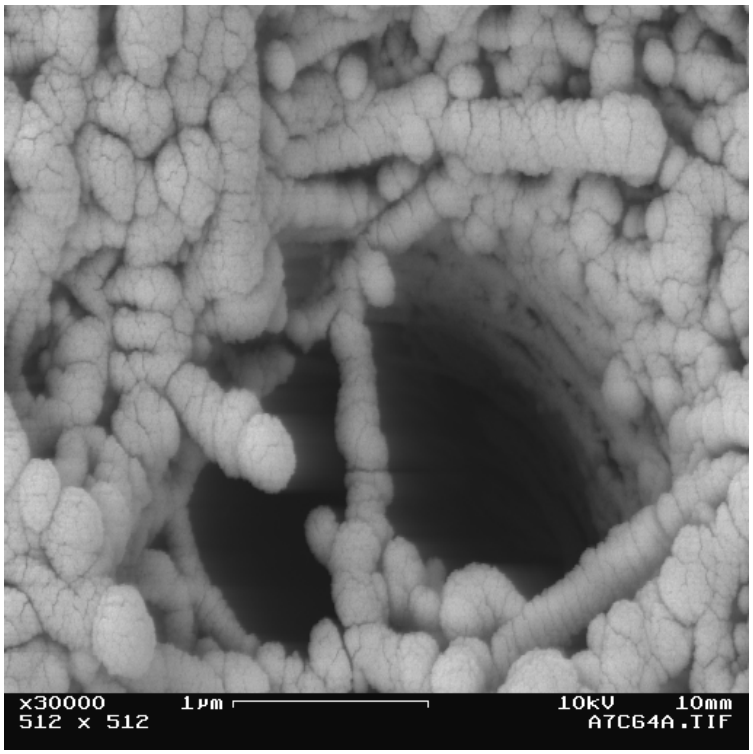

x30000 1µm 10kV 10mm
512 x 512 A7CG4A.TIF

Fig 3.8. The banding pattern was clearly visible at the collagen fibers when the surface layer was removed and when demineralization was with acetic acid (x50000).

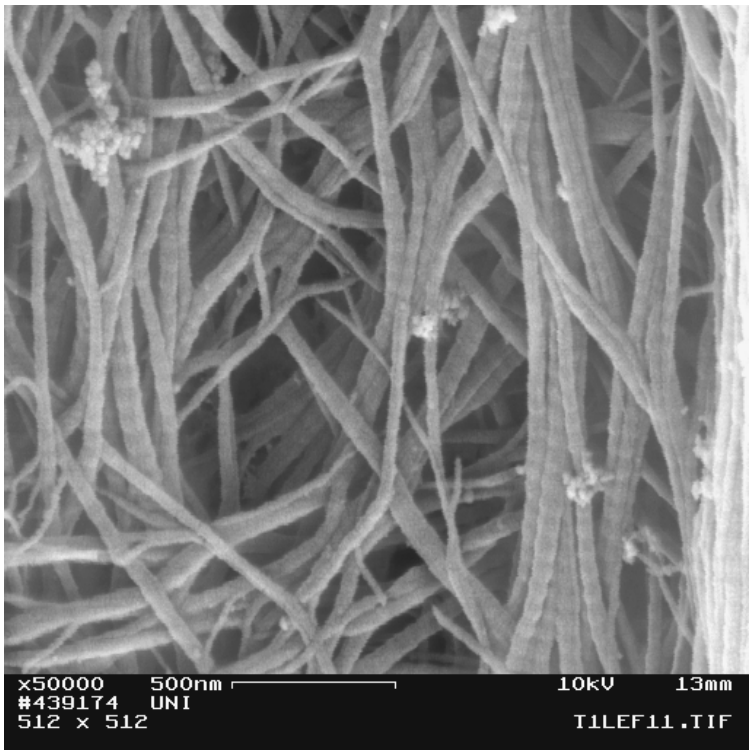
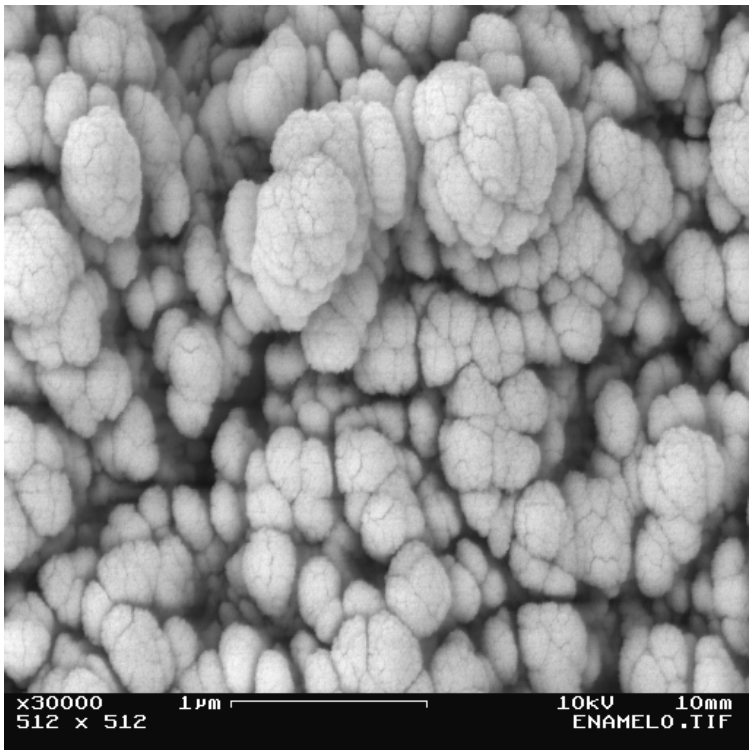


Fig 3.9. (a) Demineralized enamel hydroxyapatite (x30000). (b) Precipitates at dentin surface after 1 week of lactic acid demineralization (x30000). (c) Demineralized enamel hydroxyapatite (x50000). (d) Precipitates at dentin surface after 1 week of acetic acid demineralization (x50000).

(a)



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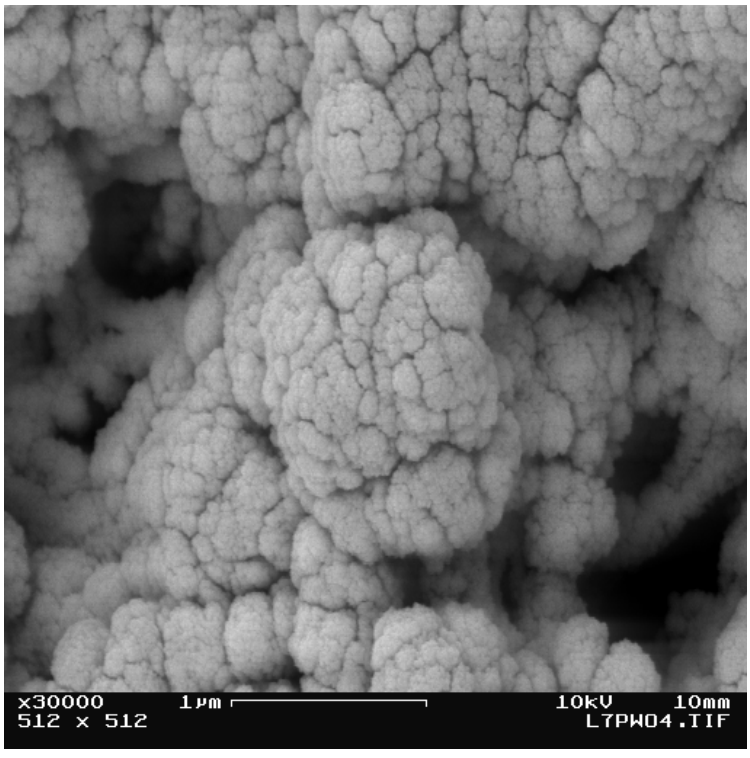
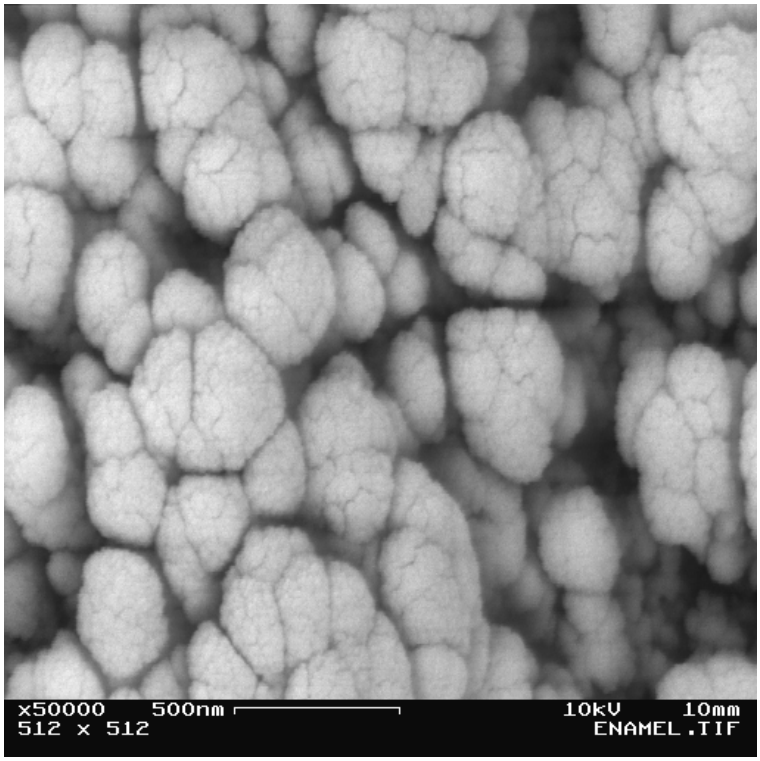


(c)


x50000 500nm
10kV 10mm
512 x 512
ENAMEL.TIF

(d)

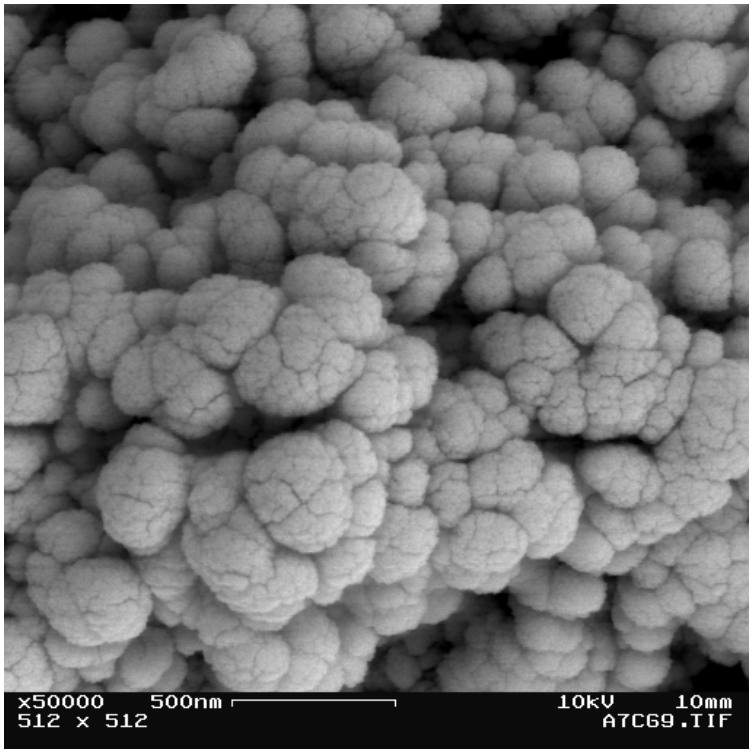
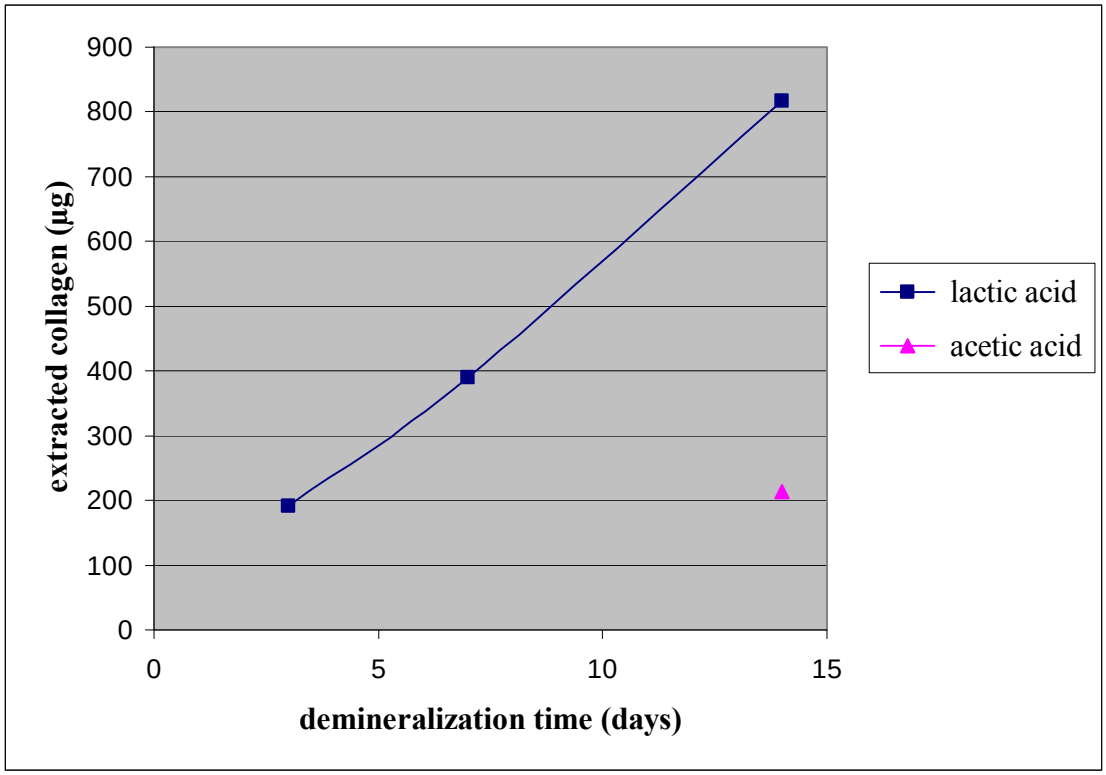

x50000 500nm
10kV 10mm
512 x 512
A7C69.TIF

Fig 3.10. The degradable collagen (µg/slice) released from coronal dentin slices during demineralization in 0.1 M lactic acid pH 4 (▪), 0.5 M acetic acid pH 5 (Δ) after 3, 7 and 14 days. The amount of the extracted collagen was calculated from the hydroxyproline concentration (µg) that was detected in the demineralizing solutions (20ml/slice). There was no hydroxyproline/solubilized collagen found at days 3 and 7 in acetic acid.



Self-Limiting Caries Therapy

Chapter 4

Introduction

The traditional guidelines for cavity preparation were outlined by GV Black in 1893. They consist of complete removal of the carious lesion, extension for prevention, outline form, resistance and retention forms (Mertz-Faihurst et al., 1998). As a consequence to the development of the adhesive dental restorative materials, the minimally invasive dentistry concept was introduced and several excavation techniques had been developed (Banerjee et al., 2000; Lager et al., 2003). Therefore, current clinical practice nowadays considers Blacks' principles too destructive for tooth structure (Banerjee et al., 2000). This disagreement with Black concerning the sufficient amount of tissue that needs to be excavated from the carious lesion is not new (Langeland and Langeland, 1968; Banerjee et al., 2000; Kidd, 2004). Authors such as Tomes (1859) (Fisher, 1981; Kidd, 2004) and Andrieu (1889) and Preiswerk (1903) (Langeland and Langeland, 1968) already advocated a more conservative attitude giving the pulp protection priority rather than running the risk of sacrificing the tooth, while Black (1908) favoured a more radical approach, even if that meant pulp exposure (Langeland and Langeland, 1968; Fisher, 1981; Kidd, 2004).

Therefore, alternatives to the mechanical excavation aim to be more selective in caries removal by removing only the infected denaturated and cariogenic layer leaving behind the affected demineralized but remineralizable one (Ericson et al., 1999; Yip et al., 1999; Banerjee et al., 2000; Beeley et al., 2000; Fure et al., 2000; Nadanovsky et al., 2001; Lager et al., 2003; Rafique et al., 2003; Flückiger et al., 2005).

Specific enzymes could be an alternative to the mechanical excavation and a minimally invasive method in which carious dentin can be selectively removed through their self limiting properties. The activity of collagenase (Kleter et al., 1994, 1997; Kawasaki and Featherstone, 1997), aqueous sodium hypochlorite (Hannig, 1999; Marshall et al., 2001; Tonami et al., 2003), Carisolv^{TM} (Hannig, 1999; Puppin-Rontani and Caldo-Teixeira, 2001; Arvidsson et al., 2002), Trypsin (Dung et al., 1994, 1995; Kleter et al., 1997), Pronase (Willey and Steinberg, 1984; Belz et al., 1999) and Pepsin (Kleter et al., 1997; Tonami and Ericson, 2005) on dentin degradation have been previously studied for different purposes several times.

The aim of this part of the study was to determine the extent to which artificial carious dentin can be solubilized by agents that do not seem to attack sound dentin such as pepsin, trypsin, collagenase and NaOCl, and to evaluate the effect of the enzyme pepsin as a self-limiting caries therapy in dentinal carious lesions using our model for artificial dentin caries. This part tested the hypothesis that pepsin is capable of removing just the denaturated dentin but leaving the demineralized tissue.

Materials and Methods

Sample Preparation:

Fifty-four extracted human third molars were obtained from an oral surgeons' private clinic and used within 8 months of extraction. After extraction teeth were immediately stored at room temperature in Ringer solution to which sodium-azide was added to prevent bacterial growth. All teeth were clinically sound without caries, abrasions or any mechanical traumas. Teeth were cleaned with a tooth brush aided sometimes with a scalpel to remove the periodontal ligament and intercrestal bone remnants and rinsed under running tap water. The roots were cut 2.0 mm below the

cementoenamel junction. The teeth were then embedded individually in transparent cold-curing methylmethacrylate (Technovit 4004, Kulzer GmbH, Wehrheim, Germany). To expose mid-coronal dentin each tooth was sectioned parallel to its long axis into two halves using a slow speed water-cooled diamond saw (Isomet, Beuhler, Illinois, USA), the occlusal part of each half was then removed by a cut perpendicular to the long axis of the tooth and 1.5-3.0 mm away from the pulp. Each half was divided into four slices parallel to the long axis of the tooth and extends from the middle of the tooth to the outer enamel with a thin saw microtome (Leica SP 1600, GmbH, Nußloch, Germany) under tap water. Dentin exposed surfaces were then polished flat with water proof silicon carbide abrasive paper (P500-grit) with a Leco VP 100 (GmbH, Neuss, Germany) device. Subsequently they were polished using wet polishing paper with silicone paste of polycrystalline diamonds of size 9 µm (DAP-7, Struers, Copenhagen, Denmark).

Dentin surfaces together with the surrounding Technovit were covered with two coats of nail varnish (Keyte, GmbH, Munich, Germany) to avoid the penetration of the solutions' molecules in any marginal gaps that could exist between the tooth and the acrylate, leaving one window of exposed mid-coronal dentin per slice. Adhesive paper was cut into 1x1.5 mm^2 pieces and attached to the dentin surfaces before applying the nail varnish to standardize the window. Two of the four exposed surfaces were perpendicular to the dentinal tubules and the other two were parallel to the tubules. The samples were then kept in air for about fifteen minutes to allow the nail varnish to dry. After the removal of the adhesive strips each window was etched for 20 s with 37% phosphoric acid gel (Total etch, Ivoclar Vivadent GmbH, Schaan, FL) to remove the smear layer.

