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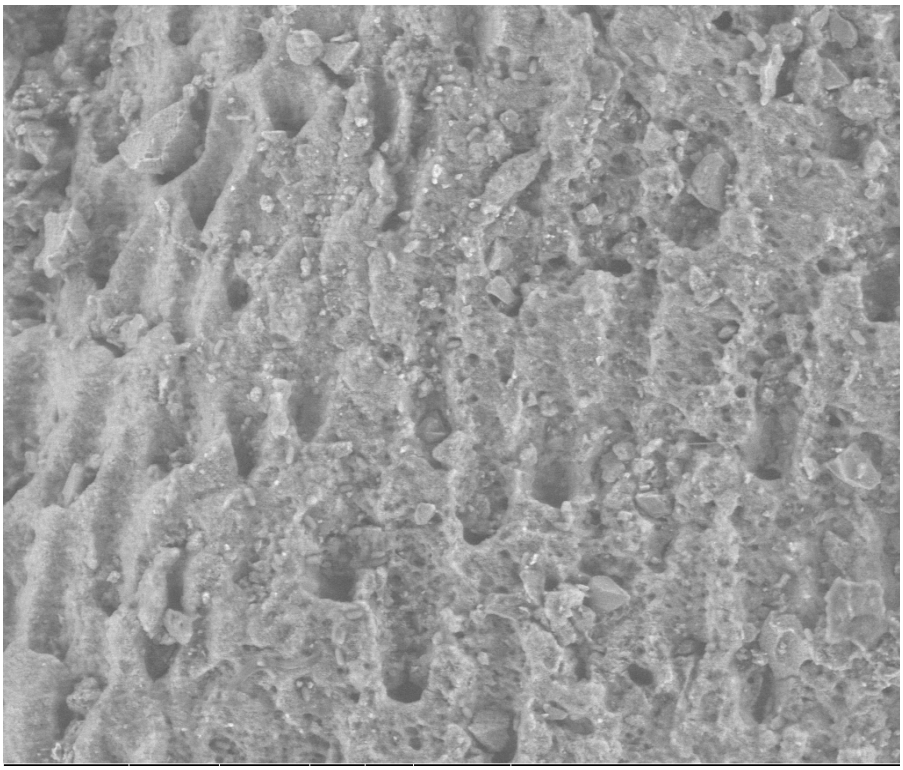
Dentin Remineralization Around Ceramir Restoration



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Introduction

Overview:

Teeth are the most heavily mineralized tissues in the human body. Demineralization and remineralization processes coexist in teeth during the entire life of an individual. In pathological conditions, demineralization outweighs remineralization [1]. Fermentation of dietary carbohydrates by acidogenic bacteria results in the production of acids such as lactic acid, acetic acid and propionic acid that demineralize enamel and dentin. As the carious lesion progresses into dentin, activation of endogenous, bound matrix metalloroteinases and cysteine cathepsins will lead to the degradation of collagen fibrils and decrease in the mechanical properties of dentin [2,3]

In the last decade, the focus of caries research has shifted from only restoring missing tooth structure to the development of methodologies for the detection of early caries lesions and the non-invasive management of caries lesions through remineralization to preserve tooth structure.[4] Fluoride is generally known to promote remineralization, but its remineralization process relies on calcium and phosphate ions from saliva. Therefore, several new remineralizing agents have been introduced. These agents supplement and enhance the ability of fluoride to restore tooth minerals by increasing availability of these ions.[5,6] .

Fluoride is the cornerstone of the non-invasive management of non-cavitated caries lesions, but its ability to promote net remineralization is limited by the availability of calcium and phosphate ions (Reynolds *et al.,* 2008)[7]. Fluoride ions can drive the remineralization of extant non-cavitated caries lesions if adequate salivary or plaque calcium and phosphate ions are available when the fluoride is applied. For fluorapatite or fluorhydroxyapatite to form, calcium and phosphate ions are required, as well as fluoride ions. Several authors have now shown that enamel remineralization *in situ* and the retention of fluoride in plaque are dependent on the availability of calcium ions (Chow *et al.,* 2000; Whitford *et al.,* 2005; Reynolds *et al.,* 2008; Vogel *et al.,* 2008)[8,9,10].

Hence, on topical application of fluoride ions, the availability of calcium and phosphate ions can be the limiting factor for fluoride retention and net enamel remineralization. Under hyposalivation conditions the lack of available calcium and phosphate ions is highly exacerbated (Reynolds *et al.,* 2008)[7]. When adequate levels of calcium and phosphate ions are present together with the fluoride ions, it has been shown *in vitro* that this combination can produce substantial remineralization of lesions of enamel and even those penetrating the underlying dentin in pH-cycling experiments (ten Cate, 2001; ten Cate *et al.,* 2008)[11,12]. Therefore, the challenge now is to achieve this clinically, since salivary remineralization of enamel promoted by topical fluoride (particularly high concentrations) has been shown to give rise to predominantly surface remineralization (Arends and Ten Cate, 1981; ten Cate *et al.,* 1981; Ögaard *et al.,* 1988; Willmot, 2004).[13,14,15,16] Surface-only remineralization does little to improve the aesthetics and structural properties of the deeper lesion. Ideally, a remineralization system should supply stabilized bioavailable calcium, phosphate, and fluoride ions that favor subsurface mineral gain rather than deposition only in the surface layer. [17]

In recent years, biomimetic treatment of early caries lesions by the application of various types of nano-sized hydroxyapatite or calcium carbonate has received considerable attention (Huang S *et al.,* 2009, 2010, 2011; Nakashima *et al.,* 2009). [18,19,20,21] An experimental dentifrice containing 1% nano-sized amorphous calcium carbonate particles (several tens to hundreds of nm), applied twice a day over 20 days, yielded statistically significant mineral gain and remineralization of artificial caries lesions in an *in vitro* system that used collagen-coated wells as a model for oral mucosal surfaces (Nakashima *et al.,* 2009) [21]. The authors concluded that the experimental dentifrice has the potential to remineralize incipient enamel lesions due to the unique properties of the nano-sized calcium carbonate, which had been retained on the collagen-coated surfaces in the *in vitro* model system and thus might also be retained on oral surfaces, thereafter releasing Ca ions into oral fluids for remineralization (Nakashima *et al.,* 2009).[21]

Caries-Preventive Nano fillers

Several studies have indicated that nano- apatite, in principle, has the potential to remineralize, at least in part, initial enamel caries lesions under dynamic pH-cycling conditions *in vitro* (Huang S *et al.,* 2009, 2010, 2011).[18,19,20,20] A 10% suspension of nano-hydroxyapatite particles (10-20 nm diameter, 60-80 nm length) promotes preferential remineralization of the superficial layer of artificial caries lesions, and thus might be effective in reversing lesion progression in the outer surface layer of initial caries lesions measuring 20 to 40 μm (Huang S *et al.,* 2010).[19] However, little remineralization could be obtained by nano-hydroxyapatite in the body of the lesion (Huang S *et al.,* 2010, 2011).[19.20] Interestingly, hydroxyapatite nanoparticles promote remineralizing effects under *in vitro* conditions, in contrast to a control solution containing an equivalent concentration of free ions as provided by the nano-HA solution at equilibrium (Huang *et al.,* 2011).[20] These observations suggest that intrinsic characteristics of the nano-HA, such as size and structure or chemical composition, may be of considerable relevance for the remineralization process (Huang *et al.,* 2011).[20]Apparently, not only the size of the apatite nanoparticles used for remineralization purpose but also the pH of the remineralizing agent will affect the process of mineralization (Huang *et al.,* 2011). More mineral was deposited in the body part of the lesion if the pH-value was reduced from 7.0 to 4.0 (Huang *et al.,* 2011).[20]

Recurrent Caries

Secondary caries and restoration fracture are still the main reasons for dental restoration failure, thus limiting the longevity of (resin composite) restorations. Recurrent caries around composites is strongly linked to leakage through marginal gaps caused by polymerization shrinkage.[22] To control caries-induced demineralization at the resin composite-tooth interface, calcium and phosphate ion-releasing nanofillers have been developed, such as nanoparticles of dicalcium phosphate anhydrous (112 nm in

size) or of amorphous calcium phosphate (116 nm in size) (Xu *et al*., 2007a,b, 2010b, 2011; Moreau *et al.,* 2011).[23,24,25,26] These additives enable the resin composite to release calcium and phosphate when the pH is dropped down under *in vitro* conditions, providing caries-inhibiting properties (Xu *et al.,* 2007a,b, 2010b, 2011; Chen, 2010).[23,24,25,27]Nanocomposites containing 40% nanoparticles of amorphous calcium carbonate have been shown to rapidly neutralize a lactic acid solution of pH 4.0 by increasing the pH to 5.69 within 10 min (Moreau *et al.,* 2011).[26] The mechanical properties of the calcium- and phosphate-releasing experimental composites match those of commercial hybrid composites (Chen, 2010; Moreau *et al.,* 2011; Xu *et al.,* 2011). [25,26]

Most recent developments are novel nanocomposites which contain antibacterial agents, such as chlorhexidine (10%) and quaternary ammonium dimethacrylate (7%) alone or in combination with silver nanoparticles (0.028%), in addition to calcium and phosphate ion-releasing nanofillers (Cheng *et al.,* 2012a,b,c).[28,29,30] Incorporation of these antibacterial components into nanocomposites has been shown to yield antibacterial capabilities, thereby reducing the biofilm colony-forming unit counts, the metabolic activity, and lactic acid production of *Streptococcus mutans* biofilms under *in vitro* conditions (Cheng *et al.,* 2012a,b,c).[28,29,30] In the presence of marginal gaps caused by polymerization shrinkage.However, the effectiveness of all these strategies for the control of demineralization processes still needs validation, on the one hand, by *in vitro* studies focusing on the caries-inhibiting potential of ion-releasing and antibacterial resin composites, as well as by subsequent clinical studies, on the other. [31]

Enamel Anatomy and Remineralization

Dental Enamel is the outermost covering of teeth. It is the hardest mineralized tissue present in the human body. Enamel faces the challenge of maintaining its integrity through periods of demineralization and remineralization within the oral environment and it is vulnerable to wear, damage, and caries. Enamel is composed of crystalline calcium phosphate of 96% mineral with the remaining 4% consisting of organic

components and water. The organic content consists of breakdown products of major enamel protein amelogenin [32]. The main component of enamel includes rods, which are bundles of aligned crystallites that are woven into intricate architecture that are 3-5 μm in diameter [32]. The second component of the enamel matrix is inter-rod enamel which surrounds and packs between the rods [33]. The third structure, aprismatic enamel, refers to the structures containing HA crystals that show no mesoscale or macroscale alignment.

The mature enamel is acellular and does not regenerate itself unlike other biomineralized tissues such as bone and dentin [34]. To replace enamel that is damaged by dental caries, dentistry has formulated artificial replacement materials that mimic the hardness of enamel [35]. But none of these materials could mimic all the physical, mechanical, and aesthetic properties of enamel [36]. Recently scientists have shown much interest in the direction of synthesizing artificial enamel [34]. Thorough understanding of structure and pattern of ameloblast gene products, control of protein self-assembly and simultaneous hydroxyapatite crystallization allows one to design biomimetic approaches to create synthetic enamel [34]. There is now a transition of emphasis from traditional synthetic biomaterials toward biological materials [37]. Advances in tissue engineering methods paves a way for enamel regeneration.

Based on the understanding of biological process involved in amelogenesis and advances in nanotechnology, Chen et al., fabricated fluoapatite nanorods, which resembles enamel prism like structures from a supersaturated chemical solution under physiological condition. These nanorods have similar characteristics to those of natural enamel crystals isolated from rat incisor enamel [38]. Yin et al., regenerated enamel like microstructures using a simple chemical approach, which may have a potential clinical application to repair enamel damage in dental clinics [39]. Zhang et al., have achieved an ordered dental enamel-like structure of hydroxyapatite (HAP) through a solution mediated solid-state conversion process with organic phosphate surfactant and gelatin as the mediating agent [40].

Stephen Mann and colleagues prepared electrospun hydrogel mats of amorphous calcium phosphate and polymer nano and micro fibres. Mats generated HAP crystals as

an immediate layer, which covers the enamel surface. Hence, it could be used for re-growing enamel surfaces that have been lost due to erosion/or wear [41]. Ying et al., used an agarose hydrogel method, which mimics the natural enamel at secretory or matrix formation stage. This biomimetic mineralization model regenerates enamel like prismatic structure with hardness similar to natural enamel [42].

Hontsu et al., successfully fabricated a freestanding flexible HAP sheet, which was directly attached to enamel surface of extracted teeth using a calcium phosphate solution. The interface between sheet and surface was not completely adhered. [43] To improve the adhesiveness HAP sheet coated with a tricalcium phosphate layer. The adhesive strength of the HAP/TCP sheet was markedly higher than that of the HAP sheet that indicates sheet may be used for restoration [44,45].

Dentin Anatomy and Remineralization

Dentin is a complex mineralized tissue arranged in an elaborate 3-dimensional framework composed of tubules extending from the pulp to the dentin–enamel junction. The mineral portion is composed of carbonate apatites. Fibrillar type I collagen accounts for 90% of the organic matrix, while the remaining 10% consists of non-collagenous proteins, such as phosphoproteins and proteoglycans. The peri-tubular dentin, i.e., dentin surrounding the tubules, is highly mineralized (95 vol% of mineral), while most organic content is localized at the inter-tubular dentin (30vol% of mineral) [46]. Dentin undergoes modifications by physiological aging and disease processes to produce different forms of dentin [47]. This process affects the biomechanics and biochemistry of the tissue.

Although similar in composition to bone, dentin does not share the same ability to remodel. This limits site regenerative therapies. An advantage of dentin over enamel is the presence of a collagen based scaffold that provides an appropriate cell- free backbone for tissue repair and regeneration. The presence of such a scaffold is a key to advance new concepts in tissue engineering approaches to the treatment of missing hard tissue.

Recently, bio- modification of dentin has been investigated as a biomimetic strategy therapy to mechanically strengthen the existing collagen network and also control biodegradation rates of extracellular matrix (ECM) components. [48]

Different strategies have been employed for remineralizing demineralized dentin. For instance, fluoride, amorphous calcium phosphate (ACP)-releasing resins or resin-based adhesives containing bioactive glass have been used to improve the resistance of bonded restorations to secondary caries [49,50] However, most of these studies focused on remineralizing partially demineralized carious dentin, which was based on the epitaxial deposition of calcium and phosphate ions over existing apatite seed crystallites [51].

With these traditional ion-based strategies, remineralization does not occur in locations where seed crystallites are absent [52]. Thus, the classical ion-based crystallization concept may not be applicable for remineralizing completely demineralized dentin within hybrid layers created by etch-and-rinse adhesive systems or the superficial part of a caries-affected dentin lesion left behind after minimally invasive caries removal, due to the unavailability of seed crystallites in those regions for accomplishing homogeneous nucleation of apatite crystallites [53,54].

Concepts of Calcium Phosphate Biomineralization

Biomineralization is the process by which living organisms secrete inorganic minerals in the form of biominerals (e.g. magnetite, silica, oxalates, various crystalline forms of calcium carbonate and carbonated apatite) within cell cytoplasm, shells, teeth and bony skeletons [55,56]. This process exhibits a high level of spatial and hierarchical control as mineralization usually takes place in a confined reaction environment under ambient temperature and pressure conditions. Calcified human tissues consist of the collagen matrix and the hierarchically arranged carbonated apatite inorganic phase; deposition of the latter is regulated by non-collagenous proteins [57,58]. It is generally believed that non-collagenous proteins, along with specific matrix metalloproteinases and other important enzymes secreted by odontoblasts, play critical roles to orchestrate

dentin mineralization. They possess carboxylic acid and phosphate functional groups that act as preferential sites for Ca/P nucleation and subsequent apatite crystallization. [59,60]. As the therapeutic use of native or recombinant non-collagenous proteins for in situ biomineralization is not yet economically viable, research scientists have resorted to the use of polyelectrolyte and poly(acid) macromolecules to mimic the functional domains of these naturally occurring proteins, in biomimetic mineralization [61,62]. In the past few years, this field of research has attracted a lot of attention, resulting in changing concepts of calcium phosphate biomineralization.[63]

Biomimetic Remineralization of Dentin

Biomimetic remineralization represents a different approach to this problem by attempting to backfill the demineralized dentin collagen with liquid-like amorphous calcium phosphate nano-precursor particles that are stabilized by biomimetic analogs of non-collagenous proteins [63].

Several nano-technological approaches have been reported for remineralization of early caries lesions. Casein phosphopeptide-amorphous calcium phosphate nano-complexes (CPP-ACP) have been shown to promote remineralization and provide anti-cariogenic activity in laboratory, animal, and human experiments. The casein phosphopeptides stabilize calcium and phosphate ions by formation of amorphous nano-complexes. The calcium phosphate from these complexes is biologically available for remineralization of initial lesions. [31]

This is achieved by adopting the recently discovered, non-classical particle-based crystallization concept utilized by Nature in various biomineralization schemes, ranging from the mineralization of sea-shells (calcium carbonate), siliceous shells of diatoms and sponges (amorphous silica) to the deposition of calcium phosphate salts in fish scales and bone [64,65].

Intra-fibrillar mineralization of fibrillar collagen not only significantly increases its mechanical properties [66,67], but also protects the collagen molecules from external

challenges, such as temperature, endogenous enzymes, bacterial acids and other chemical factors. Using this biomimetic remineralization strategy, both hybrid layers created by etch-and-rinse adhesives and moderately aggressive self-etch adhesives [53,68,69], as well as 250–300 nm thick completely demineralized dentin lesions can be remineralized [70,71]. This bottom-up remineralization strategy does not rely on seed crystallites, and may be considered as a potentially useful mechanism in extending the longevity of resin–dentin bonds [72] via restoring the dynamic mechanical properties of the denuded collagen within the hybrid layer to approximate those of mineralized dentin [73].