Lesion Formation:

Twenty-seven teeth (216 slices) were demineralized in 0.1 M lactic acid (pH 4), while the other twenty-four teeth (192 slices) were immersed in 0.5 M acetic acid (pH 5) for 3, 7 and 14 days. The demineralization process was always with agitation of the solution with 150 rpm at 37°C (Forma Orbital Shaker, Thermo Electron Corporation, Ohio, USA) with 20 ml per slice. After the first week the demineralizing solutions were refreshed to avoid changes of the solution's pH of more than half a pH unit.

Enzymatic Treatment:

Each of the four slices from one tooth was treated either with pepsin, trypsin, collagenase or sodium hypochlorite while the other four slices from the opposite half of the same tooth served as controls. The experimental procedure is schematically illustrated in fig. 4.1.

- Pepsin: 1 mg of the enzyme pepsin (pepsin from hog stomach, 77152, Fluka, Biochemika, Sigma-Aldrich, Steinheim, Germany) was dissolved in either 1 ml of 0.5 M acetic acid pH 1.5 or pH 2.5 or 1 ml of 0.01 M HCl acid pH 2. Each slice was incubated individually in 1.5 ml of the enzymatic buffer for 10 min at 37°C with agitation 150 rpm. The slices were then washed with 0.2 M HCl tris buffer pH 8.6 at 4°C to stop the reaction and then washed with distilled water at 4°C.
- Trypsin: 1 mg of the enzyme trypsin (trypsin from hog pancreas, 93614, Fluka, Biochemika, Sigma-Aldrich, Steinheim, Germany) was dissolved in 1 ml of HEPES buffer (50 mM HEPES, 5 mM $CaCl_2.H_2O$, 0.15 M KCl, 5 mM sodium azide), pH 7.4. Each slice was incubated individually in 1.5 ml of the enzymatic buffer for 10 min or 24 h at 37°C with agitation 150 rpm. The slices were then washed with distilled water at 4°C.
- Collagenase: 1 mg of the enzyme collagenase (collagenase, Clostridiopeptidase A from Clostridium Histolyticum Type I, Sigma-Aldrich Chemie, GmbH, Steinheim, Germany) was dissolved in 1 ml of

HEPES buffer (50 mM HEPES, 5 mM $CaCl_2.H_2O$, 0.2 M NaCl, 5 mM sodium azide), pH 7.8. Each slice was incubated individually in 1.5 ml of the enzymatic buffer for 10 min or 24 h at 37°C with agitation 150 rpm. The slices were then washed with 0.001 M phosphate buffer pH 7 at 4°C to stop the reaction and then with distilled water at 4°C.

- Aqueous sodium hypochlorite: Each slice was incubated in 1.5 ml of 2.5% of sodium hypochlorite for 10 min at 37°C with agitation 150 rpm. The slices were then washed with distilled water at 4°C. Additional slices (32 slices) were etched with 37% phosphoric acid gel (Total etch, Ivoclar Vivadent GmbH, Schaan, FL) for 100 min and treated with 1.5 ml of 2.5% of sodium hypochlorite for 5 min. The experiment design is shown in table 4.1.

The 10 min period was chosen as being clinically relevant and the 24 h duration was chosen for both trypsin and collagenase according to the references. All the enzymatic buffer solutions were prepared at the same day they were used.

FE-SEM:

To obtain information on the morphology of the demineralized dentin surfaces a high resolution FE-SEM was used. Samples were fixed in 0.25 M Glutaraldehyde in 0.1 M Cacodylatebuffer pH 7.4 for 1 h, washed with 0.1 M Cacodylateuffer pH 7.4, then immersed in 50% alcohol for 20 min, subsequently in 70%, 80% and 90% alcohol, each for 20 min, and they were kept finally overnight in 96% alcohol. According to Perdigao et al. (1995) samples were put in Hexamethyldisilazane for 10 min and air dried at room temperature. Each sample was then fixed with carbon paste on the SEM sample holder. Gold sputtering was done for 1 min, with 1.0 kV, 0.3 mbar and 40 mA (Edwards Sputter Coater S15OB, Sussex, UK) and the

pictures were then made with a Leo FE-SEM (Leo DSM 982, Carl-Zeiss NTS GmbH, Oberkochen, Germany).

Through out the whole experimental procedure care was taken to avoid dentin desiccation particularly after the lesion was formed to avoid collapsing of the demineralized and denaturated collagen fibrils. Exposing the demineralized fibrils to air drying before critical point drying would lead to collapsed collagen fibrils and protinacious precipitates which would mask the morphological appearance of the surface.

Results

The surface of the demineralized human dentin samples after 3 days of demineralization, as shown in the SEM pictures, were similar for both acetic and lactic acids. The peri-tubular dentin was dissolved, the inter-tubular collagen fibres were partially demineralized and surface precipitates were formed. When acetic acid was the demineralizing regime, the surface precipitates were scattered and partially covering the demineralized surface. While demineralization with lactic acid led to large amounts of precipitates that covered large areas of the demineralized surface and occluded many of the dentinal tubules.

After 1 week of demineralization the surface precipitates were almost similar in quantity and morphological appearance for both acetic and lactic acids. They were scattered over the surfaces but they did not totally cover the inter-tubular dentin. Therefore the partially demineralized collagen fibres were clearly visible. However, with lactic acid there were always areas within the same surface that were covered with a thick layer of surface precipitates.

After two weeks the samples which were demineralized in acetic acid showed scattered patches of precipitates. With 2 weeks of demineralization in lactic acid the surface precipitates were much more than those at the

acetic acid demineralized surfaces and they totally covered the underlying demineralized inter-tubular dentin surface and occluded mostly all the dentinal tubules (fig. 4.2).

Although the precipitates covered the demineralized collagen fibres, partially demineralized collagen fibres within the inter-tubular dentin and totally demineralized collagen fibres within the dentinal tubules were sometimes observed under the precipitates.

There was no difference in the morphological appearance of the lesions which were formed perpendicular to the dentinal tubules and those which were parallel to the dentinal tubules.

Treating the acetic acid demineralized surfaces after 3 and 7 days with pepsin for 10 min did not show any significant difference in comparison to the control samples (fig. 4.3a). However, after 14 days of acetic acid demineralization small amounts of the surface precipitates were digested by the enzyme. Treating the lactic acid demineralized surfaces with pepsin for 10 min removed many of the surface precipitates (fig. 4.3b). Where the precipitates were digested the completely demineralized collagen fibres were clearly visible (table 4.2). With these samples we observed that the capability of the enzyme pepsin to remove the precipitates was decreased when the demineralization period was longer. Moreover, the capability of the enzyme in digesting the precipitates was significantly higher when the diluting buffer was 0.01 M HCl Tris buffer.

The samples which were treated with either trypsin or collagenase for 10 min showed no significant difference in the appearance of the treated surfaces in comparison to the controls. These observations were similar for both lactic and acetic acids. Although small amounts of precipitates were digested at the surfaces which were demineralized with lactic acid (fig. 4.4).

24 h of trypsin incubation of the samples, which were demineralized for 1 week in acetic acid, could not remove any of the surface precipitates (fig. 4.5a, 4.5b). While after 24 h of trypsin incubation of the samples, which were demineralized for 1 week in lactic acid, large amounts of the surface precipitates were removed. Again completely demineralized collagen fibres were observed after the precipitates were removed (fig. 4.5c). After 2 weeks of demineralization the enzyme trypsin did not remove as much as it removed after the first week in case of the lactic acid. In contrast to this it removed more than it did in the first week in case of acetic acid demineralization.

Treating the demineralized dentin surfaces with collagenase for 24 h resulted in complete removal for the surface layer exposing the underlying partially demineralized dentin. No totally demineralized fibres were seen but single mineralized fibres were always observed for both acetic and lactic acid demineralization (fig. 4.6).

Sodium hypochlorite (NaOCl) treatment for 10 min resulted in complete digestion of the surface layer exposing the underlying structures. After NaOCl treatment the acetic acid demineralized samples showed partially demineralized inter-tubular dentin while the lactic acid demineralized samples showed, that all fibres were totally demineralized (fig. 4.7).

5 min of NaOCl treatment after 100 min of phosphoric acid etching resulted in total removal of the surface and exposing deep partially demineralized dentin (fig. 4.8).

There was no difference in the morphological appearance of the treated surfaces which were formed perpendicular to the dentinal tubules and those which were parallel to the dentinal tubules.

Discussion

Results from the present study indicated that short term application of the enzyme pepsin is capable of removing just the denaturated collagen. Pepsin proved to be more efficient than trypsin and collagenase and less aggressive than NaOCl at removing denaturated dentin collagen fibers.

Acetic acid is a weak organic acid that is incapable of solubilyzing dentin collagen (Van Strijp et al., 1992). Lactic acid is a stronger organic acid (Featherstone and Rodgers, 1981), although it did not show capability of denaturating human dentin collagen after short incubation periods (Dung et al., 1994). However, it is suggested that dentin collagen is likely to denaturate during long-term acid exposure (Klont and ten Cate, 1991a; Kleter et al., 1998). Both acids are well known to be produced in major proportions by dental plaque and play an important role in carious dentin (Featherstone and Rodgers, 1981; Hojo et al., 1991).

The dentin caries process is initiated by demineralization of the mineral phase followed by the break down of the organic matrix. The organic material in dentin is protected by the hydroxyapatites so that neither the bacterial nor the endogenous proteolytic enzymes (Dung et al., 1995) have access to the organic matrix without dissolving the extra-fibrillar or intra-fibrillar, or both, apatite crystals first. The organic matrix of dentin is composed of collagen and noncollagenous compounds (NCCs). Phosphoproteins and proteoglycans are the major NCCs. Collagen type I is the dominant dentin collagen and it constitutes 90 wt% of dentin organic matrix. The triple-helix of collagen fibrils is resistant to most proteases and its degradation is initiated by cleavage of the three forming polypeptide chains (Brodsky and Ramshaw, 1997) as a result of proteolytic enzyme activity. The triple-helix of the undenaturated collagen is considered to be resistant to degradation by enzymes other than collagenases (Klont et al., 1991). Therefore pepsin, which is a carboxylic protease, is expected to act

only on the non-helical and denaturated collagen segments (Kleter et al., 1997; Tonami and Ericson, 2005). Pepsin was used to investigate its potential to remove the denaturated collagen fibrils of the demineralized matrix. Our results, as shown in the SEM pictures, indicated the capability of pepsin to remove a lot of the surface precipitates. Large proportions of these precipitates are thought to be denaturated collagen fibres. Our hypothesis is supported by our observations that the same enzyme preparation was incapable of removing these surface precipitates when the demineralizing pre-treatment was acetic acid and not lactic acid. It is known that acetic acid lacks the potential to solubilize either dentin collagen (Van Strijp et al., 1992) or dentin phosphorylated phosphoprotein (Klont and ten Cate, 1989). Consequently, we suggest that the surface layer precipitates at the lactic acid demineralized dentin surfaces is formed due to two steps. First the acid diffuses in an un-uniform pathway leaving remnants of hydroxyapatite at the surface together with calcium and phosphate reprecipitations (Moreno and Zahradnik, 1974; Featherstone et al., 1983; Fejerskov et al., 2003). Second the degraded denaturated collagen fibres together with the released NCCs remain in the demineralized tissue (Klont and ten Cate, 1990). It was argued that mineral precipitates from the demineralizing solutions could also form some of these surface precipitates

10 min of pepsin incubation at 37°C with agitation was enough to remove large amounts of surface precipitates and denaturated collagen after 3 days and 1 week at the lactic acid demineralized surfaces. This potential, however, was decreased with increasing the demineralizing period to two weeks, most probably because the amount of the demineralized matrix increased, aggregated, and partially blocked the enzyme diffusion and/or action (Kleter et al., 1994). In all lactic acid samples the ten minutes incubation was not enough to remove all the surface precipitates. So far it

was not determined wither this was a matter of quantity in which the degraded matrix needed more than ten minutes to be completely digested by the enzyme, or was it a matter of quality in which the remaining precipitates were other than denaturated collagen such as hydroxyapatites and NCCs. On the other hand a slight increase in the pepsin capacity in removing small amounts of the surface precipitates from the two weeks acetic acid demineralized surfaces was observed. In the present study the enzyme potential was optimum at pH 2 in 0.01 M HCl Tris buffer in comparison to pH 1.5 and pH 2.5 in 0.5 M acetic acid. Preliminary tests in our laboratory indicated that the acidic medium of the enzyme has a limited capacity to remove these precipitates in such a short duration.

Trypsin was used in comparison to pepsin, it is a serine protease that is also able to digest denaturated collagen, but at a neutral pH 7.4 (Kleter et al., 1997). After 24 h incubation trypsin was superior to pepsin in removing the surface layer at the lactic acid demineralized surfaces. The ten minutes treatment did not show any significant effect in decreasing the amount of the surface precipitates compared to the control samples. These results are in agreement with Kleter et al., (1997) who stated that collagen degradation during trypsin incubation was fastest during the first 24 h. Neither of the two trypsin incubations periods acted at the surface precipitates of the 3 days and 1 week acetic acid demineralized surfaces. The 24 h trypsin treatment of the two weeks acetic acid demineralized surfaces showed a very limited capacity in affecting the degraded dentin. Moreover, we observed that while pepsin had a very light effect on removing the degraded matrix from the 1 week acetic acid demineralized surfaces; trypsin could not cause any morphological differences comparable to the control group. We hypothesize that although both enzymes behaved very similarly in digesting the non helical and denaturated segments of collagen

(Kleter et al., 1997) the acidic environment of the pepsin with pH 2 was responsible for the surface etching observed with pepsin.

Collagenase is a specific metalloproteinase that is capable of hydrolyzing collagen at multiple cleavage sites (Van Strijp et al., 1992; Ho et al., 2005). In the present study the enzyme collagenase at a neutral pH 7.8 did not act effectively within 10 min at both acetic and lactic acid demineralized surfaces but it digested the entire degraded matrix and demineralized collagen fibrils during 24 h of incubation in both groups, which is in accordance with the literature. Interestingly, all the collagenase treated surfaces showed single mineralized collagen fibres which resisted its proteolytic action. We expected after 24 h of collagenase treatment to see mineralized or partially demineralized dentin surfaces into which collagen fibres are embedded, similar to the SEM pictures of Gwinnett et al., (1996) for example. However, although the mode of the collagenase-collagen interaction that determines cleavage specificity is yet to be elucidated (Brodsky and Ramshaw, 1997) it was stated that the collagenase binding and cleavage sites along the collagen triple-helices has to be accessible to the enzyme in order to bind and cleave the fibres. This means that the hydroxyapatite crystals covering the single mineralized fibres inhibited the cleaving action of the collagenase (Klont and ten Cate, 1991b). Our findings based on the collagenase action suggest that the intrafibrillar mineralization does not inhibit the collagenase proteolytic action while the extrafibrillar mineralization blocks the collagenase binding sites and prevent collagen break down by the enzyme.

Preliminary tests in our laboratory led to SEM pictures similar to those of Willey and Steinberg (1984), in which five minutes of collagenase treatment after three minutes of citric acid application provided -in the mentioned study- visually apparent matrix clearing, exposing more collagen fibres with a smaller mean diameter. Such results could be

provided with short incubations of either collagenase or NaOCl after short demineralizing periods in which increasing the treatment incubation time led to gradual digestion of the exposed demineralized collagen. On the other hand increasing the amount of the collagenase to 5 mg and 10 mg did not enhance the digestion strength.

In the present study all the tested agents' efficiency in removing the surface precipitates and digesting the degraded matrix was decreased with both, increasing the demineralizing period and/or decreasing the treatment duration.

NaOCl is a non specific proteolytic agent which is used widely in various dental procedures (Marschall et al., 2001). It was introduced as a chemomechanical method to remove carious dentin after mixing it with three amino acids in the Carisolv^{TM} system. It was reported that these amino acids are responsible for the selective carious dentin tissue removal observed with the Carisolv system in comparison to the pure NaOCl (Hanning, 1999; Tonami et al., 2003). In our study 2.5% of pure NaOCl was chosen to study the extent of its effect at both demineralized and denaturated dentin. Our results were in agreement with Hannig (1999). NaOCl effectively removed both the denaturated and demineralized dentin layers for acetic acid and lactic acid demineralized surfaces. In addition, when phosphoric acid gel etching was used instead of acetic and lactic acids the NaOCl effect was enhanced due to the decreased amount of degraded organic matrix that is needed to be removed. The pure NaOCl in our study was the most aggressive agent comparable to the other agents used to remove denaturated and demineralized dentin. There were no single mineralized collagen fibres at the surface after NaOCl treatment. These results suggest that extra-fibrillar mineralization could not resist the NaOCl action.

In conclusion:

1- Our model produced both denaturated and demineralized dentin only when lactic acid was used. Therefore lactic acid should be used to evaluate self-limited enzyme-based experiments.

2- The type of acid and the demineralization time affect the ratio of denaturated/demineralized dentin.

3- The pre-treatment and treatment type and time influenced the quality and quantity of the digested substrate.

4- The aggressiveness of the enzymes and agents used in the present study can be ranked (from least effective to most effective) Trypsin < Pepsin < Collagenase < NaOCl. The enzyme pepsin, with its acidic pH optimum, was more aggressive in removing the disintegrated dentinal organic matrix than the neutral enzyme trypsin. We hypothesize that although both enzymes behaved very similarly in digesting the denaturated collagen, the acidic environment of the pepsin with pH 2 was responsible for the surface etching observed with pepsin. We believe that the pepsin acidic medium is advantageous, in which the enzyme can be inactivated by washing it and thus neutralizing its pH.

5- Based on the working principle of the treating solutions we suggest that artificial dentinal caries in the presence of lactic acid exhibits four successive layers with respect to the collagen fibres: denaturated collagen fibres, all fibres totally demineralized, single mineralized fibres and partially demineralized fibres (fig. 4.9). Table 4.2 clarify the definitions we used for the mentioned layers. The criteria were selected according to our observations from the SEM pictures.