Bottom-up Remineralization Strategy

Nanotechnologies involved in the fabrication of biomaterials may be classified as top-down or bottom-up approaches [74]. The top-down approach starts from a bulk material that incorporates critical nanoscale details. In this approach, a biomaterial is engineered by scaling down a complex entity into its component parts, such as creating small crystals from a bulk mineralized hard tissue via acid-etching. By contrast, the bottom-up approach assembles materials from the nanoscopic scale, such as molecules and atoms, to form larger structures [75]. Examples of the bottom-up approach include self-assembly and molecular patterning [74]. Biomineralization, which involves the spatial regulation of amorphous mineral phases via matrix proteins and organization of nanoscopic crystal-line mineral units into hierarchical structures, represents the perfect example of a bottom-up approach [76,77]

Biominerals found in bone and teeth are carbonated apatites with dimensions that are small enough to fit within the gap zones of type I collagen molecules. This hierarchical arrangement of over-lapping platelets can be identified as cross-banded patterns in unstained, non-demineralized ultrastructural sections [78,79]. Partial demineralization of a mineralized collagen matrix by acids derived from bacteria or clastic cells represents an example of a top-down approach in generating seed crystallites [74] as nidi of heterogeneous nucleation [80]. The orientation of those mineral lattices is determined by

the lattice of the seed crystallites [81]. Conventional mineralization strategies often involve the use of metastable calcium and phosphate ion-containing solutions or gels [82,83]. This example of a top-down mineralization approach [75] does not occur by spontaneous nucleation of minerals on the organic matrix but rather by epitaxial growth over existing seed crystallites [84]. Throughout the evolution of biomineralization, matrix proteins play a pivotal role in the regulation of mineral nucleation and growth [85,86]. In the absence of seed crystallites, matrix protein–mineral interactions are responsible for overcoming the thermodynamic barriers in homogeneous nucleation [87].

The precise control of crystal growth at the nanoscale and the creation of natural structures based on bottom-up self-assembly mechanisms have inspired scientists to mimic these non-classical strategies in the fabrication of novel biomaterials [87]. The literature abounds with examples of the use of bottom-up, self-assembly approaches in the fabrication of hybrid nanostructures [88]. A biomimetic mineralization strategy has been developed [89] based on the use of polyanionic molecules to mimic the functions of matrix proteins in biomineralization [90,91]. In this strategy, a polycarboxylic acid-based biomimetic analog is employed as a sequestration agent [92] to stabilize amorphous calcium phosphate (ACP) derived from set Portland cement and simulated body fluid (SBF) in the form of nanoparticles that are moldable enough to infiltrate the water compartments of a collagen fibril. Another phosphorus-based analog mimicking the collagen-binding function of matrix phosphoproteins [93] is used as a template to promote nanoprecursor recruitment to the gap zones of the collagen fibrils, where they nucleate and self-assemble into hierarchically arranged apatite nanocrystals within the fibril. This example of a particle-mediated [94], bottom-up [77] crystallization strategy differs from the classical top-down crystallization approach in two aspects. Firstly, it recapitulates the progressive dehydration mechanism in natural biomineralization [95] by replacing free and loosely bound water within the internal compartments of a collagen fibril by apatite crystallites [96] Secondly, this self-assembly approach proceeds in the absence of apatite seed crystallites and natural matrix phosphoproteins in a collagen matrix and can be duplicated using reconstituted collagen [97].

The mineral phase in collagenous hard tissues such as bone and dentin is classified as intrafibrillar apatites, which are deposited within or immediately adjacent to gap zones of the collagen molecules and extend along the microfibrillar spaces within the fibril; and extrafibrillar apatites, which are located within the interstitial spaces separating the collagen fibrils [78,98]. Previous studies have shown that intrafibrillar apatites play a significant role in the mechanical properties of mineralized tissues [99,100]. Thus, biomimetic mineralization must recapitulate both the dimension and hierarchical arrangement of apatites present in natural mineralized tissues [101,102].

Non-collagenous matrix proteins which serve as promoters or inhibitors of crystal nucleation or growth play an important role in intrafibrillar mineralization [103]. In the absence of biomimetic analogs of those matrix proteins, there should be no intrafibrillar apatite deposition using the top-down mineralization approach.[104]

Amorphous calcium phosphate (ACP) Development

Generally, it is believed that ACP was firstly described by Aaron S. Posner [105] in the mid 1960s. It was obtained as an amorphous precipitate by accident when mixing high concentrations (30 mM) of calcium chloride and sodium acid phosphate (20 mM) in buffer [106]. In X-ray diffraction, it was shown to have only two broad and diffuse peaks, with maximum at 25° 2θ. No other features were obvious and it was clearly not apatite. This pattern is typical for substances that lack long range periodic regularity. It was found that immediately after being mixed, the spontaneously formed precipitate was a non-crystalline, or amorphous, calcium phosphate with calcium to phosphorus molar ratio (Ca/P) of 1.50. After several hours, it could convert to poorly crystalline apatite on ageing. Afterwards, this solid converts slowly to crystalline apatite (Ca/P = 1.67) by an autocatalytic mechanism [107].

In 1965, Eanes et al. identified ACP as a bone component [106]. ACP in bone, along with the apatite, might account for the broad diffraction pattern and variable composition of bone minerals. An age-dependent change in the ACP content of bone was also

described, with the proportion of ACP decreasing with age [107]. In 1975, ACP was found in the mineralized cytoplasmic structure isolated from the blue crab hepatopancreas, with a very similar short-range atomic structure to synthetic amorphous calcium phosphate [108].

Amorphous calcium phosphate (ACP) is the initial solid phase that precipitates from a highly supersaturated calcium phosphate solution, and can convert readily to stable crystalline phases such as octacalcium phosphate or apatite products. Its morphological form, structural model and X-ray diffraction patterns are typical for non-crystalline substances with short-range periodic regularity. ACP has been demonstrated to have better in vivo osteoconductivity than hydroxyapatite (HAP), better bio-degradability than tricalcium phosphate, good bioactivity but no cytotoxicity [105]. These excellent biological properties make ACP widely used in dentistry, orthopedics and medicine.

Amorphous calcium phosphate (ACP) and its role in forming Hydroxy appatite

After the discovery of amorphous calcium phosphate, the early studies were focused on the structure of ACP. It was suggested that synthetic ACP particles, which appear as 300- 1000 Å spheres in the electron microscope, consist of a random assembly of ion clusters 9.5 Å in diameter, dimensions consistent with the chemical composition of $Ca_9(PO_4)_6$ [108]. And the 15-20% of water found in synthetic amorphous calcium phosphate was shown to be mostly in the interstices between, and not within, the individual $Ca_9(PO_4)_6$ clusters [109]. Aggregated ACP particles readily dissolve and crystallize to form apatite, a thermodynamically stable phase. The typical radial distribution of noncrystalline ACP cluster structures, calculated from the x-ray diffraction patterns, is only two broad and diffuse peaks showing the rapid drop-off of atomic periodicity. Short-range order exists in these amorphous structures but no long-range order such as that in crystalline hydroxyapatite [109]. Infrared analysis showed a similar lack of crystalline order about the PO_4 anions in the ACP structure

[110]However, Wuthier et al reported that ACP, with Ca/PO4 molar ratio as low as 1.15 precipitated at more acidic preparative pHs, i.e.6.9 [111].

More importantly, it has been shown that ACP particles are nanometer particles. Primary particle sizes of ACP is about 40-100 nm. The morphology of ACP solids appears to be a curvilinear shape when viewed by TEM, rather than the faceted, angular shape of crystalline calcium phosphates. However, this curvilinear appearance has only been clearly established with dried ACP [112]. The initial flocculates collected immediately after precipitation of highly hydrated ACP have a low-contrast disk-shaped appearance. High-contrast spherical particles begin to appear as ACP suspensions age, and become the dominant shape with time [113].

The ACP precipitate, with little long-range order, is a highly unstable phase and hydrolyzes almost instantaneously to more stable phases. In the presence of other ions or under in vivo conditions, ACP may persist for appreciable periods due to kinetic stabilization [114]. Although the exact mechanism of stabilization of ACP is not understood, the presence of Mg^{2+}, F^{-}, carbonate, pyrophosphate, diphosphonates, or polyphosphorylated metabolites or nucleotides, in sufficient quantity will prevent the transformation of synthetic ACP to hydroxyapatite [115,116].

Amorphous calcium phosphate (ACP) and its application in dentistry

ACP has been widely applied in biomedical field due to its excellent bioactivity, high cell adhesion, adjustable biodegradation rate and good osteoconduction [117,118]. As discussed above, the first quantitative studies on synthetic ACP were done in the mid 1960s [105]. From then on, more and more attention has been attracted in the development and the application of ACP-containing products, especially in orthopedic and dental fields. It is also used as filler in ionomer cements to fill carious lesions or as a colloidal suspension in toothpastes, chewing gums or mouthwashes to promote demineralization of carious lesions and/or to prevent tooth demineralization .[119]

CPP-ACP

CPP-ACP is an acronym for a complex of casein phosphopeptides (CPP) and amorphous calcium phosphate (ACP). Caesins are a heterogenous family of proteins predominated by alpha 1 and 2 and beta caesins. CPPs are phosphorylated casein-derived peptides produced by trypsin digestion of caesin. This protein nanotechnology combines specific phosphoproteins from bovine milk with nanoparticles of ACP.