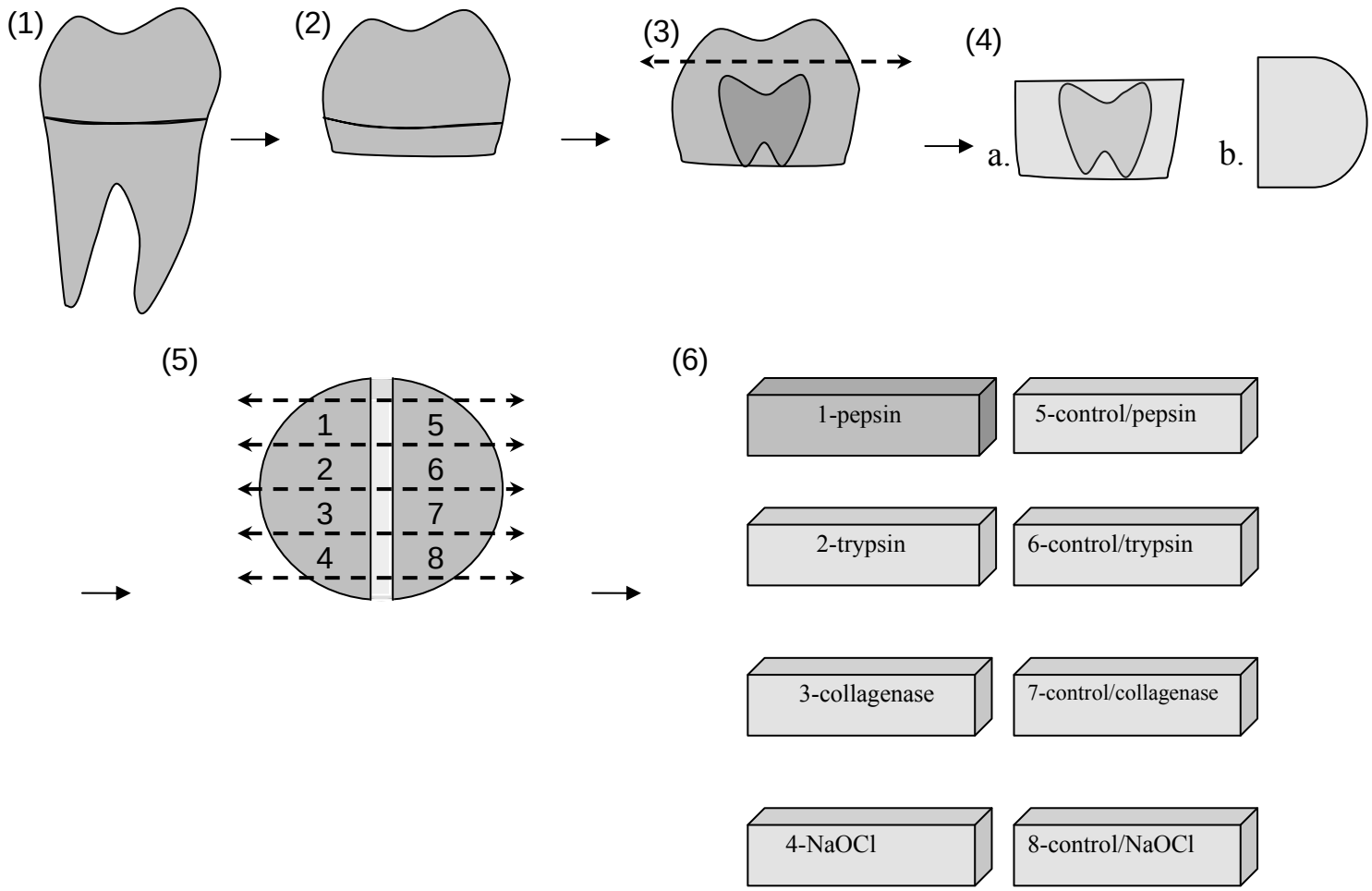


Fig 4.1. A schematic drawing illustrating the experimental procedure. (1) Caries free molar. (2) The root was abraded short below the cemento-enamel junction and the tooth was cut longitudinally from the middle. (3) The occlusal part of each half was removed to expose deep dentin. (4) a. Longitudinal section of one half of the crown after the occlusal part was removed, b. cross section. (5) Each half was sliced into four slices (1, 2, 3, 4) and (5, 6, 7, 8). (6) Every two opposable slices (1,5), (2,6), (3,7) and (4,8) were demineralized separately either in lactic or acetic acid. One of every two slices (5,6,7 and 8) serves as a control and the other (1,2,3 and 4) was treated with one of the treating regimes.

Table 4.1. The experiment design (N is the number of slices).

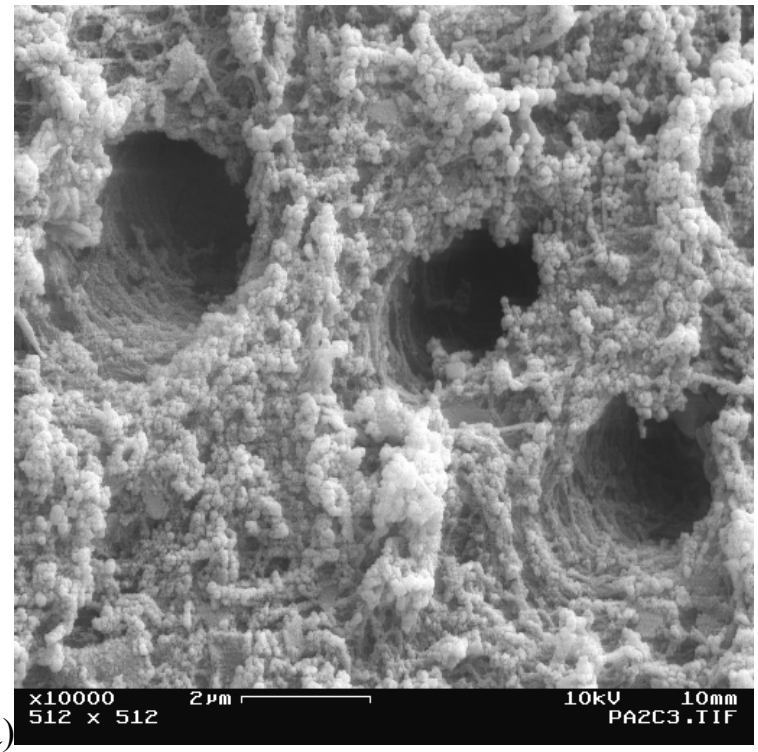
Demineralising regime / Treatment type and time	Acetic acid, pH 5 N= 192			Lactic acid, pH 4 N= 216			Phosphoric acid N= 32
	3days n= 48	7days n= 72	14days n= 72	3days n=48	7days N=96	14days n=72	100 minutes n=32
Pepsin in acetic acid buffer, pH 1.5or 2.5, 10 min N= 34	n= 4	n= 6	n= 6	n= 4	N= 8	n= 6	-
Pepsin in HCl acid pH 2.0, 10min N= 27	n= 3	n= 5	n= 5	n= 3	N= 6	n= 5	-
Trypsin, 10 min N= 24	n= 3	n= 4	n= 4	n= 3	N= 6	n= 4	-
Trypsin, 24hrs N= 34	n= 4	n= 6	n= 6	n= 4	N= 8	n= 6	-
Collagenase,10min N= 24	n= 3	n= 4	n= 4	n= 3	N= 6	n= 4	-
Collagenase, 24hrs N= 34	n= 4	n= 6	n= 6	n= 4	N= 8	n= 6	-
NaOCl, 10 min N= 27	n= 3	n= 5	n= 5	n= 3	N= 6	n= 5	-
NaOCl, 5 min N= 32	-	-	-	-	-	-	n= 32

Collagen status	Definition (according to the observations)
Denaturated collagen	- The extrafibrillar and intrafibrillar minerals are dissolved. - The internal structure of the fibres is destructed. - The cross-links are broken. - Globular and random coils are formed.
All fibres totally demineralized (completely demineralized fibres)	- The extrafibrillar minerals are dissolved; there are no hydroxyapatites between the fibres or alongside the single fibre. - The intrafibrillar minerals are not affected. - The internal structure of the fibres is not destructed. Therefore, the tropocollagen triple helix composed of the three polypeptide α chains is preserved. - The cross-links are not broken. - They are rod-like in shape, 300 nm long and 1.5 nm in diameter.
single mineralized fibres	- They are the same as the completely demineralized fibres but the extrafibrillar minerals which lie in the interstitial spaces between the fibres were dissolved while each single fibre was still covered with hydroxyapatites.
Partially demineralized fibres	- These are the same as the single mineralized fibres but the extrafibrillar minerals which lie in the interstitial spaces between the fibres were not totally dissolved. The hydroxyapatites covered the single fibre and partially remained between the fibres.

Table 4.2. The terms which were used in this article were based on our SEM observations and the definitions we made were according to the mentioned criteria.

Fig 4.2. (a) Human coronal dentin after two weeks demineralization with acetic acid. The peri-tubular dentin is dissolved and the inter-tubular dentin is partially demineralized (x10000). (b) Human coronal dentin after two weeks demineralization with lactic acid. A thick surface layer covered the demineralized surface and occluded the dentinal tubules(x10000).

(a)



(b)

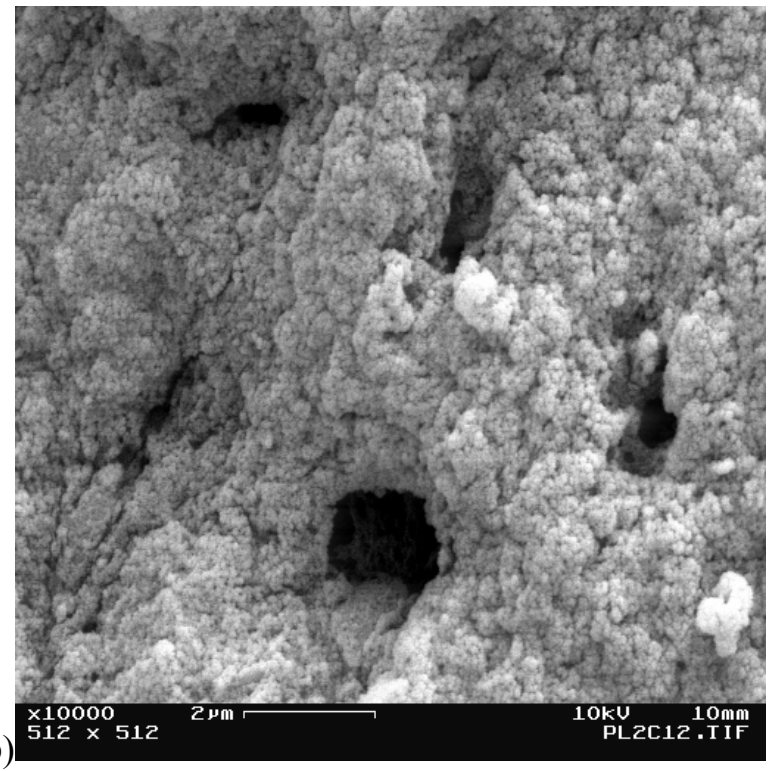
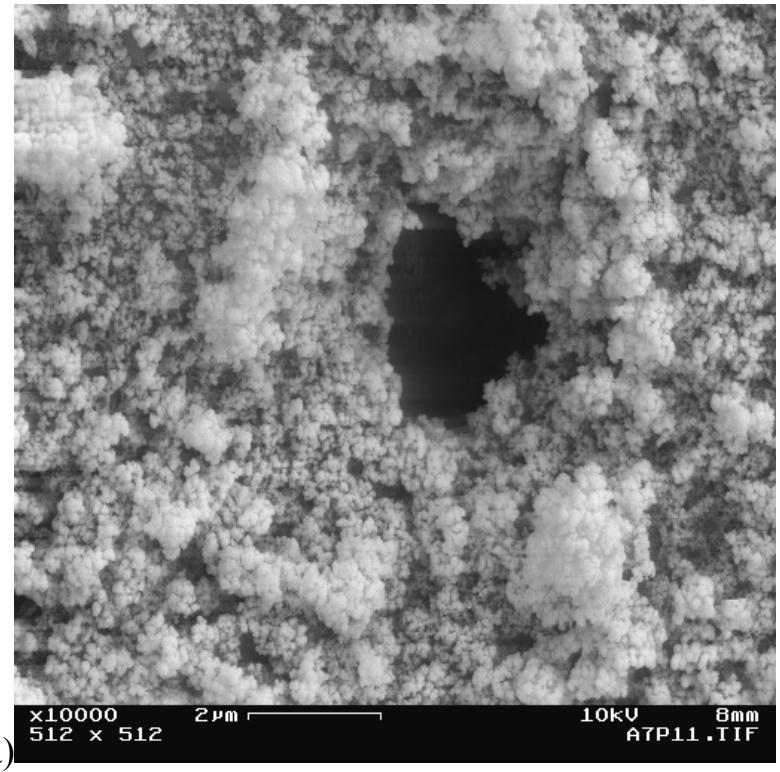


Fig 4.3. (a) Pepsin-treated surface after 1 week demineralization with acetic acid (x10000). (b) Pepsin-treated surface after 1 week demineralization with lactic acid (x10000). 1.5 mg pepsin in 1.5 ml of 0.01 M HCl Tris buffer, pH 2, at 37°C, with 150 rpm, for 10 min.

(a)



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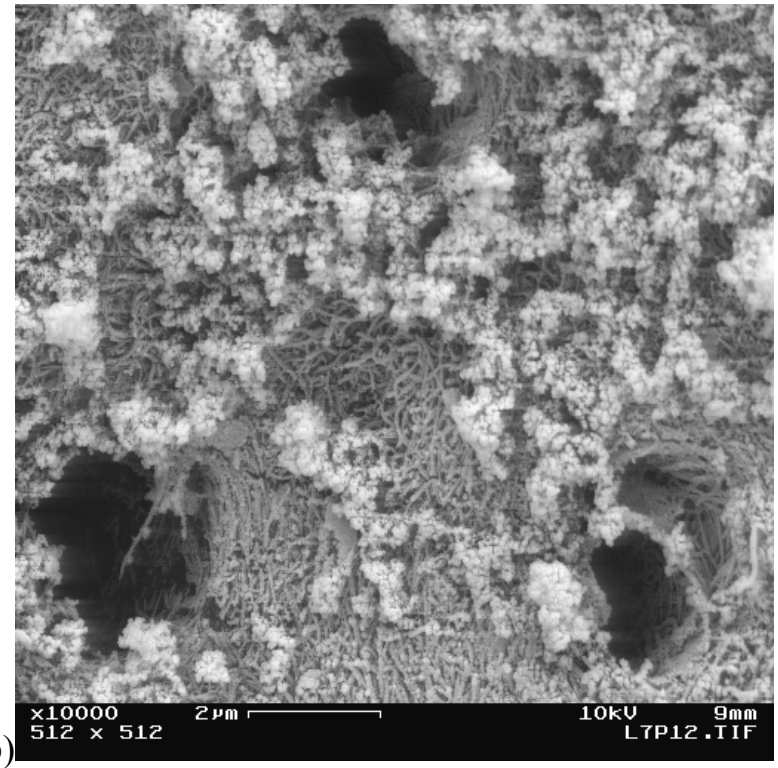
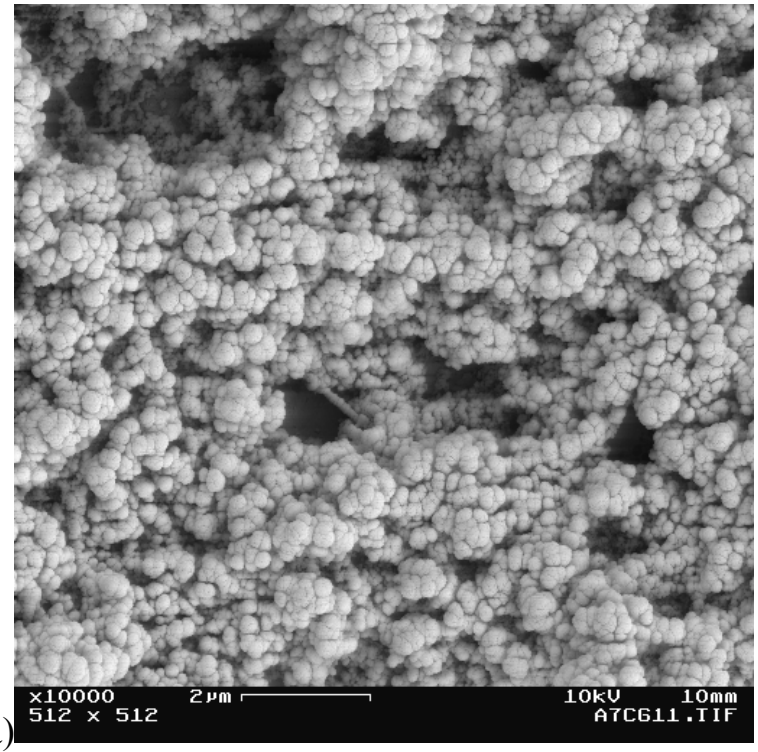


Fig 4.4. (a) Collagenase-treated surface after 1 week demineralization with acetic acid (x10000). (b) Collagenase-treated surface after 1 week demineralization with lactic acid (x10000). 1.5 mg collagenase in 1.5 ml of 50 mM HEPES buffer, pH 7.8, at 37°C, with 150 rpm, for 10 min.

(a)



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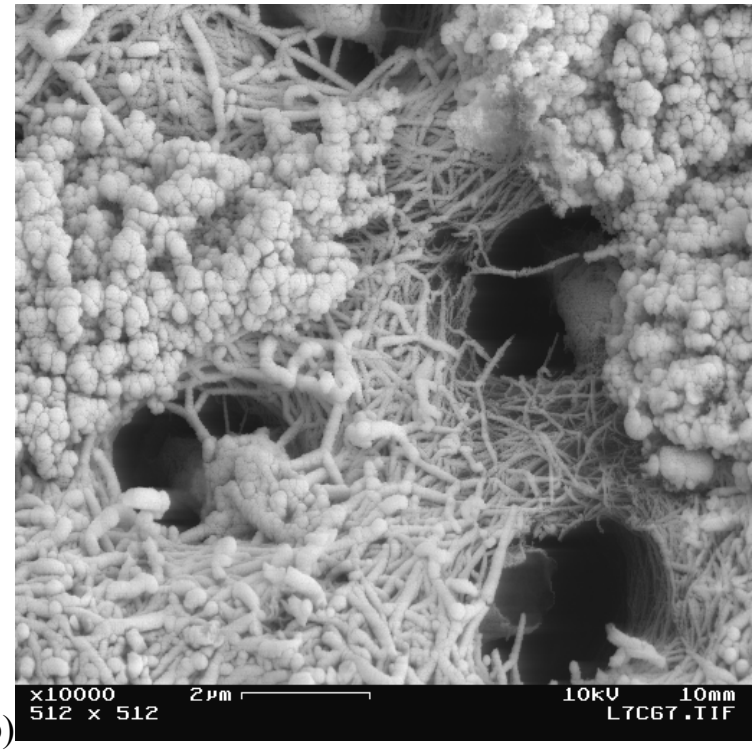
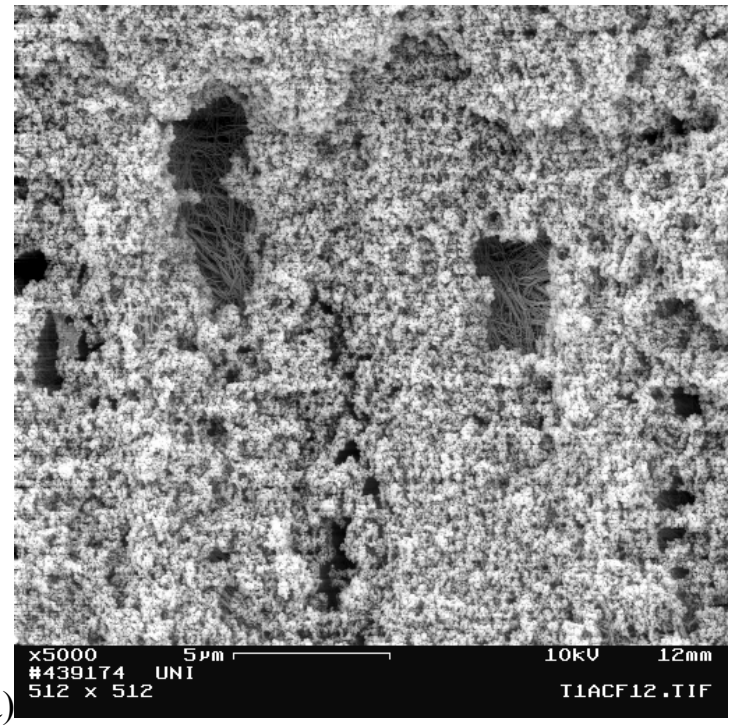


Fig 4.5. (a) 1 week of demineralization with acetic acid (pH 5), the window is parallel to the dentinal tubules (x10000). (b) Trypsin-treated surface after 1 week demineralization with acetic acid (x10000). (c) Trypsin-treated surface after 1 week demineralization with lactic acid (x10000). 1.5 mg trypsin in 1.5 ml of 50 mM HEPES buffer, pH 7.4, at 37°C, with 150 rpm, for 24 h.