The precise ratio is 144 calcium ions; 96 phosphate ions; and six peptides of CPP. [119]

The possible cariostatic potential of dairy products is the subject of many reports in the literature.[120,121] .In 1991, the complex CPP-ACP, derived from a major protein found in milk called casein, was patented in the United States.[122]The complex is presented as an alternative remineralizing agent that is remarkably capable of stabilizing calcium phosphate, maintaining a state of supersaturation of these ions in the oral environment. As a consequence, the tooth structure would benefit from the high levels of calcium phosphate in the biofilm,and remineralization would occur.[123,124]CPP-ACP nanocomplexes have been shown to prevent demineralization and promote remineralization of enamel subsurface lesions in animal and in-situ caries models.By stabilizing calcium phosphate in solution, the CPP maintains high-concentration gradients of calcium and phosphate ions and ion pairs in the subsurface lesion and, thus, causes high rates of enamel remineralization. The calcium phosphate in these complexes is biologically available for intestinal absorption and remineralization of subsurface lesions in tooth enamel.[125]

CPP-ACP Mechanism of Action

CPP has been shown to stabilize calcium and phosphate, preserving them in an amorphous or soluble form called amorphous calcium phosphate (ACP). ACP (Ca3H.,0) is postulated as a precursor in the formation of hydroxyapatite (HA). The ACPs exhibit a very high solubility and are readily converted to HA, which makes them suitable mineralizing agents. The main advantage of ACP is its facile, single solid phase

phosphate formulation and its biocompatibility with both hard and soft tissues, which is equal to that of HA and various di-, tri-, and tetracalcium phosphates.[125]

The following mechanism is responsible for a consistent level of remineralization through CPP-ACP.Casein phosphopeptide-amorphous calcium phosphate is a technology based on amorphous calcium and phosphate (ACP) stabilized by casein phosphopeptides (CPP). CPP containing the cluster sequence -Ser(P)- Ser(P)-Ser(P)-Glu-Glu- stabilizes ACP in metastable solution. Through the cluster sequence, the CPP binds to forming clusters of ACP, preventing their growth to the critical size required for nucleation and phase transformation.[125]

Rose and Hogg investigated the affinity and capacity of *Streptococcus mutans* for CPP-ACP. Using the equilibrium dialysis system they described, their results demonstrated that CPP-ACP binds with about twice the affinity of the bacterial cells for calcium up to value of 0.16g/g wet weight cells. Application of CPP-ACP to plaque may cause a transient rise in plaque fluid-free calcium, which may assist remineralization. Subsequently, CPP-ACP will form a source of readily available calcium to inhibit demineralization. Hence, CPP-ACP binds well to plaque, providing a large calcium reservoir, which is likely to restrict mineral loss during a cariogenic episode and provide a potential source of calcium for subsequent remineralization. In short, once in place, CPP-ACP will restrict the caries process. [125]

In the United States, up to now, this product is primarily used for abrasive prophylaxis pastes and secondarily used for the treatment of tooth sensitivity especially after in-office bleaching procedures, ultrasonic scaling, hand scaling or root planing. However, its use for remineralizing dentin and enamel and preventing dental caries is an off-label application. Outside the United States, this product is marketed as GC Tooth Mousse [126,127].

Clinical Safety of CPP-ACP Usage

No serious side effects were reported in studies assessing the clinical safety of CPP-ACP usage. [128,129,130,131] In Morgan's two-year follow-up study, no significant differences were found in the incidence of side effects (i.e. nausea, headache, and diarrhoea) between the intervention and control groups.[128]

No allergies or serious side effects were recorded in Rao's and Bailey's trials as well.[128,130] Sitthisettapong also confirmed by email corre-spondence that no extra calculus formation had occurred on the primary teeth in their experimental group.[131]

ACP-filled polymeric composites

ACP has been evaluated as a filler phase in bioactive polymeric composites [132]. Skrtic has developed unique biologically active restorative materials containing ACP as filler encapsulated in a polymer binder, which may stimulate the repair of tooth structure because of releasing significant amounts of calcium and phosphate ions in a sustained manner [133,134]. In addition to excellent biocompatibility, the ACP-containing composites release calcium and phosphate ions into saliva milieus, especially in the oral environment caused by bacterial plaque or acidic foods. Then these ions can be deposited into tooth structures as apatitic mineral, which is similar to the hydroxyapatite found naturally in teeth and bone [135,136].

Compared with more commonly used silanated glass or ceramic filler, more hydrophilic and biodegradable ACP-filled composites exhibited inferior mechanical properties, durability and water sorption characteristics [137]. The uncontrolled aggregation of ACP particulates along with poor interfacial interaction plays a key role in adversely affecting their mechanical properties [138]. Their clinical applicability may be compromised by relatively poor filler/matrix interfacial adhesion and also by excessive water sorption that occurs in both resin and filler phases of these composites [139,140].

Enhanced Glass Ionomer - Ceramir

Recently Doxa Dental AB,(Uppsala, Sweden) introduced a bioceramic, Ceramir with a modified GI composition. The cement has been shown to form apatite on storage in simulated body fluids (SBF)and saliva. [141]. The powder contains approximately 50% Calcium aluminate in place of Calcium aluminosilicate glass. The high Calcium aluminate content is responsible for apatite formation in SBF and saliva which has been reported to contain enough phosphate ions to promote this effect. The setting reaction is a combination of a glass-ionomer reaction and an acid-base reaction of the type occurring in hydraulic cements. The set cement has an acidic pH of 5 and gradually increases to 8.5 after 3-2 hours of setting. The set cement is reported to form hydroxyapatite on the surface when in contact with phosphate containing solutions. Initial setting time of 3mins and final setting time of 6 mins have been reported. [142]. Although Vickers hardness increased gradually from 30-110 MPa over a 28 days period after mixing. The set cement is alkaline and releases calcium and fluoride ions. The incorporation of Calcium Aluminate is believed to fix the GIC structure and hinders the ionomer glass from continuous leaching over time. (Parmeijer 2007) When in contact with phosphate solutions, such as saliva or body fluids it first forms precursors of HA, which transform to apatite which can integrate with bone and dentin. The high pH of the set cement inhibits bacterial growth. A comparative study showed that GI cement modified by addition of calcium aluminate (Ceramir) induced hydroxyapatite formation when immersed in SBF solution. This represents a significant development because use of this material could potentially remineralize adjacent hard tissues. Other study investigated HA formation of Ceramir in saliva and concluded that saliva contained adequate amounts of calcium and phosphate for HA formation and demonstrated HA formation on samples stored in saliva. [141]

Surfactant and HA formation

Hydroxyapatite (HA) is the principal inorganic constituent of bones and teeth [143]. HA is used mostly as powders and its usefulness depends on the powder properties such as particle size, surface area, and morphology. Nano-structured HA particles with a higher surface area would be more desirable for their use in many fields including separation processes. [144].

HA can be synthesized by a variety of methods including conventional routes such as solid-state reactions [145] and wet chemical routes [146] based on precipitation at low temperature.

These conventional methods, however, mostly prepare irregular forms of powders. Hydrothermal method, which has been proved to be a convenient way to prepare materials, including salts, metal oxides, etc., has also been applied, but the control on morphology is poor [147]. Nevertheless, the size and morphology would largely determine the behavior of a certain material, that's why a biomineralization process usually involves complicated mediation and the final products generally have a delicate microstructure [148]. Bone itself is a composite consisting of HAP nanorods embedded in the collagen matrix [149].

Synthetic HAP crystals are usually prepared as rods or needles, which are similar in structure and composition to HAP found in human bone.

Hence, HAP nano-rods are desirable when biocompatibility is considered [150]. Although control over microstructure seems too big a challenge to traditional methods, the biological process itself has given some clues to achieve this: the controlled nucleation and crystal growth process mediated by macromolecule control and cell organization would finally result in uniform products. A method called biomimetics is then aroused [151,152]. Macromolecules, such as stearic acid, monosaccharides and related molecules were explored and the molecule addition has exerted significant control on the morphology. [152]. Bose and Saha synthesized HAP nano-powders with a controlled surface area and particle size by using nonionic surfactant emulsion. [153].

Another surfactant-templating approach has been extensively used in the preparation of various nanoporous or nano tubular frame-work materials including mesoporous silica[154] and many other metal oxides.[155] Y. Wang et al. (2006) shows that the size and morphology of precipitated hydroxyapatites can be affected by adding adequate a surfactant, Cetyltrimethylammonium bromide (CTAB) and control of the precipitation temperature and pH.

One current hypothesis is that the use of remineralizing materials in dentistry would prevent secondary caries due to a natural formation of apatite between material and tooth, leading to a stable interface. [141]

Aim of This Study

This study aimed to evaluate the effect of Ceramir and the addition of mono-n-Dodecy phosphate to Ceramir as a surfactant in dentin remineralization.

Dentin remineralization is expected to occur in two mechanisms : 1. the classical ion-based crystallization concept. 2. Locations where seed crystallites are absent. The surfactant can act a biomemitic analogue OF non-collagenous proteins. To modulate remineralization, the latter can lead to increase the calcium precipitation and modify the HAP particle size to improve dentin remineralization ability of the Ceramir.

This, might be applicable for remineralizing completely demineralized dentin within hybrid layers created by etch-and-rinse adhesive systems or the superficial part of a caries-affected dentin lesion left behind after minimally invasive caries removal . This method would also prevent secondary caries due to a natural formation of apatite between material and tooth structure, leading to a stable interface.

Dentin remineralization will be determined by the alteration in micro-hardness of dentin surface around Ceramir. The null hypothesis is that none of the filling using including: plain Ceramir or Ceramir with mono-n-Dodecyl phosphate would significantly affect the Knoop micro-hardness of demineralized dentin surface. [156]

Materials and methods:

Sample Selection:

In this study, 45 permanent, intact, non-carious, non-hypoplastic, unfractured and non-malformed, upper and lower, anterior and posterior human teeth, freshly extracted for orthodontic or periodontic reasons were selected and stored in 0.5% chlorothymol solution. Teeth with any defect were excluded.
All selected teeth were used within three months of extraction as recommended by Occupational Safety & Health Administration (OSHA).