(a)



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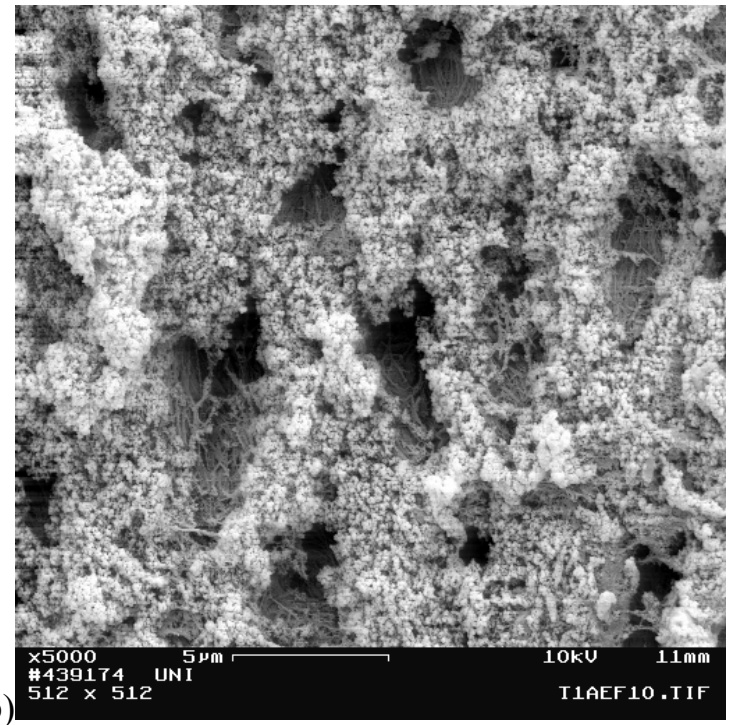


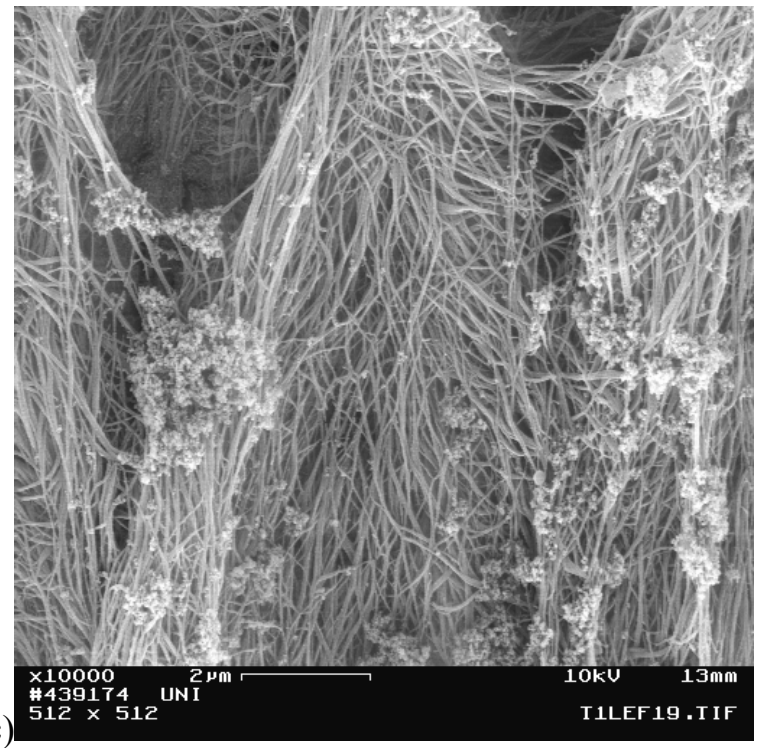
(c) 

Fig 4.6. Collagenase-treated surface after 1 week demineralization with acetic acid (x10000). 1.5 mg collagenase in 1.5 ml of 50 mM HEPES buffer, pH 7.8, at 37°C, with 150 rpm, for 24 h.

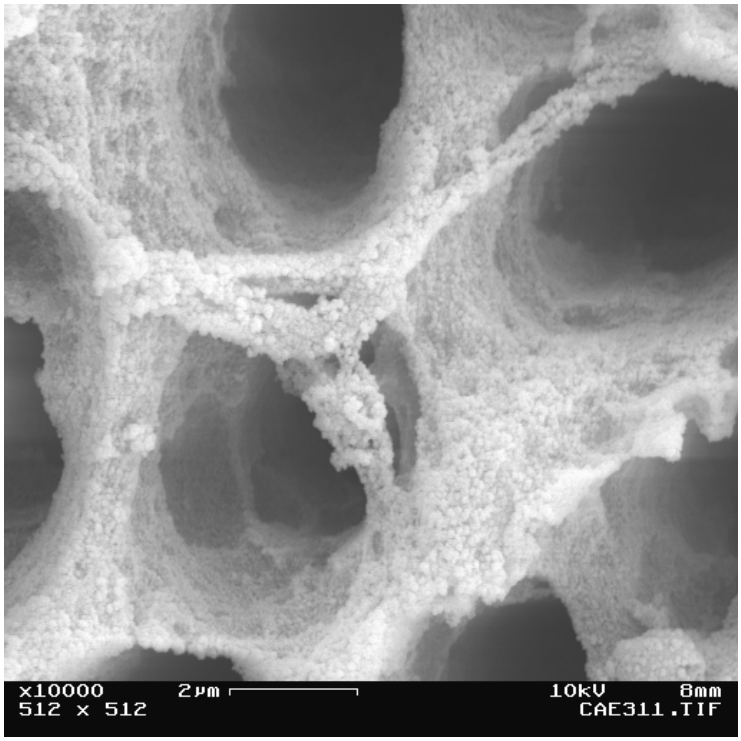
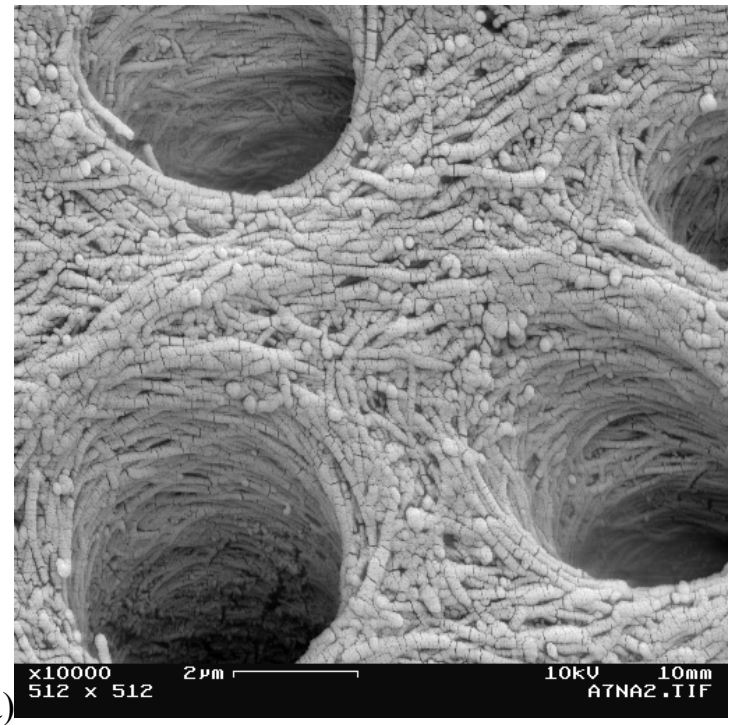


Fig 4.7. (a) NaOCl-treated surface after 1 week demineralization with acetic acid. Note the thick partially demineralized collagen fibres (x10000). (b) NaOCl-treated surface after 1 week demineralization with lactic acid. Note the thin totally demineralized collagen fibres (x10000). 1.5 ml of 2.5% NaOCl, at 37°C, with 150 rpm, for 10 min.

(a)



(b)

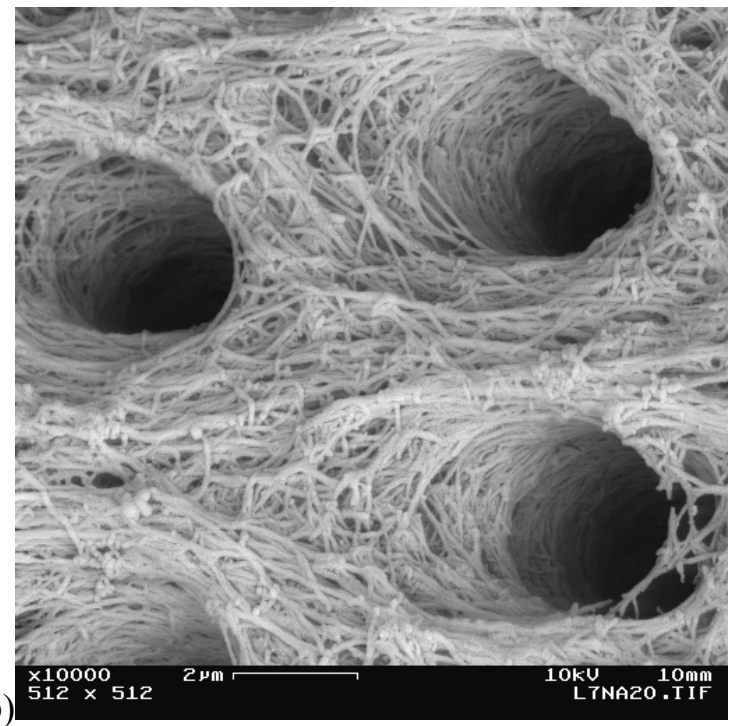
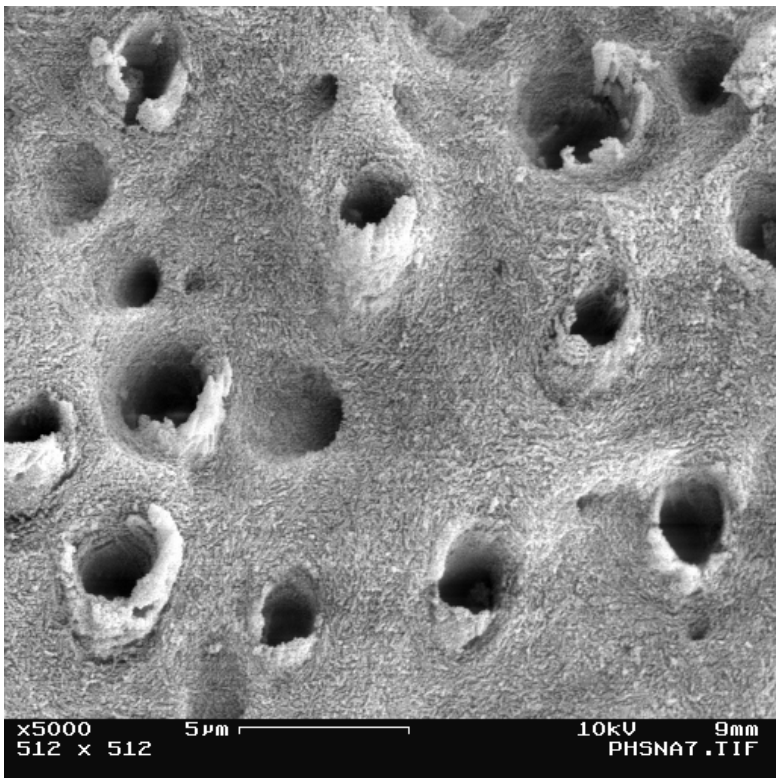


Fig 4.8. NaOCl-treated surface after 100 min of 37% phosphoric acid gel etching (x5000). 1.5 ml of 2.5% of sodium hypochlorite for 5 min.



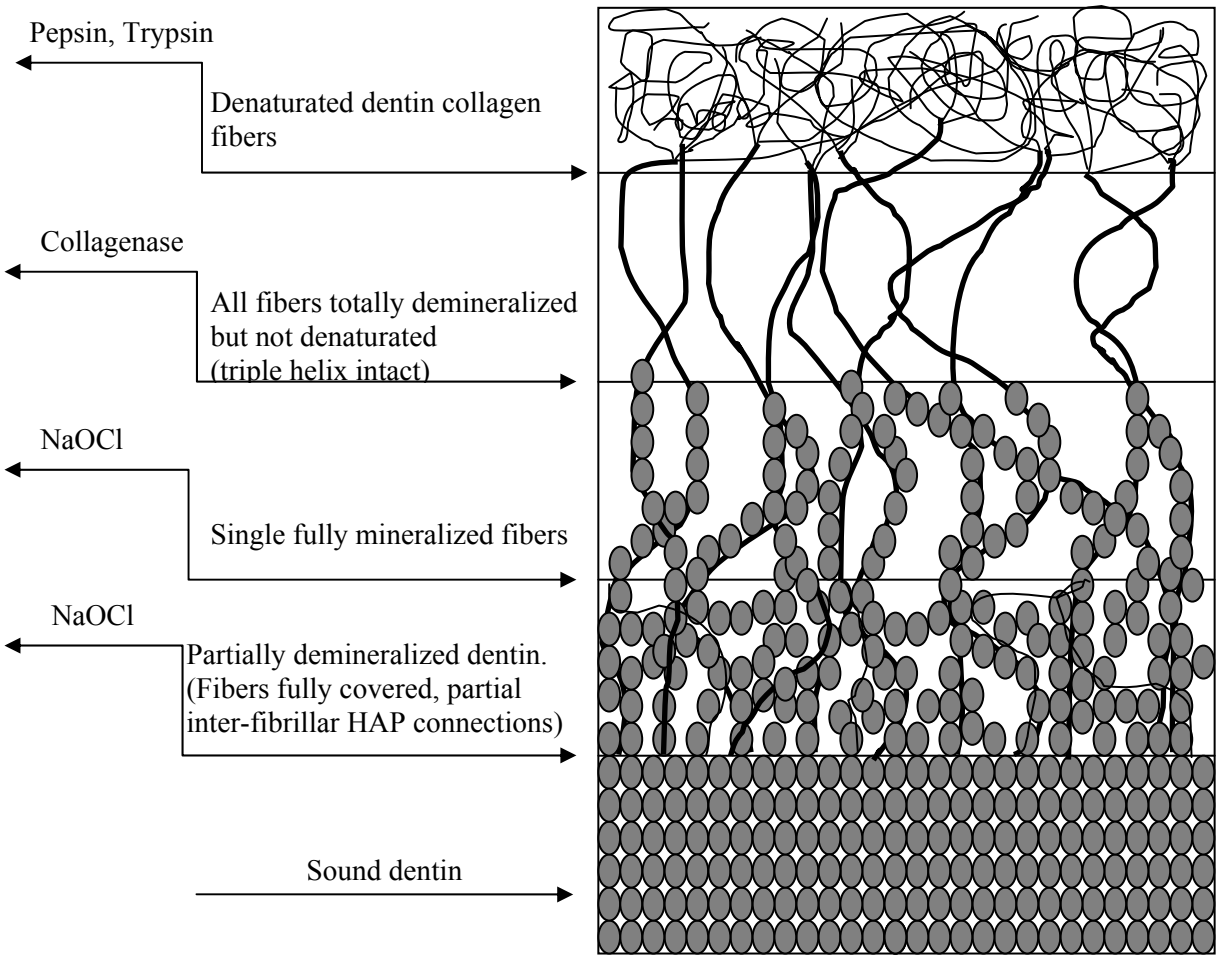

Pepsin, Trypsin
Denaturated dentin collagen fibers
Collagenase
All fibers totally demineralized but not denaturated (triple helix intact)
NaOCl
Single fully mineralized fibers
NaOCl
Partially demineralized dentin. (Fibers fully covered, partial inter-fibrillar HAP connections)
Sound dentin

Fig 4.9. The effect of various enzymes and agents on the four successive layers of the artificial dentinal lesions produced with lactic acid pH 4. Both trypsin and pepsin are capable of digesting just the denaturated collagen fibres. Collagenase action is stronger in which denaturated and demineralized dentin is digested but it is resisted by single mineralized fibres. NaOCl is able to remove these mineralized fibres and even the partially demineralized dentin.

Dentin Remineralization Enhancement under Dental Restoration

Chapter 5

Introduction

As a consequence to the development of the adhesive dental restorative materials, the minimally invasive dentistry concept was introduced and several excavation techniques had been advocated (Banerjee et al., 2000; Lager et al., 2003). Several studies tried to evaluate the clinical outcome of leaving the affected dentinal tissue behind after excavation before restoring the tooth (Bjorndal et al., 1997; Weerheijm et al., 1999; Maltz et al., 2002; Lager et al., 2003; Foley and Blackwell, 2003). They assume that elimination of the harmful microbial mass at the lesion surface would permit the underlying layer to heal gradually through the biological properties of the tissue after sealing it with a restorative material, at the same time they preserve the remineralizable tissue, maintain the pulp vitality by avoiding its exposure and arrest caries progression (Ostrom, 1980; Nikiforuk, 1985; McComb, 2000; Heinrich-Weltzien and Kneist, 2001; Fejerskov et al., 2003).

However, deep dentinal lesions can remineralize under certain conditions which favour crystal growth on partially demineralized dentin (Levine and Roweles, 1973; Kuboki et al., 1977; Arends et al., 1989; Lussi and Linde, 1993; Inaba et al., 1996; Saito et al., 1997, 2003; Ritchie et al., 1998; Butler, 1998; Damen et al., 1998; Kawasaki et al., 1999, 2000; Mukai and ten cate, 2002). According to the mentioned references these conditions can be summarized as following; the internal structure of the collagen fibres has to be intact, they are unable to be remineralized if they

are destructed or denaturated (Fusayama et al., 1966), the non-collagenous proteins such as phosphophoryns have to be in low amounts and in phosphorylated insoluble bounded form which covalently cross-linked to the collagen, so they would be able to bind calcium and initiate crystal nucleation and hydroxyapatite formation (Lussi et al., 1988; Saito et al., 1997, 1998, 2000; Clarkson et al., 1998; Milan et al., 2006). Moreover, ultrastructural studies on dentinal lesions have shown that remineralization neither occurred by spontaneous precipitation nor by nucleation of mineral on the organic matrix but by growth of residual crystals in the lesions (Klont and ten Cate, 1991b).

Therefore, alternatives to the mechanical excavation aim to be more selective in caries removal by removing only the infected denaturated and cariogenic layer leaving behind the affected demineralized but remineralizable one (Ericson et al., 1999; Yip et al., 1999; Banerjee et al., 2000; Beeley et al., 2000; Fure et al., 2000; Rafique et al., 2003; Flückiger et al., 2005).

The caries removal method using specific proteolytic enzymes (Beltz et al., 1999; Bussadori et al., 2005; Tonami and Ericson, 2005; Kappler et al., 2006) could be an alternative to the conventional methods, effective and more conservative.

After we applied different enzymes and preolytics to the artificially demineralized dentin, we studied the capability of the remaining demineralized dentin to be remineralized, because the remineralization of this remaining layer would be valuable for clinical purposes. The superficial infected layer that is composed mainly of denaturated collagen is enzymatically digested, and the affected layer which contains demineralized dentin can be preserved to avoid pulpal exposure and to protect the pulp from further mechanical, chemical and thermal injuries.

The purpose of this part of the study was to investigate whether the proteolytic treatment would interfere with the in vitro remineralization of advanced human coronal dentinal lesions. Based on our findings in chapter 2, 5.0 ppm fluoride was optimal for dentin lesion remineralization. The hypothesis to be tested was that the topical application of proteases during dentin caries excavation followed by fluoride application could be of a clinical significance in dentin remineralization.

Materials and Methods

Sample Preparation:

Thirty-six extracted human third molars were obtained from an oral surgeons' private clinic and used within 9 months of extraction. After extraction teeth were immediately stored at room temperature in Ringer solution to which sodium-azide was added to prevent bacterial growth. All teeth were clinically sound. Teeth were cleaned with a tooth brush aided sometimes with a scalpel to remove the periodontal ligament and intercrestal bone remnants and rinsed under running tap water. The roots were cut 2.0 mm below the cementoenamel junction. The teeth were then embedded individually in transparent cold-curing methylmethacrylate (Technovit 4004, Kulzer GmbH, Wehrheim, Germany). To expose mid-coronal dentin each tooth was sectioned parallel to its long axis into two halves using a slow speed water-cooled diamond saw (Isomet, Beuhler, Illinois, USA), the occlusal part of each half was then removed by a cut perpendicular to the long axis of the tooth and 1.5-2.0 mm away from the pulp. Each half was divided into four slices parallel to the long axis of the tooth and extends from the middle of the tooth to the outer enamel with a thin saw microtome (Leica SP 1600, GmbH, Nußloch, Germany) under tap water. Dentin exposed surfaces were then polished flat with water proof silicon carbide abrasive paper (P500-grit) with a Leco VP 100 (GmbH, Neuss, Germany) device. Subsequently they were polished using wet

polishing paper with silicone paste of polycrystalline diamonds of size 9 µm (DAP-7, Struers, Copenhagen, Denmark).