Sample Preparation and Baseline Measurement:

The teeth were cleaned using an ultrasonic scaler (cavitron). The teeth were then embedded in self-cured dental acrylic resin to expose buccal or lingual surfaces. The buccal/lingual surfaces were wet ground starting with 800 grit silicon carbide paper through 1200 and1600 under water cooling to obtain a smooth dentin surface, final polishing was accomplished with alumina powder using 9 , 3 and 1 micron grit to obtain highly polished surface.

The Knoop hardness tester (Leco M-400-G1 Hardness Tester). was calibrated with standard test blocks provided by the manufacturer. Baseline surface micro-hardness of sound dentin was measured using Knoop indenter. A loading force of 50 g and dwell time 10 seconds were used. For each sample four indentations were performed in the dentin surface and the mean Knoop microhardness value (KHN) was calculated.

Demineralization and Cavity Preparation:

The dentin surfaces of all specimens were then etched using 37% phosphoric acid for 5 seconds to demineralize dentin and expose dentin collagen. The dimensions of the acid etched area ranged from 7.5 to 8 mm. After the demineralization process was

completed, all the specimens were rinsed in deionized water and blotted dry. The Knoop indenter microhardness of the etched dentin was measured. The mean Knoop microhardness of etched dentin was calculated.

Round cavities were prepared with 1/4 round carbide bur in the middle of the etched area to obtain 6.35 mm width round cavity and 3 mm depth as shown in figure .1.

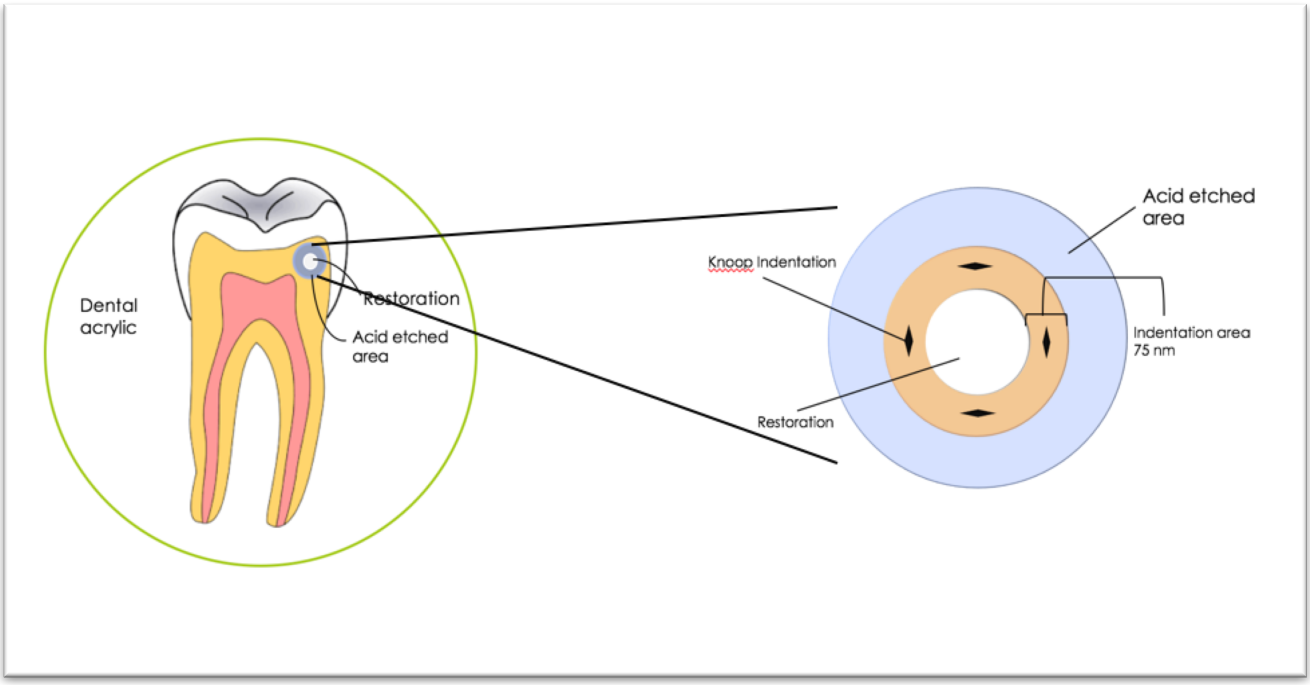

Figure 1. Study Sample, showing: acid etched area, restoration, Knoop indentation and Knoop indentation area in dentin.

The forty-four specimens were randomly divided into four equal groups:

Group 1: control group, samples were demineralized and cavity prepared but had no restoration placed in them.

Group 2: cavities prepared in etched dentin and restored with plain Ceramir.

Group 3: cavities prepared in etched dentin and were restored with Ceramir containing 2% mono -n-Dodecyl phosphate.

Group 4: cavities prepared in etched dentin and restored with Ceramir containing 5% mono -n-Dodecyl phosphate.

Ceramir capsules were mixed using an amalgamator for 5 seconds following the manufacturer's instructions. To fabricate experimental samples containing 2% and 5% surfactant one Ceramir capsule was mixed and emptied and the weight of the paste measured in grams to calculate the 2% and 5% surfactant. Then, the powder of mono -n-Dodecyl phosphate was measured and added to the Ceramir paste to obtain Ceramir with 2% and 5% concentration of surfactant.

Simulated body fluid (SBF) was made according to the International Organization for Standardization (ISO) for evaluation of apatite forming-ability. Table 1 lists the reagents contained in the SBF solution. The SBF was then transferred to a plastic bottle for storage and was kept in a refrigerator set between 5 - 10°C. Each sample was then placed in a small container and completely immersed in simulated body fluid (SBF) and stored in an incubator at 37°C to simulate human body environment.

Reagent	Amount for 1 L of SBF
sodium chloride	8.035 g
sodium bicarbonate	0.355 g
potassium chloride	0.225 g
potassium phosphate dibasic trihydrate	0.231 g
magnesium chloride hexahydrate	0.311 g
1 M hydrochloric acid	39 mL
calcium chloride	0.292 g
sodium sulfate	0.072 g
tris(hydroxymethyl) aminomethane	6.118 g

Table 1. Reagents for Preparing the 1L of Updated Simulated Body Fluid (SBF)

Knoop indenter micro-hardness measurements were taken at 10, 20 and 38 days. At each test interval samples were wiped dry with tissue paper and four indentations were performed per sample within an area of approximately 75 μm away from the filling margin in the dentin surface.

The mean KHN value of all teeth in each group was calculated at each interval, we calculated the change in the means from both baseline and the pre-filling, etched interval.

Dentin surface was evaluated by FEI Quanta 600 environmental scanning electron microscope after each testing period. Environmental electronic microscope permits wet and insulating samples to be imaged without prior specimen preparation. The use of the environmental microscope eliminated the need for specimen preparation by drying and sputters coating with gold or carbon that could alter the dentine surfaces.

One sample in each of the groups restored with 2% and 5% surfactant were excluded because the fillings were dislodged after 10 days.

Data were expressed in Knoop Hardness Number (KHN) and statistically analysed running a Repeated Measures ANOVA Analysis and pair-wise multiple comparison procedures on the difference of means for each data point using Sigma Stat version 3.5 (Systat software , Point Richmond, California ,USA). Statistical significance was determined at p value of 0.05.

Results:

SEM pictures were taken throughout the study period using (FEI Quanta 600 Environmental Scanning Electron Microscope). Samples were imaged in low vacuum mode without any additional surface treatment. Figure 2 shows SEM of the polished dentin surface with dentinal tubules partially blocked by smear layer. Figure 3 shows micrographs of dentin after etching with phosphoric acid with widened dentin tubules that appear interconnected. Figures 4, 5 and 6 show progressive formation of crystal precipitates that are blocking the dentin tubules that were opend by acid etching.

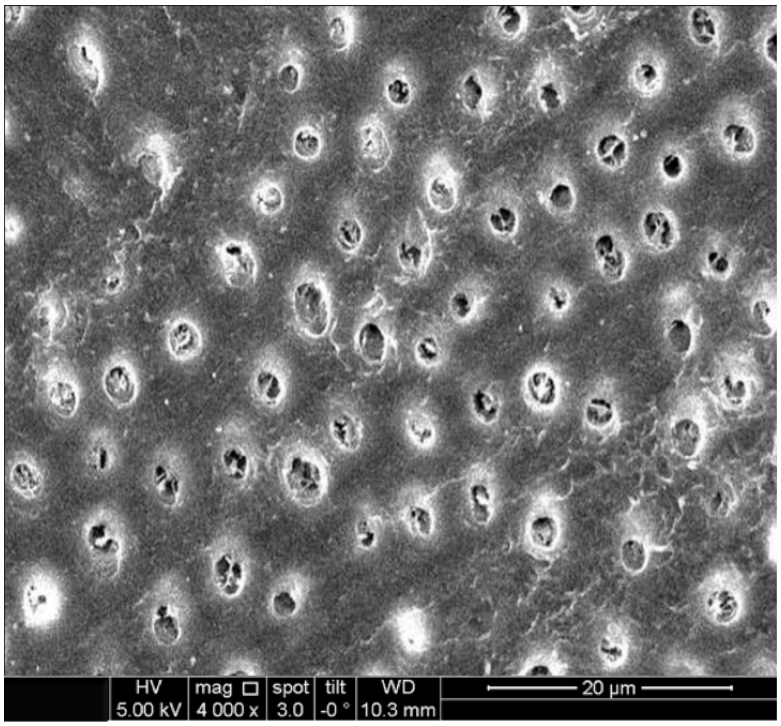

Figure 2. SEM of non-etched dentin. The dentinal surface revealed smear layer blocking dentinal tubules.