Dentin surfaces together with the surrounding Technovit were covered with two coats of nail varnish (Keyte, GmbH, Munich, Germany) to avoid the penetration of the solutions' molecules in any marginal gaps that could exist between the tooth and the acrylate, leaving one window of exposed mid-coronal dentin per slice. Adhesive paper was cut into 1x1.5 mm² pieces and attached to the dentin surfaces before applying the nail varnish to standardize the window. The samples were then kept in air for about fifteen minutes to allow the nail varnish to dry. After the removal of the adhesive strips each window was etched for 20 s with 37% phosphoric acid gel (Total etch, Ivoclar Vivadent GmbH, Schaan, FL) to remove the smear layer.

Lesion Formation:

Based on the protocol derived in chapters 3 and 4, 288 slices were demineralized in 0.1 M lactic acid (pH 4) for 14 days, at room temperature, without agitation, with 10 ml per slice. Every two slices were demineralized together.

After demineralization the 216 slices were treated as follows; 108 slices were treated with either pepsin, trypsin, collagenase or sodium hypochlorite (see below) and then transferred to a flask containing 10 ml of remineralizing solution composed of 20 mM HEPES, 1.5 mM $CaC_{l2}.2H_2O$, 0.9 mM $KHPO_4$, 130 mM KCl, and 3.08 mM sodium-azide and 5.0 ppm fluoride as NaF with the pH adjusted with KOH to 7. The other 108 slices were remineralized immediately without an intermediate treatment.

Samples for Lesion Assessment:

The remaining 72 slices were analysed immediately without enzymatic treatment or remineralization and used for lesion assessment.

They were polished flat with wet silicon carbide abrasive paper (800-grit) to obtain a plano-parallel slice of 120-150 μm before imbibition in quinoline (Quinoline 22650, Fluka chemie GmbH, Hamburg, Germany) for at least 4 h. They were then mounted for microscopic examination. Lesion depth was measured along a vertical line perpendicular to the tooth surface extending from a point at the lesion surface to a point at the non-demineralized surface through out the lesion body to the inner most border of the lesion.

Enzymatic Treatment:

Each of the four slices from one tooth was treated with either pepsin, trypsin, collagenase or sodium hypochlorite while the other four slices from the opposite half of the same tooth served as controls.

- Pepsin: 1 mg of the enzyme pepsin (pepsin from hog stomach, 77152, Fluka, Biochemika, Sigma-Aldrich, Steinheim, Germany) was dissolved in 1 ml of 0.01 M HCl acid pH 2. Each slice was incubated individually in 1.5 ml of the enzymatic buffer for 10 min at 37°C with agitation 150 rpm. The slices were then washed with 0.2 M HCl tris buffer pH 8.6 at 4°C to stop the reaction and then washed with distilled water at 4°C.
- Trypsin: 1 mg of the enzyme trypsin (trypsin from hog pancreas, 93614, Fluka, Biochemika, Sigma-Aldrich, Steinheim, Germany) was dissolved in 1 ml of HEPES buffer (50 mM HEPES, 5 mM $CaCl_2.H_2O$, 0.15 M KCl, 5 mM sodium azide, pH 7.4). Each slice was incubated individually in 1.5 ml of the enzymatic buffer for 10 min or 24 h at 37°C with agitation 150 rpm. The slices were then washed with distilled water at 4°C.
- Collagenase: 1 mg of the enzyme collagenase (collagenase, Clostridiopeptidase A from Clostridium Histolyticum Type I, Sigma-Aldrich Chemie, GmbH, Steinheim, Germany) was dissolved in 1ml of HEPES buffer (50 mM HEPES, 5 mM $CaCl_2.H_2O$, 0.2 M NaCl, 5 mM

sodium azide, pH 7.8). Each slice was incubated individually in 1.5 ml of the enzymatic buffer for 10 min or 24 h at 37°C with agitation 150 rpm. The slices were then washed with 0.001 M phosphate buffer pH 7 at 4°C to stop the reaction and then with distilled water at 4°C.

- Aqueous sodium hypochlorite: Each slice was incubated in 1.5 ml of 2.5% of sodium hypochlorite for 10 min at 37°C with agitation 150 rpm. The slices were then washed with distilled water at 4°C. Additional slices were etched with 37% phosphoric acid gel (Total etch, Ivoclar Vivadent GmbH, Schaan, FL) for 100 min and treated with 1.5 ml of 2.5% of sodium hypochlorite for 5 min.

The 10 min period was chosen as being clinically relevant and the 24 h duration was chosen for both trypsin and collagenase according to the references. All the enzymatic buffer solutions were prepared at the same day they were used.

Analytical Tools:

Microscopy:

Quinoline with polarized light (Axioskope 2, MAT, Carl Zeiss Jena GmbH, Göttngen, Germany) was used for the visual qualitative analysis of the lesions before and after remineralization. Digital images were taken with the image analysis software Axiovision (Rel. 4.4, SP2, Carl Zeiss Jena GmbH, Göttngen, Germany).

Microhardness:

Testing the microhardness of the remineralized dentin was done with a Vickers pyramid diamond indenter under 30 mN/mm² with the automatic microhardness tester Fischerscope H100C (Helmut Fischer GmbH, Sindelfingen, Germany). Three lines per lesion were made in which each line composed of 12-22 points which were spaced by 25-30 μm. Each line extended vertically through the lesion from a point just beneath the lesion

bottom up to the surface to determine cross surface microhardness (CSMH) through out the lesion.

FE-SEM:

To obtain information on the morphology of the demineralized dentin surfaces a high resolution FE-SEM was used. Samples were fixed in 0.25 M Glutaraldehyde in 0.1 M Cacodylatebuffer pH 7.4 for 1 h, washed with 0.1 M Cacodylatebuffer pH 7.4, then immersed in 50% alcohol for 20 min, subsequently in 70%, 80% and 90% alcohol, each for 20 min, and they were kept finally overnight in 96% alcohol. According to Perdigao et al. (1995) samples were put in Hexamethyldisilazane for 10 min and air dried at room temperature. Each sample was then fixed with carbon paste on the SEM sample holder. Gold sputtering was done for 1 min, with 1.0 kV, 0.3 mbar and 40 mA (Edwards Sputter Coater S15OB, Sussex, UK) and the pictures were then made with a Leo FE-SEM (Leo DSM 982, Carl-Zeiss NTS GmbH, Oberkochen, Germany).

Through out the whole experimental procedure care was taken to avoid dentin desiccation particularly after the lesion was formed to avoid collapsing of the demineralized and denaturated collagen fibrils. Exposing the demineralized fibrils to air drying before critical point drying would lead to collapsed collagen fibrils and protinacious precipitates which would mask the accurate morphological appearance of the surface.

Results

Lesion depth before and after remineralization with and without protease treatment was measured by polarized light microscope (PLM) and is shown in table 5.1.

According to the PLM observations, there was no decrease in lesion depth after 1 week of remineralization with 5.0 ppm of fluoride.

Remineralization did not show to take place at the lesion depth with and without topical application of the protealytic agents.

Surface erosion after 2 weeks of demineralization with lactic acid was observed in all the samples. The erosion at the demineralized surfaces in all of the groups increased after 1 week of remineralization. The samples which were treated with trypsin for 10 min were the only group that showed decrease in surface erosion after 1 week of remineralization in comparable to its control group (fig. 5.2). On the other hand, surface erosion was prominent in samples which were treated with collagenase for 24 h and those treated with NaOCl for 10 min. The other experimental groups showed no difference in surface erosion in comparison to the control groups.

PLM showed remineralizing bands at the surfaces which were treated with trypsin for 24 h. No banding was observed within the lesion body for other control and experimental groups.

The Vickers indentations resulted in two patterns of values for the dentinal lesions in the control groups. These patterns were similar before and after remineralization. Diagram 5.1 represents the results of the microhardness tests for the control groups before and after remineralization. The first pattern of measurements showed low values of hardness throughout the lesion body and at the lesion surface. The second pattern demonstrated low values throughout the lesion body and high values at the surface of the lesion. The surface values were equal to and sometimes even exceeded the values of sound dentin. The sound dentin values were taken from the literature (Müller-Stahl, 2006). No changes were observed in the dentin hardness throughout the lesion even after 1 week of remineralization with 5.0 ppm fluoride in comparison to the controls.

The mean values of the CSMH for the experimental groups are shown in table 5.2. The pepsin, NaOCl, trypsin (10 min) and collagenase (24 h) treated samples demonstrated high values throughout the lesion body and at the lesion surface in comparison to their control groups. Slight increase in the values throughout the lesion body was observed in the samples which were treated with trypsin for 1 day. No significant difference in the measured values between the samples which were treated with collagenase for 10 min and their control group.

The surfaces of the demineralized human dentin samples after 2 weeks of demineralization, as shown in the SEM pictures, were covered with surface precipitates that totally covered the underlying demineralized inter-tubular dentin and occluded most of the dentinal tubules. At the fractured surface of the demineralized samples, the peri-tubular dentin was completely dissolved and the collagen fibres in the inter-tubular dentin were totally demineralized (fig. 5.3).

After 1 week of remineralization, the SEM pictures of the surfaces of the control samples showed remineralization which increased the number of the occluded dentinal tubules (fig. 5.4). On the other hand, the appearance of the fractured surfaces did not changed in comparison to the fractured surfaces of the demineralized samples. The demineralized inter-tubular collagen fibres did not show remineralization throughout the lesion body. These demineralized fibres, as shown in the SEM pictures, were completely deprived from any extra-fibrillar hydroxyapatites.

After 1 week of remineralization, surface remineralization was observed at all the dentinal surfaces which were pre-treated with various proteolytic agents. The inter-tubular dentin in pepsin treated surfaces was remineralized slightly more than its control counterpart. More dentinal tubules were occluded in the experimental samples (fig. 5.5). NaOCl treated samples showed two patterns of results; where most of the lesion

was removed with the NaOCl application, the remainder of the lesion at the lesion front was remineralized (fig. 5.6,a.). The lesions, where NaOCl did not remove the demineralized collagen fibres, did not show surface remineralization (fig. 5.6,b.). Collagenase and trypsin 10 min treated samples showed surface remineralization comparable to their control groups' remineralization (fig. 5.7). Surface remineralization was dominant at the surfaces which were treated with trypsin for 24 h. Most of the dentinal tubules were occluded, and the inter-tubular dentin appearance was masked with the heavy remineralization (fig. 5.8). The lesion body of the samples, which were treated with collagenase for 24 h, was destroyed in almost all the samples during preparation.

Discussion

The results of this study clearly indicated that treating demineralized dentinal surface with proteases before remineralization would facilitate the remineralization process only to the extent they could remove the degraded organic matrix. Moreover, our results support previous studies that remineralization occurred by regrowth of residual crystals in the lesions, rather than by spontaneous precipitation or nucleation of mineral on the organic matrix.

Dentin caries involves demineralization as well as the proteolytic breakdown of organic matrix. Therefore, remineralization of dentine will be influenced by the remaining mineral, the remaining organic matrix and the dentinal ultrastructure (Arends et al., 1989).

Immersion samples which were demineralized with lactic acid (pH 4) in a remineralizing solution containing 5.0 ppm fluoride for one week did not show to affect the dentinal lesion depth nor the lesion body as shown by the PLM and FE-SEM pictures and as measured with the Vickers' indentor. In the present study no remineralization was observed on the

dentinal demineralized collagen fibres at the lesion front or in the lesion body even with high fluoride concentrations. These results were in contrast to our previous laboratory experiments which were performed with acetic acid (pH 5) to demineralize the samples. With acetic acid lesion depth decreased and lesion body remineralization occured after one week of remineralization with 5.0 ppm fluoride. Accordingly, the 5.0 ppm fluoride was chosen as an optimal fluoride concentration for effective remineralization of dentinal lesions to the innermost part. Those previous results were in agreement with other studies that concluded that the remineralization of the lesion front, body and surface of deep dentinal lesions can be possible even with very low mineral contents in their body (Mukai and ten Cate, 2002). It was suggested that the presence of high concentration of fluoride in the remineralizing solution could induce spontaneous mineral deposition on the exposed collagen matrix (Kawasaki et al., 2000) and could act as a nucleating agent at the lesion front to induce remineralization and decrease the lesion depth (Kawasaki et al., 1999). Accordingly, we speculated that the demineralizing regime and the original lesion mineral profile had influenced and controlled the mineral deposition within the lesion.

Based on the mentioned hypothesis the lactic acid which is stronger than acetic acid has more potential to dissolve hydroxyapatite crystals under the same temperature. The completely demineralized collagen fibres which were demineralized with lactic acid could not initiate remineralization while the acetic acid partially demineralized fibres demonstrated lesion remineralization. Our observations indicate and confirm the possibly different effect of acetic and lactic acids on collagen degradation and organic matrix breakdown (mentioned in chapter 3). These observations are in good agreement with previous studies stated that remineralization neither occurred by spontaneous precipitation nor by novo

nucleation of mineral on the organic matrix but by growth of residual crystals in the lesions (Levine and Roweles, 1973; Klont and ten Cate, 1991b; Kawasaki et al., 1999). Moreover, the demineralization process, the exposed collagen fibres, the release of the non-collagenous proteins and/or the activation of latent proteases during lesion formation may alter the dentin collagen in several ways (Klont and ten Cate, 1991a; Dung et al., 1994; Kleter et al., 1997, 1998), and mutilate the organic matrix so as it would not act as an effective template for crystal growth (Klont and ten Cate, 1991b).

We treated the demineralized dentin surfaces with either collagenase or NaOCl. Both agents were able to increase surface erosion depth due to their capabilities to digest the degraded organic matrix. Moreover, the microhardness tests and the SEM pictures demonstrated high values and lesion remineralization after both treatments, respectively. These results were expected due to the known potential of collagenase (Kleter et al., 1994, 1997; Kawasaki and Featherstone, 1997) and NaOCl (Hannig, 1999; Marshall et al., 2001; Tonami et al., 2003) to digest the demineralized collagen fibres. However, some of the NaOCl-treated surfaces demonstrated completely demineralized collagen fibres which were clearly exposed and did not show any changes after the remineralization period. These observations again support the proposed hypothesis that remineralization is formed due to regrowth of existing crystallites rather than de novo formation of crystallites. Therefore, we hypothesize that, the effect of the topically applied proteases on the remineralization process is related to their ability to remove the dentinal degraded organic matrix.

Remineralization of the 10 min trypsin-treated surfaces was comparable to the remineralization of the pepsin-treated surfaces for all the analytical methods. Remineralization after treating the demineralized surfaces with trypsin for 24 h resulted in the appearance of surface

remineralizing bands in the PLM. It was suggested that fluoride is responsible for the microscopic lamination phenomenon in dentin (Wefel et al., 1995; Nyvad et al., 1997). The appearance of these surface bandings in the microscope in the trypsin treated samples correlated well with a surface layer that occluded the dentinal tubules in the FE-SEM pictures. Therefore, we suggest that this surface layer could be a surface remineralization. The microhardness values of the remineralized trypsin-treated surfaces were high too. Therefore, according to these results related to the tryspin-treated surfaces, we can say that these surfaces probably showed surface remineralization, which could be, according to our interpretations, a reflection of the potential of the enzyme to digest the degraded organic matrix and thus expose the partially demineralized collagen fibres under the precipitates at the lesion surface to be remineralized.

Our observations regarding the increase of the surface erosion depth even after the removal of the samples from the demineralizing solution can be explained either by the possibility of the erosion progression even after the removal of the acidic attack (Kawasaki and Featherstone, 1997) or it could be due to shedding of the weak organic surface layer with time and manipulation. According to Kawasaki and Featherstone (1997), the surface erosion of a dentinal lesion does not occur by mineral dissolution but by the proteolytic attack on the organic matrix. Another suggestion is that the dentin collagenenous material is likely to denaturate during long-term acid exposure (Kuboki et al., 1983; Klont and ten Cate, 1991a; Kleter et al., 1998).

The high values measured with the Vickers' indentations near and at the lesion surface could be due to surface precipitations. These values correlated well with the surface remineralization which was observed in the SEM pictures of the control group. They can be explained by the presence of the partially demineralized hydroxyapatite crystals, which in turn, can

function as an initiator of mineralization. This explanation is based on the results of previous studies, in which we suggested that the surface layer precipitates at the lactic acid demineralized dentin surfaces is formed due to two steps. First the acid diffuses in an un-uniform pathway leaving remnants of hydroxyapatite at the surface together with calcium and phosphate reprecipitations (Moreno and Zahradnik, 1974; Featherstone et al., 1983; Fejerskov et al., 2003). Second the degraded denaturated collagen fibres together with the released NCCs remain in the demineralised tissue (Klont and ten Cate, 1990).

From a comparison of the hardness and the SEM results of the pepsin, and trypsin treated-groups, contradictory point emerged. The hardness data revealed high values throughout the lesion body while the SEM pictures showed the exposed collagen fibres to be highly demineralized. To understand and explain these results more studies need to be undertaken, but it could be argued that the mineral deposition within a lesion and its hardness measurements are not always well correlated with each other (ten Cate and Duijsters, 1982; Marshall et al., 2001). Moreover, the microhardness values are very proune to artefacts and errors depending on the sample preparation, the precision of the indenter loading and the precision of the determination of the points to be measured within the lesions.

In conclusion, within the limits of our in vitro studies, we suggest that enzymatic treatment during cavity preparation together with the application of fluoride, which was in our experiments optimum at 5.0 ppm, before the replacement of dental restoration might be of a significance benefit for remineralization in dentinal lesions. However, the proteolytic treatment would enhance remineralization of advanced dentinal lesions to the extent they could remove the degraded organic matrix from the demineralized surface. Nevertheless, the remineralizing property of a restorative material

is just a factor of many other vital factors such as the antibacterial, adhesive, sealing, mechanical and aesthetic properties that would determine the long term success of a restorative procedure. Therefore, more laboratory and clinical research is warranted before such a procedure can be introduced into the clinic. Moreover, the mode of application of fluoride in chapter 2 was fluoride ions in solutions and not fluoride incorporated in varnish, base or restorative material. Therefore, such results should be considered carefully when explored in clinical practice

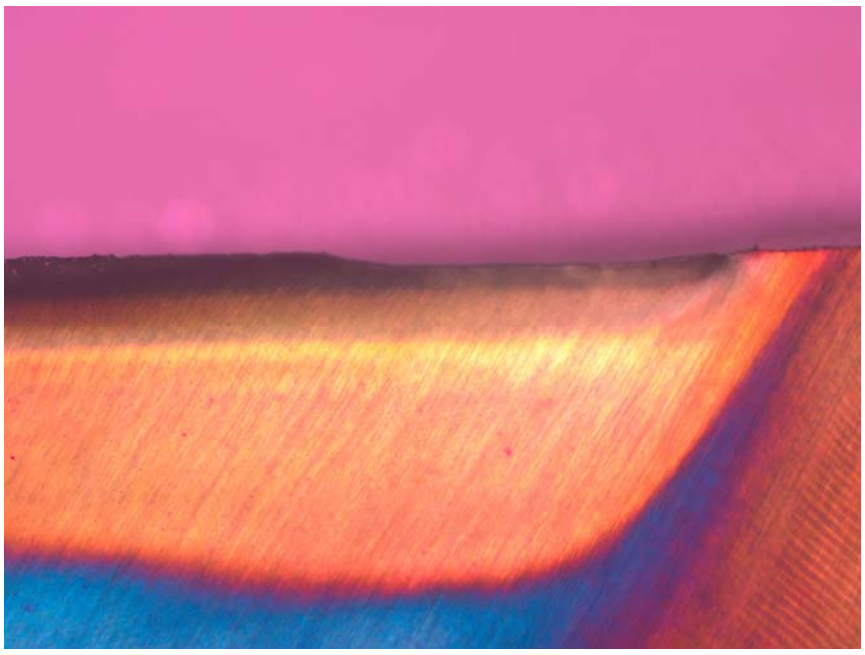
Table 5.1. Lesion depth before and after remineralization with and without various proteolytic treatments (Mean ± SD) as observed with the polarized light microscope. The lesion depth with and without the eroded surface together with the erosion depth were calculated for each sample as a mean of 3 measurements.