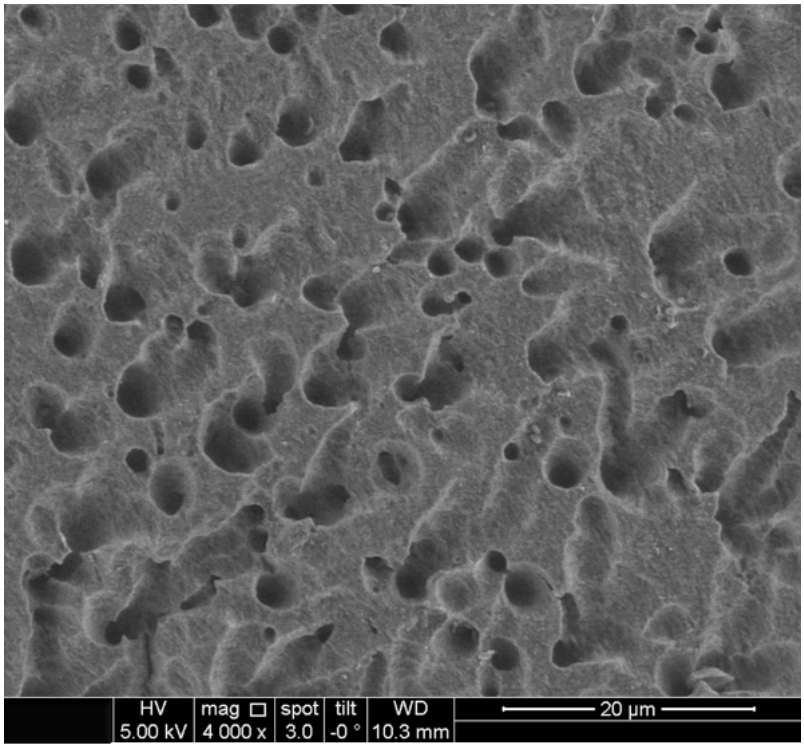

Figure 3. SEM of etched dentin surface with 37% phosphoric acid etch for 5 seconds, The micrograph depicts open dentinal tubule and exposed collagen fibers.

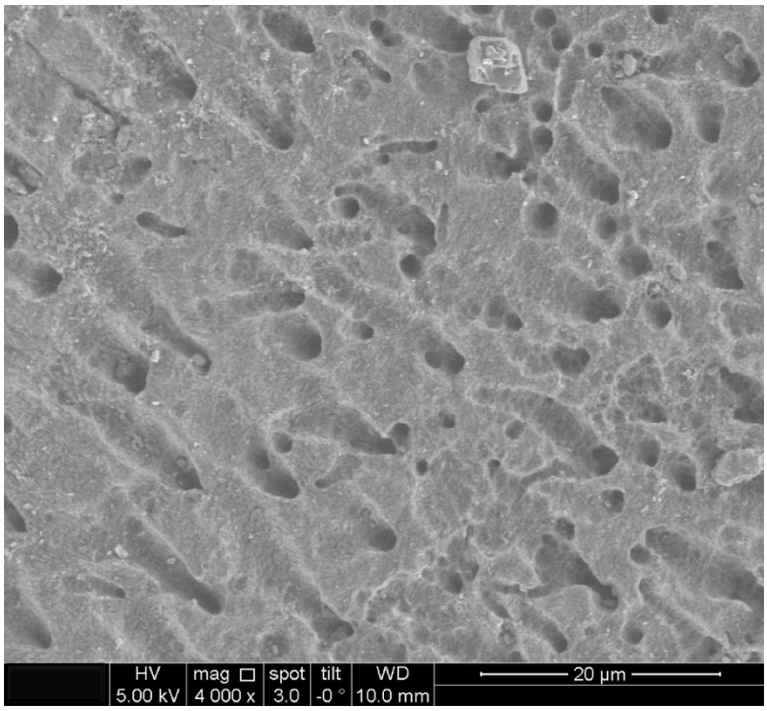

Figure 4. SEM of dentin surface after 10 days depicts minor crystal formation.

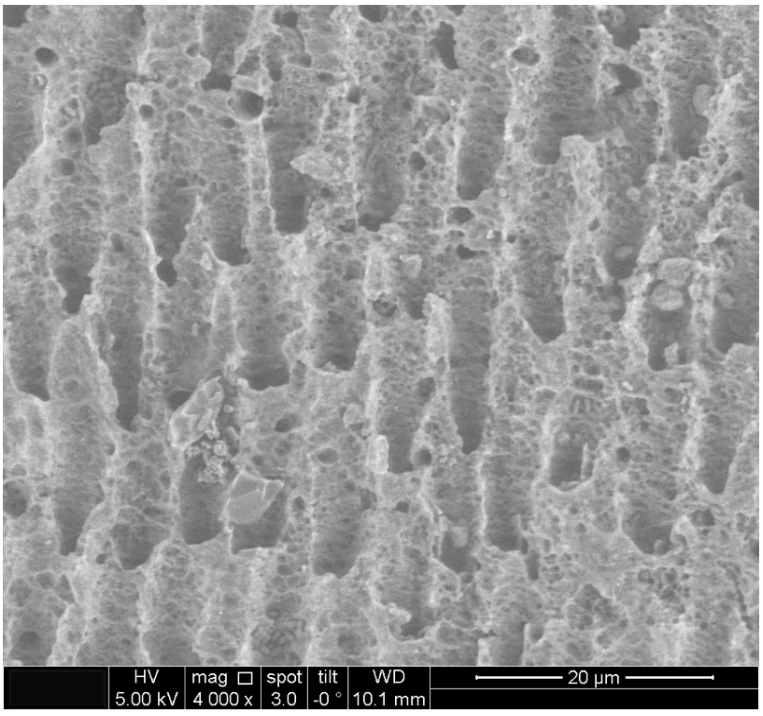

Figure 5. SEM of dentin surface after 20 days depicts more crystal formation compared to dentin surface after 10 days.

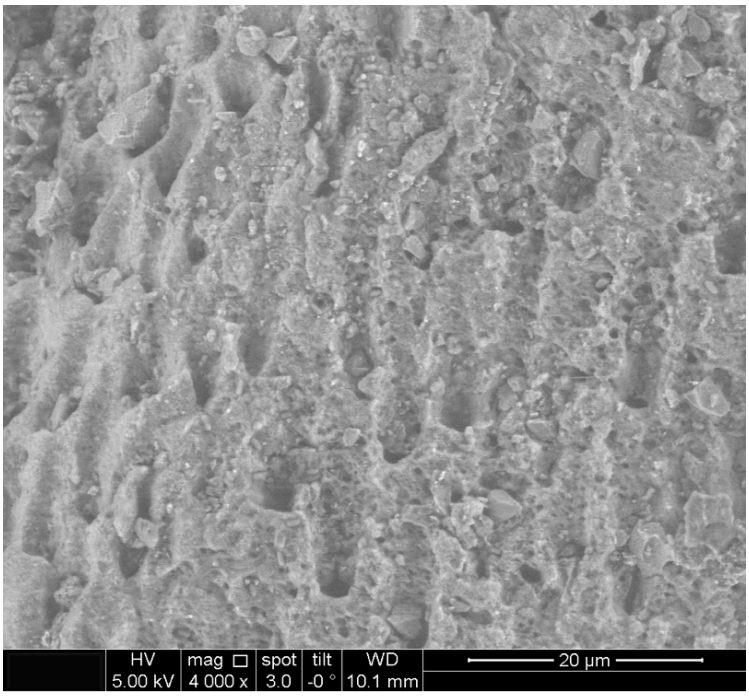

Figure 6. SEM of dentin surface after 38 days depicts crystal formation covering dentin surface and blocking dentinal tubules.

Knoop micro-hardness mean value (KHN) of polished dentin was 105.3(20). After etching KHN was reduced to 73.3(15.2) (Table 2).

Base line Knoop value for samples (SD)	**Post etching-filling Knoop value (SD)**
105.3 (20)	73.3(15.2)

Table2. Base Line and Post etching-filling Knoop mean value.

Etching significantly ($p<0.05$) reduced the micro KHN values of dentin. In samples where no restorations were placed, (Group 1), the KHN did not show a significant difference through the observation period. (Table 3). In Group 2 where cavities were restored with Ceramir, KHN was increased by 24.1, 40.6 and 48.9 KHN value at 10, 20, 38 days post restoration respectively when compared to the etched only controls. Table 3. Samples restored with Ceramir containing 2% surfactant (Group 3) increased the micro hardness as compared to etched only samples by 22.1, 45.5 and 59.2 KHN value at

10,20,38 days respectively

Samples restored with Ceramir containing 5% surfactant (Group 4) increased the micro hardness as compared to etched only samples by 24.2, 33.9 and 43.5 KHN units at 10, 20, and 38 days respectively.

Filling Type	Baseline Knoop value (SD)	Post etching-filling Knoop value (SD)		Post 20 days KHN (SD)	Post 38 days KHN (SD)
Control (no restoration)	105.3 (20)	73.3 (15.2)	73.3(15.0)	74.3(14.4)	75.2(16.2)
Plain Ceramir			101.2 (22.3)	117.7 (21.4)	126.0 (17.1)
Ceramir with 2% surfactant			90.1 (21.4)	113.5 (18.1)	127.2 (23.4)
Ceramir with 5% surfactant			93.3 (17.5)	102 (20.0)	112.5 (19.3)

Table 3. Change in Knoop values with time.