Treatment type and time.	Lesion depth with the eroded surface (μm)	Lesion depth without the eroded surface (μm)	The eroded surface (μm)
Lesion depth after 2 weeks of demineralization with lactic acid, pH 4	496.67 ± 80	451.15 ± 80	87.28 ± 30
Lesion depth after 1 week of remineralization without intermediate proteolytic treatment (controls)	502.81 ± 50	373. 67 ± 40	158.64 ± 30
Lesion depth after 10 min of pepsin treatment and 1 week of remineralization with 5.0 ppm of fluoride	527.19 ± 50	326.2 ± 40	177.89 ±20
Lesion depth after 10 min of trypsin treatment and 1 week of remineralization with 5.0 ppm of fluoride	515.1 ± 80	369.82 ± 30	135.28 ± 20
Lesion depth after 10 min of NaOCl treatment and 1 week of remineralization with 5.0 ppm of fluoride	499.88 ± 80	251.9 ± 50	239.98 ± 10
Lesion depth after 10 min of collagenase treatment and 1 week of remineralization with 5.0 ppm of fluoride	529.98 ± 80	381.13 ± 10	161.35 ± 25

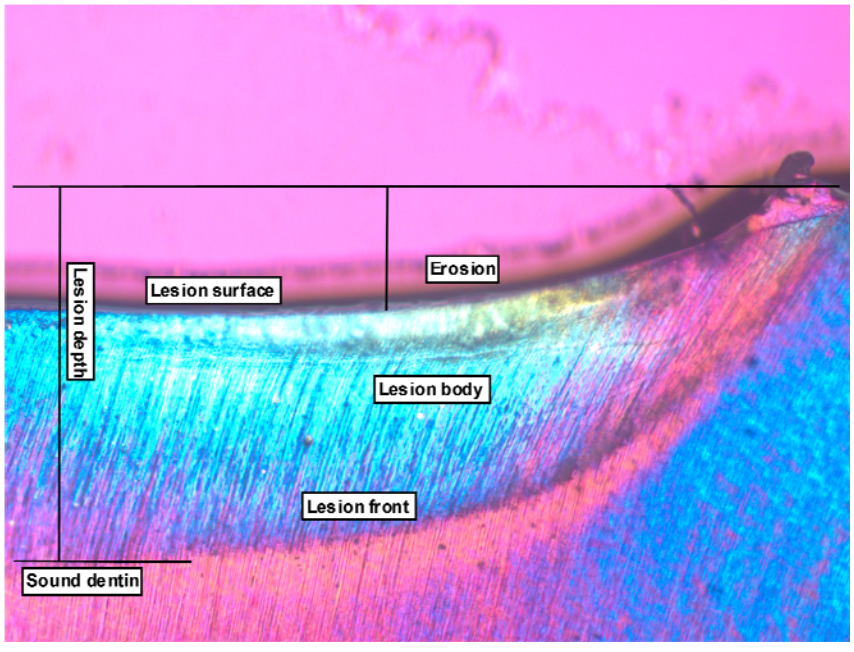
Lesion depth after 24 h of trypsin treatment and 1 week of remineralization with 5.0 ppm of fluoride	535.57 ± 80	365.34 ± 20	188.49 ±30
Lesion depth after 24 h of collagenase treatment and 1 week of remineralization with 5.0 ppm of fluoride	501.46 ± 80	237.97 ± 30	248.67 ±10

Fig 5.2. (a) Demineralized human coronal dentin after 2 weeks demineralization with lactic acid (pH 4) in the polarized light microscope (x10). (b) Sample from the control group after 1 week remineralization (x10). The method of lesion depth and surface erosion measuring is shown. (c) Sample from the trypsin treated group (24 hrs) after 1 week of remineralization with 5.0 ppm fluoride (x5). Note the remineralization band at the lesion surface (arrows).

(a)



(b)



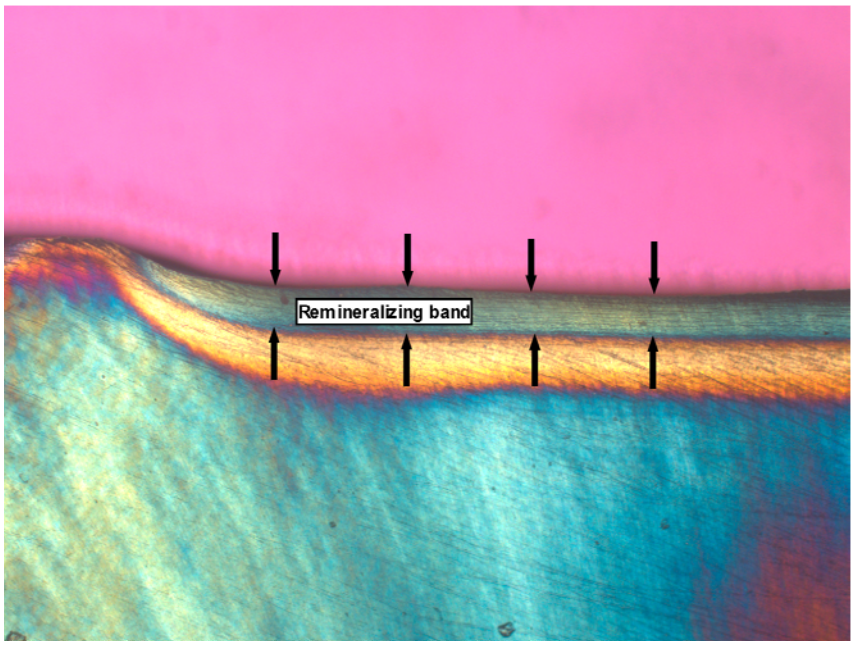
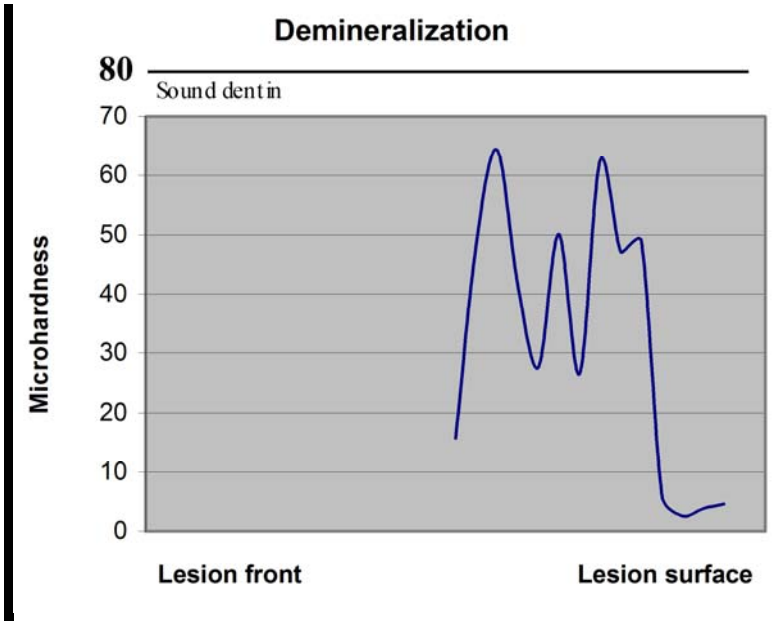
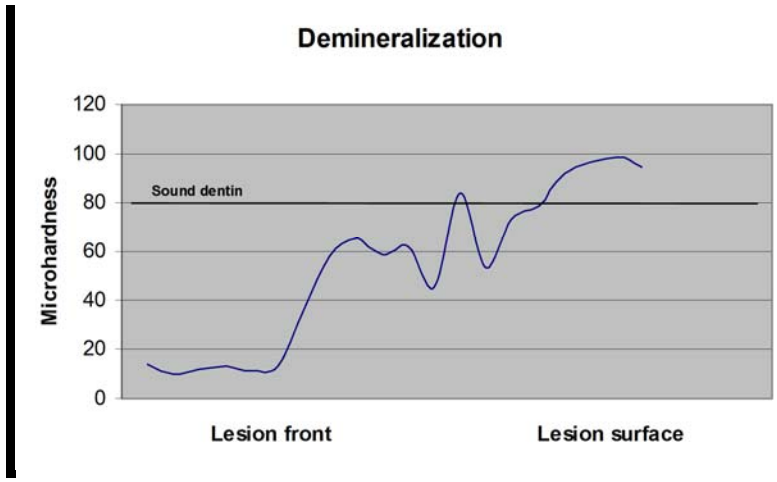
(c) 

Diagram 5.1. (a) and (b) Microhardness representative profiles for the demineralized group before remineralization. Note the two different patterns of values, in which (a) represents high values within the lesion body and (b) represents the high values near the lesion surface only. (c) and (d) represent the values measured with the Vickers indentations after 1 week remineralization. There was no significant difference between the measured values before and after remineralization.

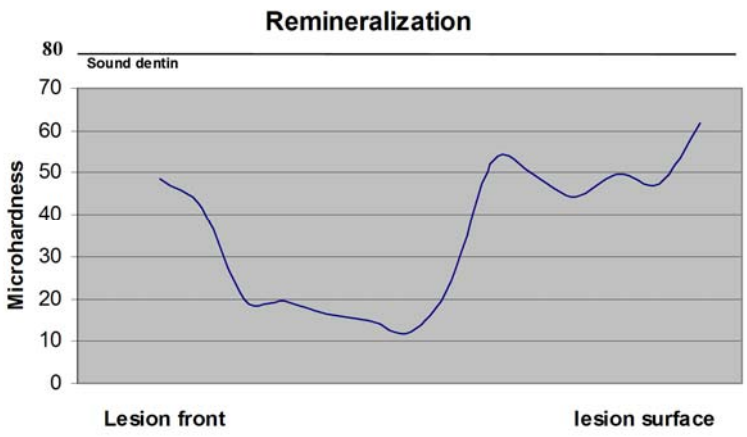
(a)



(b)



(c)


Remineralization
80
Sound dentin
70
60
50
40
30
20
10
0
Microhardness
Lesion front
lesion surface

(d)

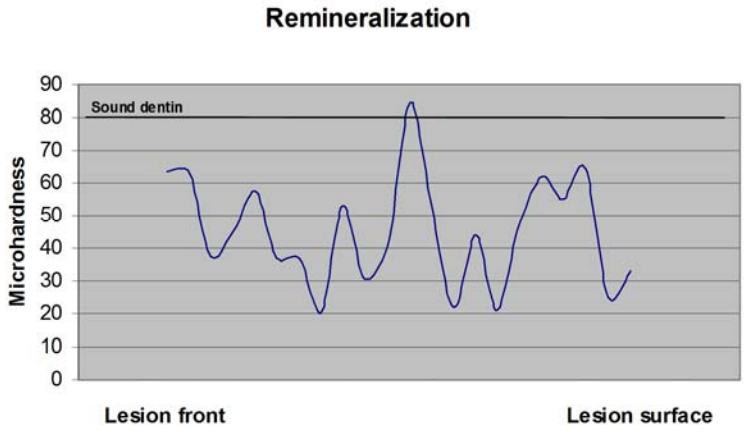
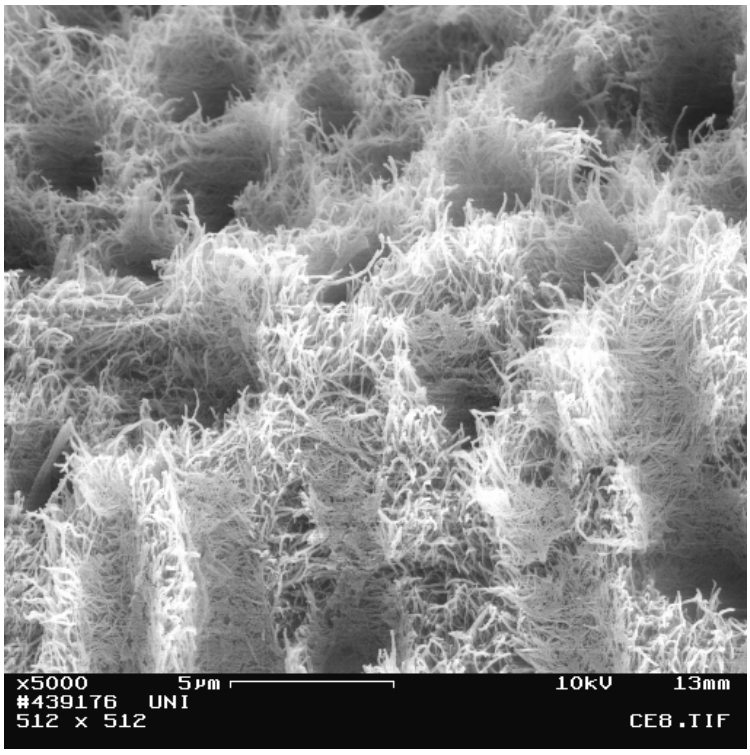

Remineralization
90
Sound dentin
80
70
60
50
40
30
20
10
0
Microhardness
Lesion front
Lesion surface

Table 5.2. The mean microhardness values measured with the Vickers indenter through out the lesion per group.

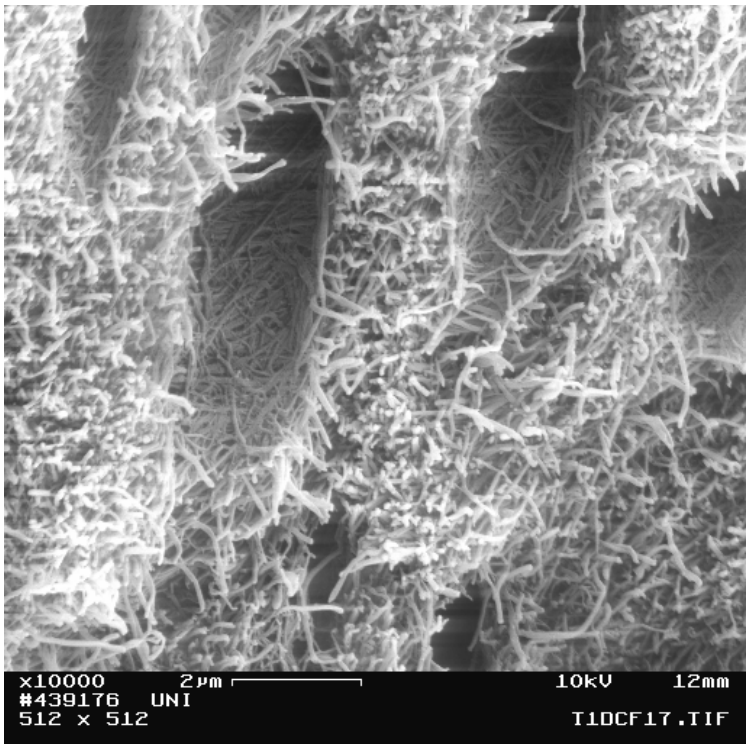
Treatment type and time	Values were measured from the lesion front to the lesion surface from the left to the right. Each value represents a mean of two consecutive values (points).										
Pepsin 10 min	55.53	33.49	46.82	29.03	59.88	35.04	89.81	61.48	55.30	58.44	45.67
Trypsin 10 min	80.74	55.49	32.89	79.82	37.05	62.91	24.37	64.59	87.36	85.30	93.35
NaOCl 10 min	62.21	36.95	82.83	45.76	48.49	28.01	26.37	71.36	50.63	83.09	58.22
Collagenase 10 min	51.10	35.00	11.77	42.72	30.55	48.64	23.90	13.19	11.21	7.59	22.43
Trypsin 24 hours	75.36	41.54	29.64	48.13	39.04	36.99	27.55	48.61	47.12	56.96	38.35
Collagenase 24 hours	74.42	52.21	41.41	55.7	42.48	35.11	51.27	42.60	55.00	42.67	72.53

Fig 5.3. (a) Human coronal dentin after two weeks demineralization with lactic acid. The peri-tubular dentin is dissolved and the inter-tubular dentin is totally demineralized (x5000). (b) The fractured surface of the demineralized samples (x10000). (c) All collagen fibres are totally demineralized (x30000).

(a)



(b)



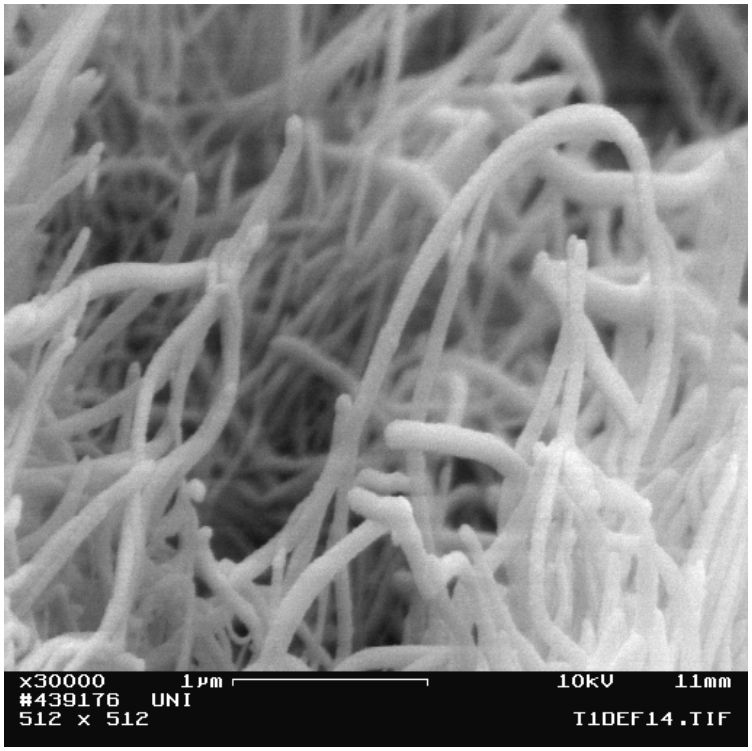
(c) 

Fig 5.4. The surface of a remineralized lesion from the control group. The inter-tubular dentin is remineralized and most of the dentinal tubules are occluded (x10000).

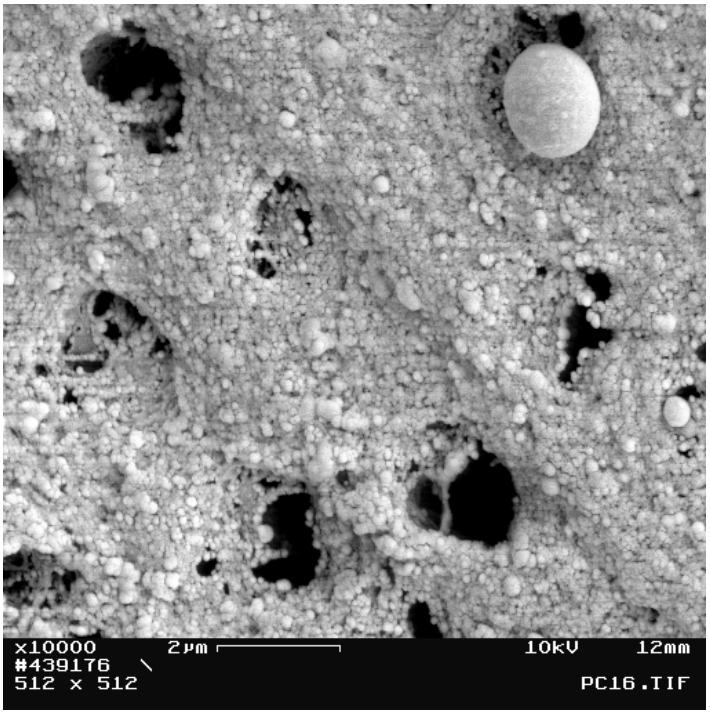
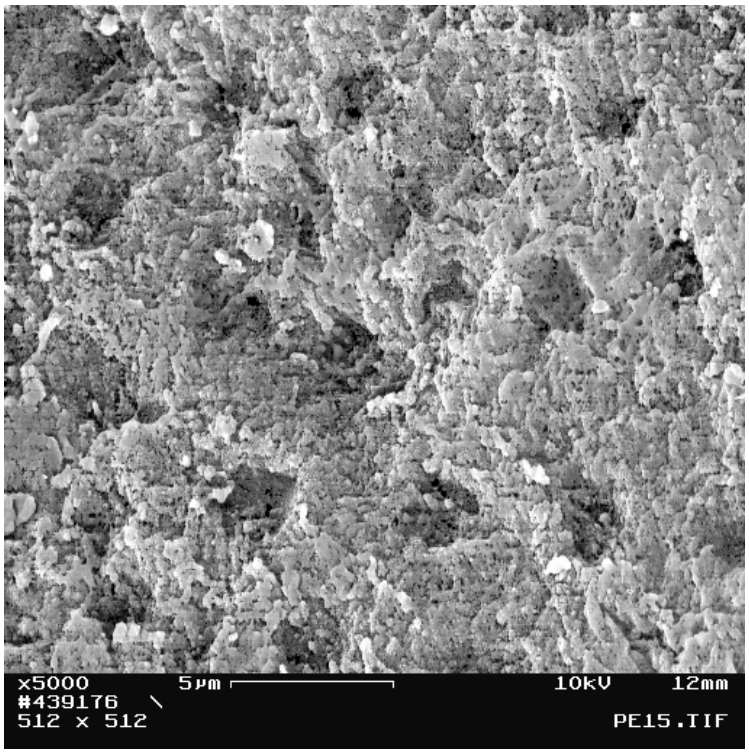


Fig 5.5. (a) and (b) Human coronal dentin was demineralized with lactic acid (pH 4) for 2 weeks. The surface was treated then with 1.5 mg pepsin in 1.5 ml of 0.01 M HCl Tris buffer, pH 2, at 37°C, with 150 rpm, for 10 min. Finally the sample was remineralized with 5.0ppm fluoride containing remineralizing solution for 1 week (x5000).

(a)



(b)

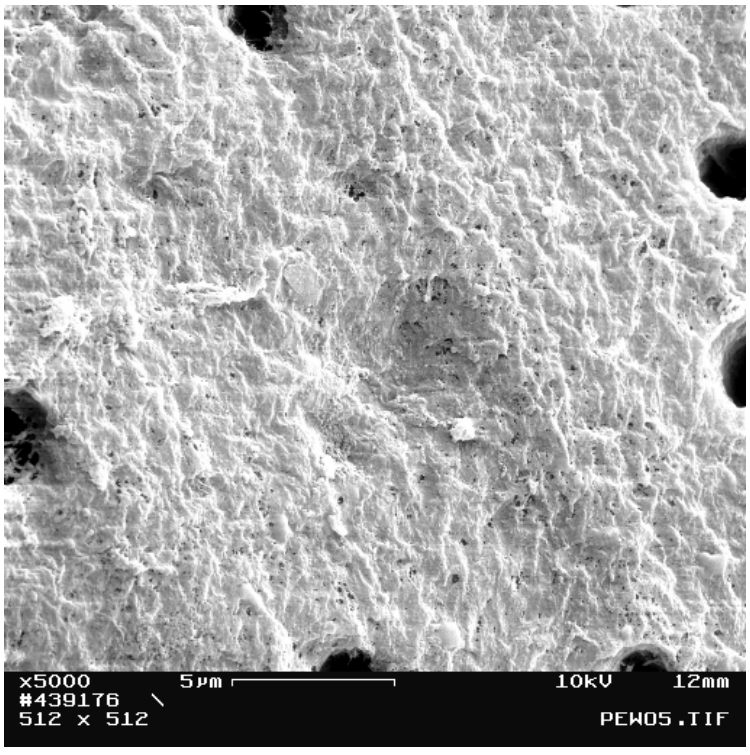
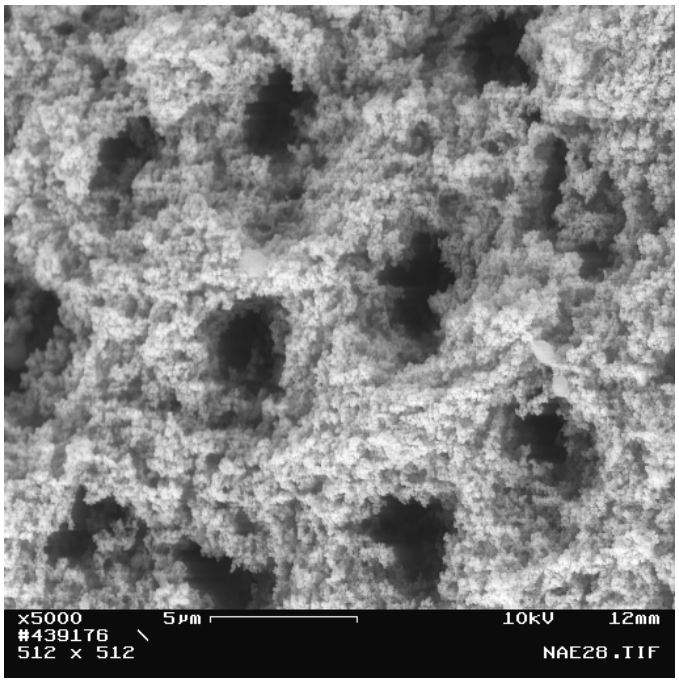


Fig 5.6. Human coronal dentin was demineralized with lactic acid (pH 4) for 2 weeks. The surface was treated then with 1.5 ml of 2.5% NaOCl, at 37°C, with 150 rpm, for 10 min. Finally the sample was remineralized with 5.0ppm fluoride containing remineralizing solution for 1 week. (a) Inter-tubular dentin at the bottom of the lesion (lesion front) was remineralized (x5000). (b) The demineralized collagen fibres after 1 week of remineralization (x3000).

(a)



(b)

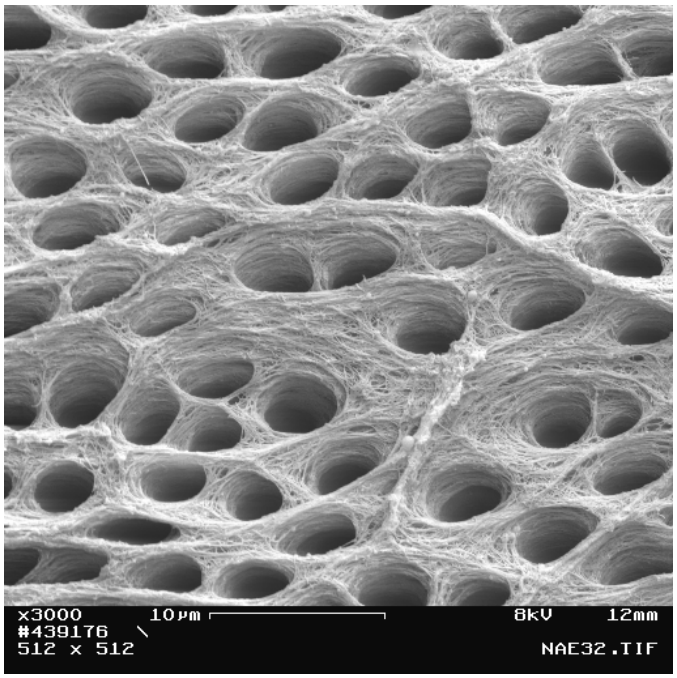


Fig 5.7. Human coronal dentin was demineralized with lactic acid (pH 4) for 2 weeks. The surface was treated then with 1.5 mg collagenase in 1.5 ml of 50 mM HEPES buffer, pH 7.8, at 37°C, with 150 rpm, for 10 min. Finally the sample was remineralized with 5.0ppm fluoride containing remineralizing solution for 1 week. The surface precipitates were removed and the inter-tubular dentin was partially remineralized (x10000).

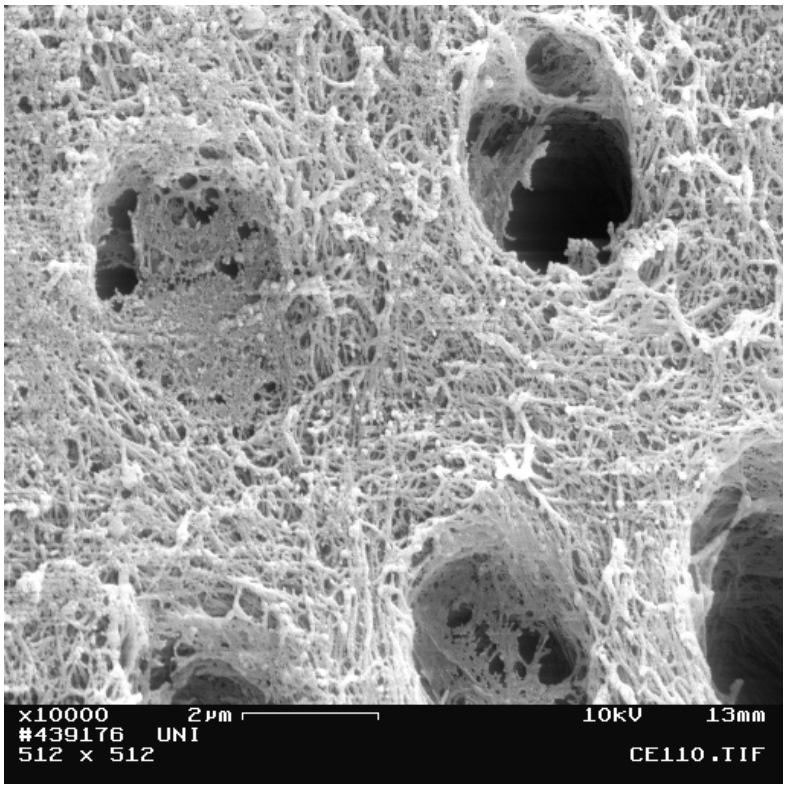
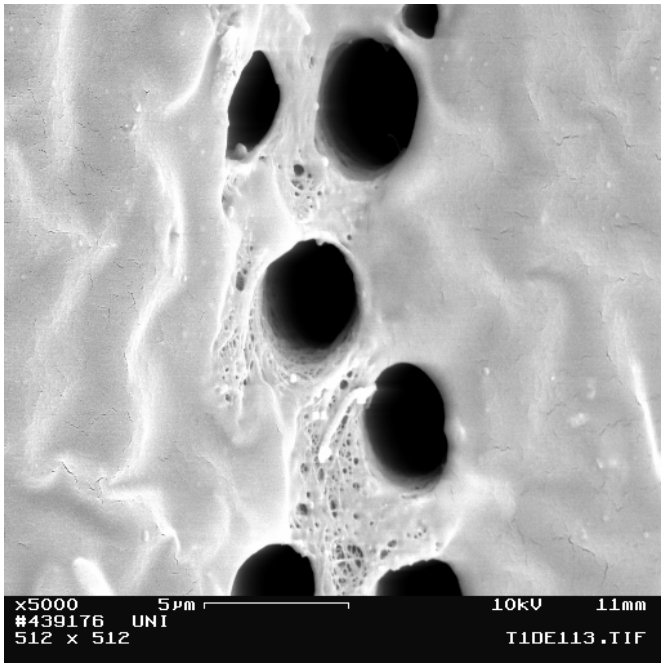
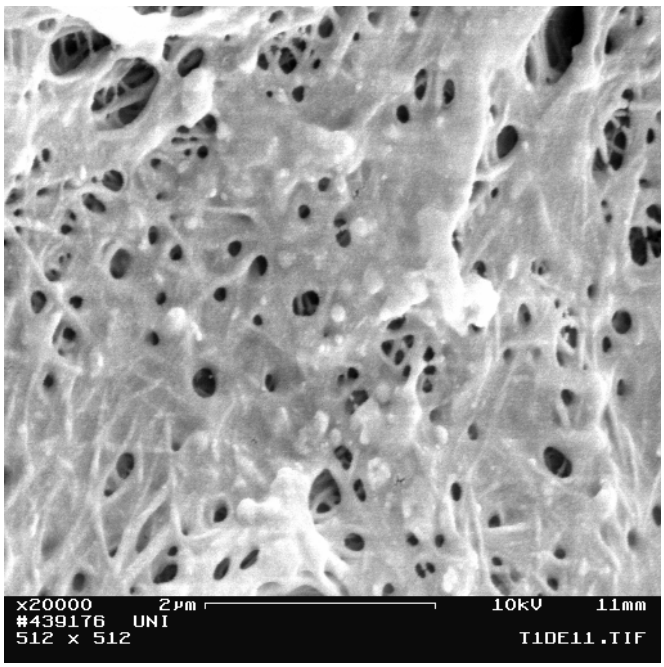


Fig 5.8. (a) Human coronal dentin was demineralized with lactic acid (pH 4) for 2 weeks. The surface was treated then with 1.5 mg trypsin in 1.5 ml of 50 mM HEPES buffer, pH 7.4, at 37°C, with 150 rpm, for 24 h. Finally the sample was remineralized with 5.0ppm fluoride containing remineralizing solution for 1 week. The inter-tubular dentin was covered with a remineralized layer which occluded most of the dentinal tubules (x5000). Higher magnification of the partially remineralized inter-tubular dentin (x20000).

(a)



(b)



Summary and Conclusions

Chapter 6

Summary

The series of studies described in this thesis were primarily designed to expose the complex environment of deep dentinal lesions. The therapeutic end point dilemma during deep dentinal lesions excavation, the high rate of restorative regimes failure, the technological advancement in dental materials and the need for more scientific information that could guide the clinician to optimize his restorative therapies outcomes when operating in carious dentin were the main ideas behind this work.

Chapter 1 reviews the multi factorial integration which contributes to the likelihood of dental restoration success from a biological prospective. Available in vivo, in situ and in vitro experiments and data were gathered in an attempt to investigate and understand reasons and differences between schools of opposing hypothesis in the area of dentinal caries excavation.

Chapter 2 describes in vitro experiments performed on artificial dentinal lesions to gain more information on the pattern of remineralization of demineralized dentin, the distribution of mineral ions in the presence of fluoride within the lesion body, the depth at which the lesion can still be remineralized and the influence of a surface mineralized layer on remineralizing the lesion body and/or front because such data are lacking in the literature. Various fluoride concentrations were used to clarify the optimal fluoride concentration which could be integrated in a restorative material, dentin bonding agent, liner or base to be used to restore such cavities.

In the course of our study, we needed to evaluate the effect of several proteolytic agents on the demineralized dentinal organic matrix. Natural caries is too variable for reproducible evalautions. Therefore, we developed an in vitro artificial dentinal caries model to be used for evaluating self-limited enzyme-based experiments. Chemically induced dentinal caries was produced with both acetic and lactic acid demineralizing solutions. We wanted to clarify the possible variable effects of both acids on organic matrix degradation and collagen denaturation.Thus, in chapter 3 we studied the morphological appearance of the lactic and acetic acids demineralized coronal dentin surfaces to gain more information and better understanding the effect of these acids on demineralizing and/or denaturating dentin collagen.

The concept of self-limiting caries therapy aims to keep the demineralized and to remove the denaturated dentin tissue. Therefore, the research in chapter 4 is targeted to investigate the individual capabilities of enzymes and proteases in removal of denaturated and/or demineralized dentin prior to restoration placement. Pepsin, which is a carboxylic protease, that acts only on denaturated collagen and trypsin, which is a serine protease, that is also able to digest denaturated collagen, but at a neutral pH 7.4, were used to digest the degraded organic material that was disintegrated during artificial dentinal lesion formation. Collagenase, which is a specific metalloproteinase, that is capable of hydrolyzing collagen at multiple cleavage sites and sodium hypochlorite, which is a non-specific proteolytic agent that was introduced as a chemomechanical method to remove carious dentin, were used as control groups. However, although the enzymes seem to be an exciting alternative method to mechanical excavation, mechanical removal of the superficial infected layer of dentin has to proceed or to be adjunct to the chemical digestion if time is to be considered in clinical practice.

In chapter 5 experiments were performed to study the capabilities of dentinal lesions to remineralize after the removal of the organic portion. The main purpose is to evaluate the post-operative effect of the treating proteases on the remaining dentin. Dentin remineralization after enzymatic treatment is investigated to evaluate this new minimally invasive caries removal method in which the superficial infected layer that is composed mainly of denaturated collagen is enzymatically digested, and the affected layer which contains demineralized dentin can be preserved to avoid pulpal exposure and to protect the pulp from further mechanical, chemical and thermal injuries. The interaction between this remaining layer of demineralized dentin and the restorative material that is placed to restore the tooth has an essential role in the long term restoration success. Remineralizing, antibacterial, adhesive and marginal adaptation properties of the restorative material are important factors in determining the longevity of the restoration and the healing potential of the pulp.

From the perspective of minimally invasive dentistry, treating deep carious lesion with a suitable enzyme preparation may represent an interesting alternative method for mechanical caries excavation. The bulk of the infected superficial layer can be removed with a hand excavator which is more self-limiting than a bur due to the sensitive tactile feedback. The lesion is treated then with an enzymatic solution or gel for couple of minutes. A soft brush or a plastic tip instrument can be used to insure the removal of any incomplete digested organic material.

On the other hand, more laboratory and clinical research is needed before such a procedure can be introduced into the clinic.

Limitations and Future Perspectives

Although our results concerning treatment of dentinal lesion with the enzyme pepsin as a self-limiting caries therapy are promising, these results have to be considered carefully when explored in clinical practice.

- The oral environment, the nature of the disease whether it is acute or chronic, active or arrested, the oral hygiene, dietary habits, general physical and mental health and other patient related factors have to be assessed individually for each patient.
- Our experiments were conducted in vitro on extracted teeth, and we already mentioned in chapter 3 the limitations of such non-vital teeth and described the differences between in vivo vital and in vitro non-vital teeth.
- Although the chemical systems we used in these studies were able to produce artificial carious lesions which are very similar to natural caries, the role of the physical bacterial presence, bacterial byproducts and proteases, carbohydrates and the associated interaction between the dietary sugars and dentin proteins (Millard reaction) could not be elucidated within the limitations of our study.

Further research is needed to answer some critical questions such as the strength of a bond between the remaining demineralized layer and a bonding agent or a restorative material, the quality of the hybrid layer formed and the efficiency of the infiltration in the remaining dentin layer. Remineralizing property of a restorative material is just a factor of many other vital factors such as the antibacterial, adhesive, sealing, mechanical and aesthetic properties that would determine the long term success of a restorative procedure.

Conclusions

In this study we evaluated the effeciency of the self-limiting caries therapy, and we investigated it together with the remineralization phenomenon, and we concluded the following:

1. There is a proportional relationship between fluoride concentration and dentinal lesion remineralization from 0.1-10.0 ppm.
2. Fluoride concentration determines the rate as well as the pattern of mineral deposition in dentinal lesion.
3. The formation of a well-remineralized surface layer inhibited remineralization at the lesion front. On the other hand the lesion front remineralization was found to be independent from fluoride concentration, most probably due to its limited remineralization capacity.
4. For effective remineralization of dentinal lesions to the innermost part, fluoride levels from 1.0-5.0 ppm have the highest efficiency.
5. Our model minimizes the biological variations between the dentin samples through the very close location between the compared treated slices and thus, enabled us to compare effectively between different variables.
6. Our model produced both denaturated and demineralized dentin only when lactic acid was used. Therefore lactic acid is to be used for evaluating self-limited enzyme-based experiments.
7. The type of acid and the demineralization time affect the ratio of denaturated/demineralized dentin and the pre-treatment and treatment type and time influenced the quality and quantity of the digested substrate.
8. The effectiveness of the enzymes and agents used in the present study were in the following order Trypsin < Pepsin < Collagenase <NaOCl. The enzyme pepsin, with its acidic pH optimum, was more aggressive in

removing the disintegrated dentinal organic matrix than the neutral enzyme trypsin. We hypothesize that although both enzymes behaved very similarly in digesting the denaturated collagen, the acidic environment of the pepsin with pH 2 was responsible for the surface etching observed with pepsin. We believe that the pepsin acidic medium is advantageous, in which the enzyme can be inactivated by washing it and thus neutralizing its pH.