According to the Repeated Measures Anova Analysis, the increase in KHN values for the plain Ceramir group were not significantly after 10 days post filling. After 20 and 38 days post filling the KHN readings show significant increase in values vs etched values. Also, significant change found to be between 10 vs 38 days as shown in table 4.

Time period	Significance of KHN value
Etched vs 10 days	not significant
Etched vs 20 days	significant
Etched vs 38 days	significant
10 days vs 20 days	not significant
10 days vs 38 days	significant
20 days vs 38 days	not significant

Table 4. Significance of KHN value over time period for group 2 (plain Ceramir).

For group 3 in which samples were restored with Ceramir containing 2% surfactant, the increase in KHN values were statistically not significant ($p<0.05$) after 10 days post filling. After 20 and 38 days post filling the KHN readings show statistically significant

increase in values vs etched values. Also, statistically significant change found between 10 vs 38 days as shown in table 5.

Time period	Significance of KHN value
Etched vs 10 days	not significant
Etched vs 20 days	significant
Etched vs 38 days	significant
10 days vs 20 days	not significant
10 days vs 38 days	significant
20 days vs 38 days	not significant

Table 4. Significance of KHN value over time period for group 5 (Ceramir containing 2% surfactant).

For group 4 in which samples were restored with Ceramir containing 5% surfactant, the increase in KHN values were statistically not significant after 10 days post filling. After 20 and 38 days post filling the KHN values were statistically significantly increased. No statistically significant differences were found between 10 vs 20 vs 38 days as shown in Table 6.

Time period	Significance of KHN value
Etched vs 10 days	not significant
Etched vs 20 days	significant
Etched vs 38 days	significant
10 days vs 20 days	not significant
10 days vs 38 days	not significant
20 days vs 38 days	not significant

Table 6. Significance of KHN value over time period for group 4 (Ceramir contains 5% surfactant).

Statistical Analysis using One Way Repeated Measures ANOVA test followed by pairwise comparison of the difference of means for each data point, showed no statistically significant difference in micro hardness between the samples restored with

plain Ceramir compared to the Ceramir with surfactant group after 10 days.

There is no statistically significant difference between the Ceramir (Group 2) and samples restored with Ceramir with 2% surfactant (Group 3) after 20 days while there is statistically significant difference between them and the Ceramir with 5% (Group 4).

There is statistically significant difference in the micro hardness between Ceramir with 2% group and Ceramir with 5% group after 38 days and no statistical difference between plain Ceramir and Ceramir with 2% groups.

Change in micro-hardness values compared to baseline and etched values with time in percentage summarized in table 7 and 8.

Filling Type	10 days	20 days	38 days
Control (no restoration)	-33.69%	-32.6%	-31.6%
Plain Ceramir	-7.57%	10.2%	19.2%
Ceramir with 2% surfactant	-14.66%	9.6%	23.96%
Ceramir with 5% surfactant	-14.77%	-4.49%	5.7%

Table 7. Change in micro-hardness values from baseline with time in percentage.

Filling Type	10 days	20 days	38 days
Control (no restoration)	0.03%	0.7%	1.39%
Plain Ceramir	18.48%	31.30%	37.7%
Ceramir with 2% surfactant	14.96%	30.6%	40.2%
Ceramir with 5% surfactant	16.56%	22.77%	29.67%

Table 8. Change in micro-hardness values from etched dentin with time in percentage.

There is no statistically significant difference between the Ceramir (Group 2) and Ceramir with 2% surfactant (Group 3) and 5% surfactant (Group 4) after etching and after 10 days while there is statistically significant difference between the Ceramir (Group 2) and Ceramir with 2% surfactant (Group 3) with the Ceramir with 5% (Group 4) after 20 days and 38 days. Change in micro-hardness over time shown in Figure 7.

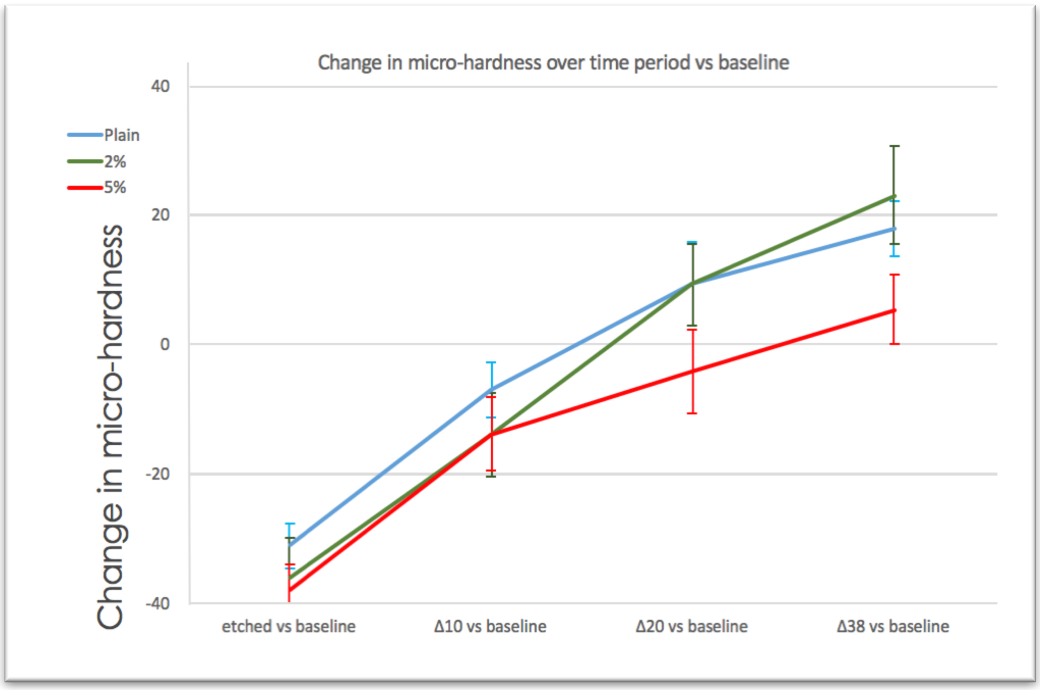

Figure 7. Change in micro-hardness over time period vs etched.

Discussion:

The results of this study show that Ceramir restorations of dentin lesions lead to remineralization of dentin within an area that is 75 µm from the margins of the restoration. Consequently, the null hypothesis is rejected.

Etching changes the micro morphological appearance of enamel and dentin surfaces independent of the type of acid, the etching time and the concentration. Many techniques have been reported for demineralization as shown in Table 9. [181] In this

study 37% phosphoric acid gel was been used for etching because it can be applied for a small area as needed also it is quick and effective in providing adequate demineralization.

The scanning electron micrograph study shows that polished dentin surface was covered by a smear layer covering which partially blocked the dentin tubules. Figure 2. Etching with phosphoric acid removed the smear layer and widened the openings of the dentin tubules. The tubules appeared interconnected due to exposure of collagen fibers in inter- tubular regions of the dentin.

The electron micrographs of samples restored with Ceramir show a sequentially increasing precipitate formation over the observation period. It is clear that **Nano** size particles were formed that covered the dentin surface and partially filled the dentinal tubules. SEM micrograph exhibited mineral depositions with large two-dimensional, plate-like structures and small three-dimensional, cubic structures The amount of precipitate particles clearly increases over time as shown in figures 4, 5 and 6.

It shows that addition of 2% surfactant to Ceramir tend to increase the remineralization over time. Toward the end of the observation period samples restored with Ceramir containing 2% surfactant appeared to remineralize at a faster rate than plain Ceramir. On the other hand addition of 5% surfactant to Ceramir was not beneficial as it led to decrease in the effect of Ceramir.

Although, the KHN values after 10 days were statistically not significant, there were significant increases in KHN in comparing with the etched KHN values.

Many different techniques have been used to evaluate dentin mineralization. Scanning and transmission electron microscopy (SEM and TEM) [157,158], Fourier transform infrared spectroscopy (FTIR) [159], Raman spectroscopy[160], X-ray diffraction (XRD)[161], energy dispersive X-ray spectroscopy (EDX)[162], micro-radiography[163,164], micro-CT scanning[165], and nano-indentation to evaluate microhardness[166].