9. Based on the working principle of the treating solutions we observed that artificial dentinal caries in the presence of lactic acid is of four successive layers in respect to collagen fibres; denaturated collagen fibres, all fibers totally demineralized, individually mineralized fibres and partially demineralized fibres.

10. Short application of the enzyme pepsin is capable of removing just the denaturated collagen. Pepsin proved to be more efficient than trypsin and collagenase and less aggressive than NaOCl at digesting denaturated dentin collagen fibers.

11. Remineralization would not occur by nucleation of mineral on the organic matrix but rather by growth of residual crystals in the partially demineralized collagen fibres.

12. When proteases are applied topically at the surface of demineralized dentin they affect the remineralization process only to the extent they can remove the dentinal demineralized and denaturated organic matrix.

13. We suggest that enzymatic treatment during cavity preparation and application of fluoride before the replacement of dental restoration might be of significance benefit for remineralization in dentinal lesions. In our study a concentration of 5.0 ppm was successfully used to validate this hypothesis.

14. This was the first time to obtain morphological images that show clearly the artificial dentinal caries with the demineralized/denaturated collagen fibres. From our SEM pictures we gained a closer view to the dentin caries process. This morphological information could be utilized as a reference for future work.

Appendix

Image 1.

Transparent cold-curing methylmethacrylate (Technovit 4004, Kulzer GmbH, Wehrheim, Germany).

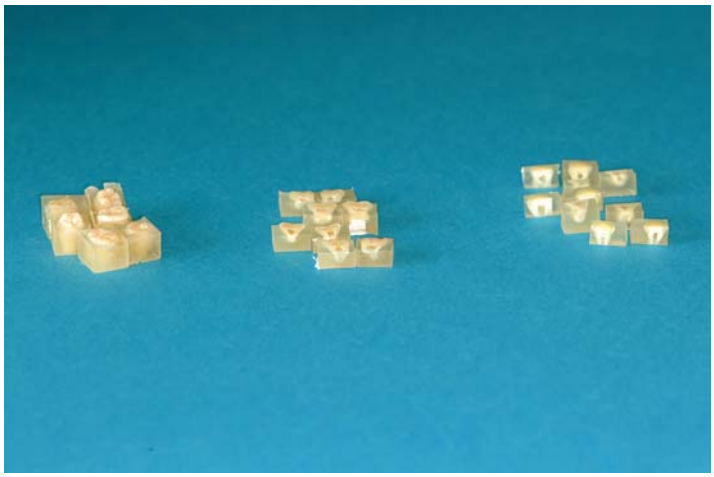


Image 2a

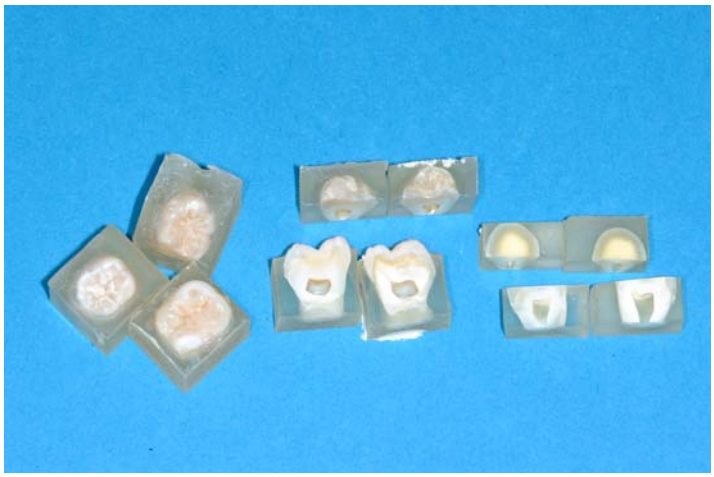


Image 2b.

Image 2c.

Images 2 (a, b, c): Sample preparation:

Caries-free human molar teeth were embeded individually in methylmethacrylate.

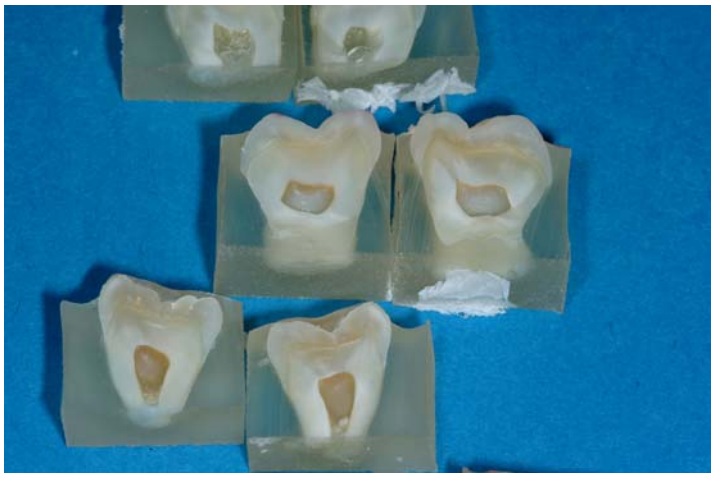


Image 3.

Each tooth was divided parallel to its long axis into two halves.

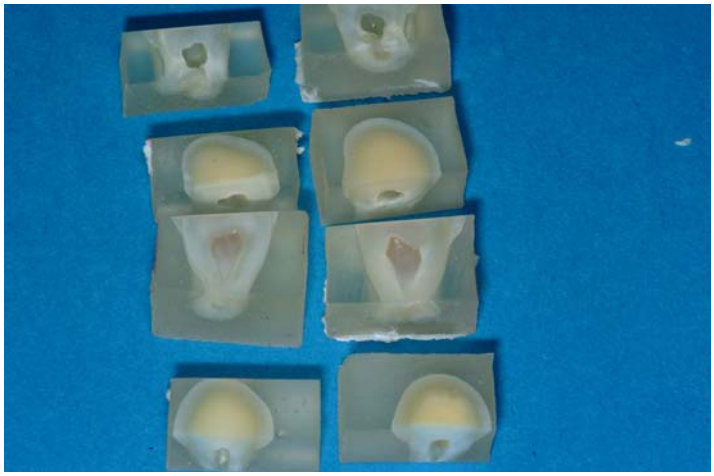


Image 4a.

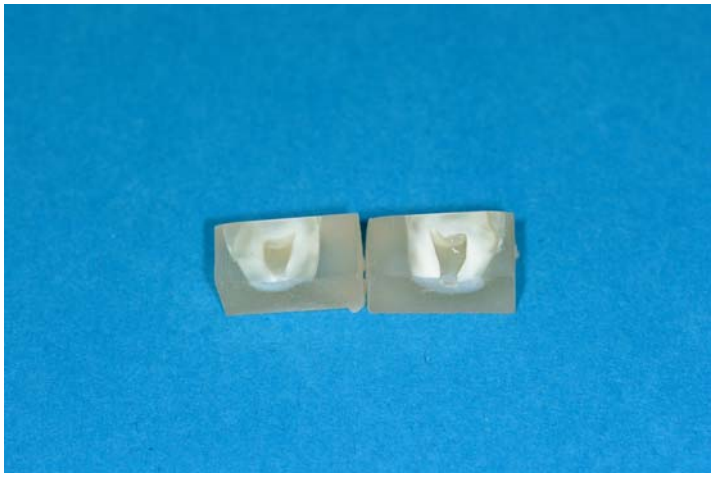
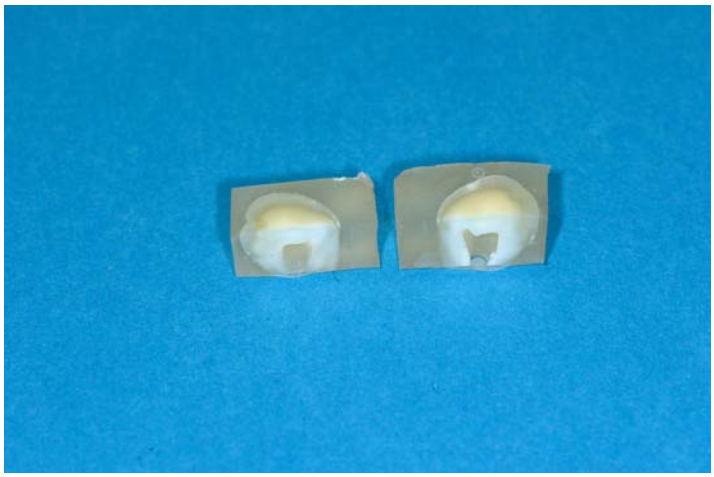


Image 4b.



Images 4c.

Images 4 (a, b, c): To expose mid-coronal dentin occlusal part was removed.

Image 5a.

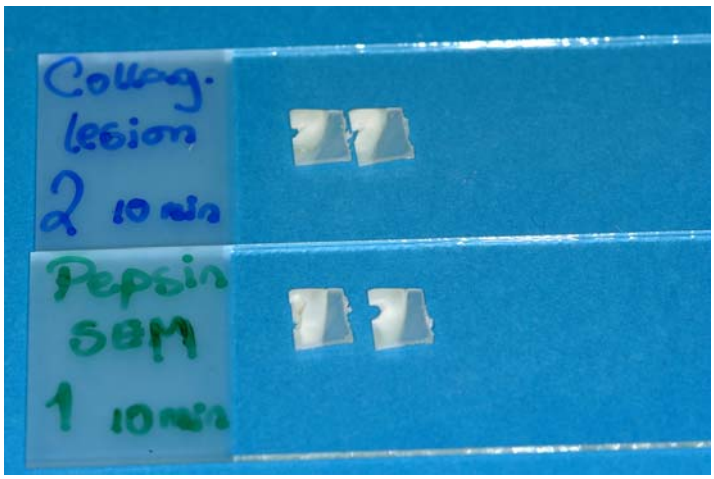


Image 5b.

Images 5 (a, b): One slice out of every two slices was treated with a specific enzyme and the other served as a control.

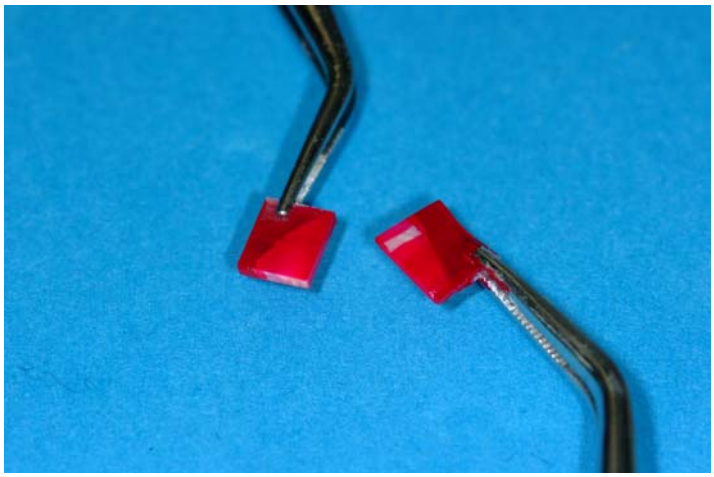


Image 6a.

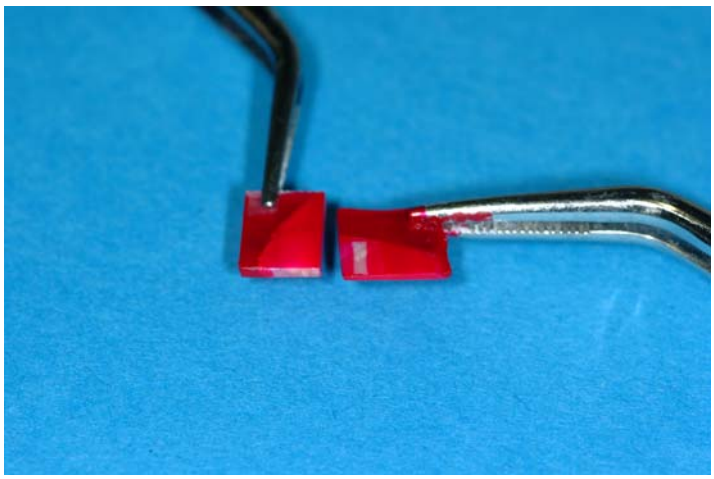


Image 6b.

Images 6 (a, b): Dentin surface together with the surounding Technovit were coated with nail varnish. Windows were made either perrpendicular at the dentinal tubules or parallel to the tubules.

Image 7a.

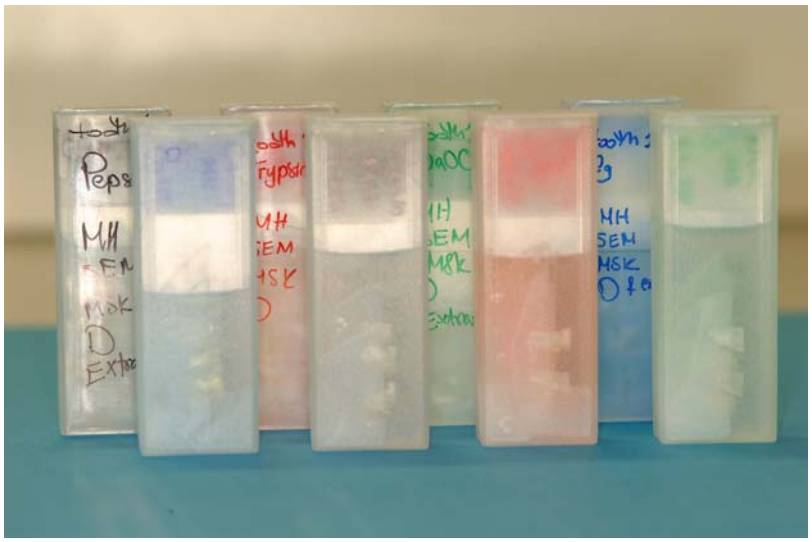


Image 7b.

Images 7 (a, b): Teeth were kept wet throughout the experimental procedures.

Image 8.

Slow speed water-cooled diamond saw (Isomet, Beuhler, Illinois, USA). To expose mid-coronal dentin each tooth was sectioned parallel to its long axis into two halves, the occlusal part of each half was then removed by a cut perpendicular to the long axis of the tooth and 1.5-2.0 mm away from the pulp.

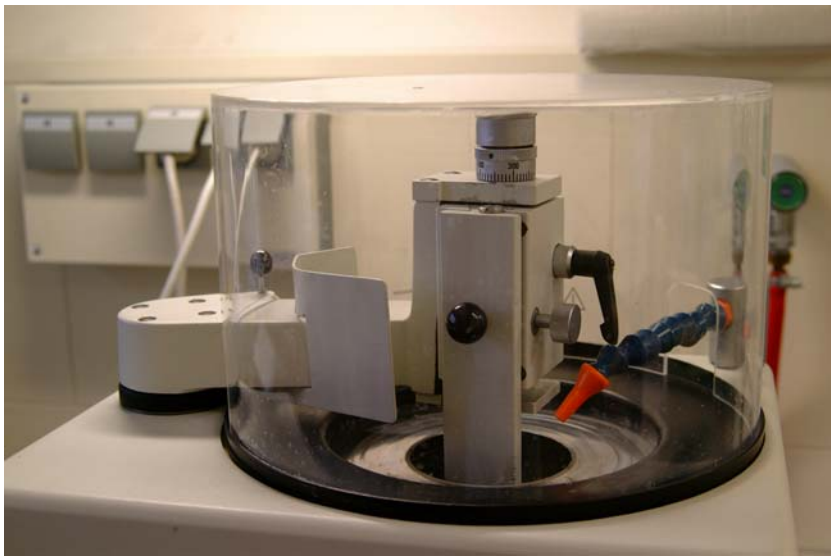


Image 9.

Saw microtome (Leica SP 1600, GmbH, Nußloch, Germany). Each tooth half was divided into four slices parallel to the long axis of the tooth, extending from the middle of the tooth to the outer enamel.

Image 10.

Polishing machine (Leco VP 100, GmbH, Neuss, Germany). The dentin exposed surfaces were polished flat with water proof silicon carbide abrasive paper (P500-grit).

Image 11.

Light Microscope (Axioskope 2, MAT, Carl Zeiss Jena GmbH, Göttingen Germany). Digital images were taken with the image analysis software Axiovision (Rel. 4.4, SP2, Carl Zeiss Jena GmbH, Göttngen, Germany), for the visual qualitative analysis of the lesions before and after remineralization (chapters 2 and 5).

Image 12.

Automatic microhardness tester Fischerscope H100C (Helmut Fischer GmbH, Sindelfingen, Germany). It was used to test the cross surface microhardness (CSMH) of the artificial demineralized dentin before and after remineralization (chapters 2 and 5).

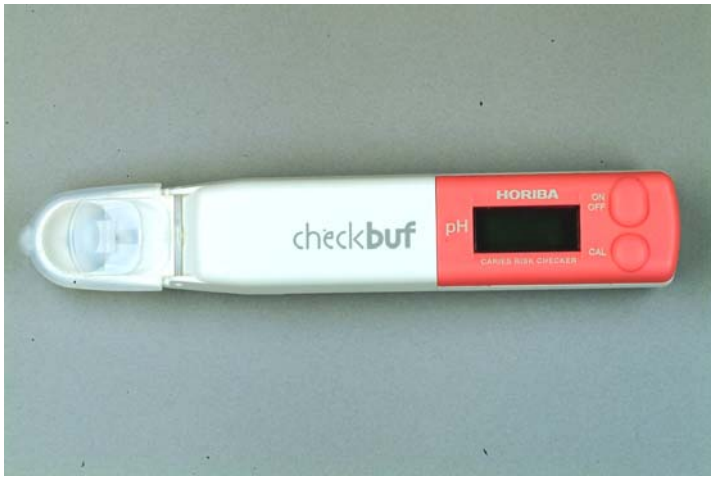


Image 13a.

Image 13b.

Images 13 (a, b): pH meter for caries risk assessment (Checkbuf, Horiba, Tokyo, Japan). It was used to measure the pH of the demineralizing solutions throughout the demineralizing period.

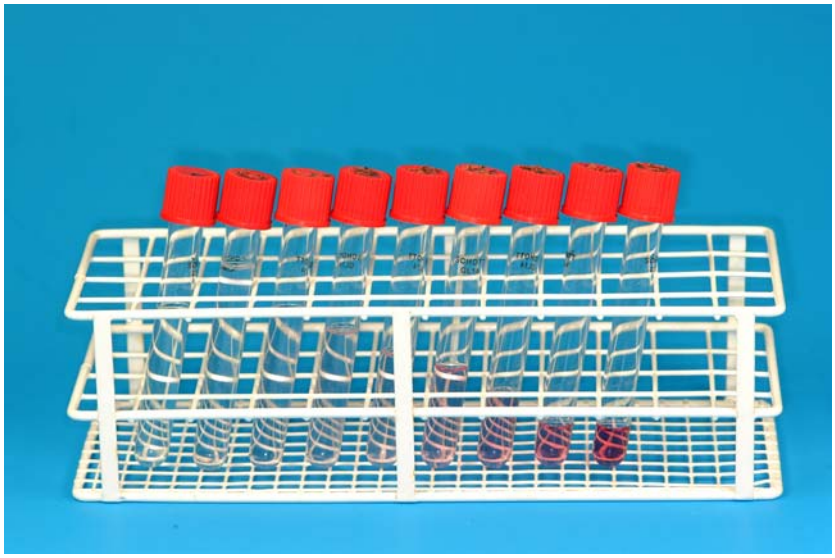


Image 14.

Standard hydroxyproline solutions starting from 1000µg/ml down to 25µg/ml were used to draw a working curve against absorbance on a spectrophotometer.

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Arends J, Christoffersen J, Buskes JAKM, Ruben J: Effects of Fluoride and Methanhydroxydiphosphate on Enamel and Dentine Demineralization. Caries Res 1992;26:409-417.

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