Microhardness is defined as the resistance to local deformation. [167] Microhardness tests are commonly used to study the physical properties of materials and they are widely used to measure the hardness of teeth. [168.169] These tests are based on the induced permanent surface deformation that remains after removal of a load. [167] Two microhardness tests, Knoop and Vickers hardness are commonly used for evaluation dental materials. Both measurements can be correlated with other mechanical properties such as fracture resistance, [170] modulus of elasticity, and yield strength. [171,172]

For our study we used a Knoop indenter (Leco M-400-G1 Hardness Tester) for the micro hardness -indentation assessment and evaluation. The dental literature shows that Knoop microhardness has been employed extensively in testing the hardness of both enamel and dentin and is an effect measure of demineralization. [174]

The Knoop micro-indentation method requires only a tiny area of specimen surface for testing. Using this technique, the specimen surfaces are impressed with a diamond indenter. The geometry of this indenter is an extended pyramid with the length to width ratio being 7:1 and respective face angles are 172 degrees for the long edge and 130 degrees for the short edge. The depth of the indentation can be approximated as 1/30 of the long dimension at a certain load for a certain period of time. After load removal, diagonals of the indentation are measured with an optical microscope. The hardness number is defined by the ratio between the indentation load and the area of the residual impression, which depends on the indenter shape. Then the hardness of materials was calculated using these equations:
KHN = 14230 (F/d^2) for Knoop microhardness or HV = 1854 (F/d^2) for Vickers microhardness. [173]
The Knoop indentation is longer and shallower than Vickers indentation and the load impression can be applied to brittle materials without cracking. Also, the longer diagonal is easier to read than the short diagonal of the Vickers. However, the advantage of the Knoop's longer diagonal is offset by the difficulty in deciding where the tapered tip ends on the surface of the dentin. [173]

The chief characteristic of the Knoop microhardness test is its sensitivity to surface

effects and textures. [175,176] For a given load, the Vickers indenter penetrates about twice as far into the specimen as the more shallow Knoop indenter, and the diagonal is about one-third the length of the longest diagonal of the Knoop indentation. Thus, the Vickers test is less sensitive to surface conditions and, due to its shorter diagonals, more sensitive to measurement errors when equal loads are applied. [175,176,177,178]

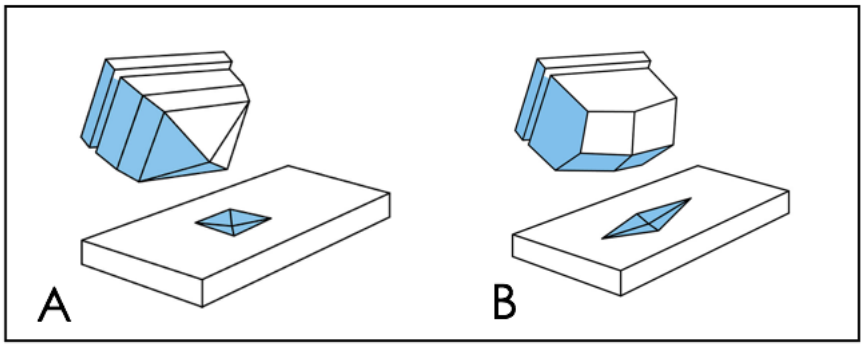

Figure 8: difference between Vickers (A) and Knoop indenter (B).

The indentation load for the micro hardness test can range from 1 to 1,000 g, and with various loading dwell times.

Dentin Knoop micro-hardness KHN values for baseline measurements ranges from 70 up to 90 depending on tooth location and area of indentation. [180][187][188] Anterior teeth tend to show lower hardness value than posterior. Victoria Fuentes et al. (2003) evaluated microhardness of superficial and deep sound human dentin using Knoop indenter. [173]

Indentations closer to the dentin-enamel junction give higher KHN and decrease as we move toward pulp direction. Because the tubules in dentin are not randomly oriented, properties may be directionally dependent. This is because the mineral content in dentin is higher at the dentin-enamel junction and as we move toward the pulp the mineral content decrease as the organic content increase. [187]

In this study KHN was measured at sites close to the DEJ (Figure 1) and found to be slightly higher than literature values reported by Huang et al. (20) Knoop micro-hardness values increase as the mineral content increase and decrease as mineral content decrease.

This can explain the results in this study. After etching which is the process in which mineral content of material removed the KHN was found to be decreased. And gradually as particles forms and mineral precipitate increase, the KHN would increase as well. [187]

Similar to our study, several studies have used microhardness measurements to evaluate both enamel and dentin remineralization process.

Manuel Toledano et al. (2004) used Knoop indenter to asess microhardness of acid-treated and resin infiltrated human dentine. The study concluded that treating dentine with either H_3PO_4 alone or H_3PO_4 followed by NaOCl caused marked reduction of its surface hardness. The removal of the mineral phase of dentine surfaces by acidic treatments modifies their surface morphology and properties, and undoubtedly their hardness. [180]

E. Bresciani et al. (2010) used Knoop indenter to evaluate dentin Microhardness beneath a calcium-phosphate cement [188]. The study reported that according to the structure of dentin, microhardness may be related to 3 different forms of mineralization, represented as: (a) plate-shaped crystals within tubule lumina, (b) uniform mineral distribution in peritubular and intertubular dentin; and (c) intra- and interfibrillar mineral in collagen. Also the study suggested that acid-etching opens dentin tubules and may assist in the mineralization of intertubular and peritubular dentin close to the interface.

Hussam Milly et al. (2014) also used Knoop indenter in their study of enamel white spot lesion remineralization using bio-active glass and polyacrylic acid-modified bio-active glass powders , and showed that increasing KHN represents increasing in the mineral content of enamel.

In our study, the mean Knoop microhardness found to show marked reduction of its surface hardness after treating dentine with 37% phosphoric acid. We assume that the removal of the mineral phase of dentine surfaces by acidic treatments modifies their surface morphology and properties, and undoubtedly their hardness. In agreement with our study Panighi and G'Sell also observed a positive correlation between hardness and

the mineral content of the tooth. They indicate that a comparable decrease in mechanical properties of dentine can be observed after acid etching treatment. [180] In this study we used the SBF to mimic saliva rule in providing phosphateions.

Authors, Year [Reference]	Surface Treatment
Forsback *et al.* 2004 [12]	0.5% NaOCl (5 min)
Vollenweider *et al.* 2007 [13]	17% EDTA (2 h)
Tay *et al.* 2008 [25]	37% PA (15 s)
Reyes-Carmona *et al.* 2009 [14]	17% EDTA (3 min), 1% NaOCl (3 min)
Gandolfi *et al.* 2011 [15]	17% EDTA (2 h)
Gu *et al.* 2011 [19]	0.5 M EDTA, 4 M GuCl
Liu *et al.* 2011 [22]	pH-cycling
Liu *et al.* 2011 [23]	pH-cycling
Gu *et al.* 2011 [26]	32% PA gel (15 s)
Xu *et al.* 2011 [27]	Demineralizing solution (72 h)
Wang *et al.* 2011 [33]	37% PA (15 s)
Zhou *et al.* 2012 [17]	37% PA (2 min)
Ning *et al.* 2012 [18]	20% PA (60 s)
Qi *et al.* 2012 [24]	pH-cycling
Li *et al.* 2013 [20]	0.5 M EDTA (30 min), 4 M GuCl
Wang *et al.* 2013 [29]	37% PA (10 s)
Cao *et al.* 2013 [31]	37% PA (60 s)
Cao *et al.* 2014 [1]	37% PA (60 s)
Osorio *et al.* 2014 [16]	35% PA (15 s)
Zhou *et al.* 2014 [21]	0.5 M EDTA (30 min), 4 M GuCl
Sun *et al.* 2014 [30]	35% PA (10 s)
Jia *et al.* 2014 [32]	37% PA (10 s)

Table 9. Demineralization Techniques.

In this study SBF was used as storage medium for samples to mimic saliva role for providing phosphate. Using human saliva in this study is not applicable for two reasons; first, it difficult to obtain large amount of saliva needed for all samples, second, the relatively long study period affects sterility of samples.

The control etched sample (group 1) showed slight increase in micro-hardness in storage in SBF.

The simulated body fluid (SBF) is widely used for the study of biomineralization. [182][183].When a material is incubated in SBF solution, the formation of apatite layer on the surface of pellet goes through a sequence of chemical reactions like spontaneous precipitation, nucleation and growth of calcium phosphate [184]. It has been suggested that surface chemistry plays an important role in this process [185] and even the functional groups of materials have a large effect on the bone-bonding property. It is well known that HAp structure consists of Ca, PO^4 and OH groups closely packed together. The OH and $PO4^{3-}$groups are responsible for negative charge of HAp surface and Ca^{2+} ions form the positive group. The process of apatite formation mainly depends on negative group, which in turn depends on the large number of negative ions (i.e. OH and $PO4^{3-}$) on the surface. During incubation period, the positive Ca^{2+} ions from SBF are attracted by the OH and $PO4^{3-}$ions present on HAp surface. Therefore, the surface gains positive charge with respective to the surrounding SBF and further attracts the negatively charged OH- and $PO4^{3-}$ ions from the SBF. This promotes formation of the apatite layer [186].

Spanos et al. (2006) conducted a study about the the precipitation of calcium phosphates in simulated body fluid (SBF) with pH 7.40 and 37°C.The crystal growth experiments in which SBF solutions of variable supersaturations were seeded with hydroxyapatite crystals showed that the precipitation of calcium phosphates took place on specific active sites provided on the surface of the synthetic seed crystals.[182]

Chavan et al (2009) showed that the Simulated Body Fluid (SBF) can support Hap formation. The ion exchange process is carried out to exchange calcium cation by sodium and potassium. The pure HAp and ion exchanged HAp pellets are used as source of nucleating agent for apatite layer formation, in SBF maintained at 37°C using incubator for different periods of time to study the bioactivity. [183]

Conclusion:

Within the limitations of this study, Ceramir was found to have a remineralization effect on demineralized dentin. Adding 2% surfactant to plain Ceramir increased the rate of remineralization. More than 2% surfactant is still questionable while adding 5% surfactant to Ceramir cement decreased the material's effect in remineralization. Our findings confirm that Ceramir can be used clinically for restorations of root lesions and remineralization of the margin of the cavity to reduce the secondary caries. Based on the remineralization effect observed, Ceramir could also have a beneficial effect in reducing root sensitivity.

Further studies of the biomimetic molecules involved in calcium fluoride phosphate stabilization and nucleation may provide improvements in the development of novel remineralization treatments. Of the remineralization technologies currently commercially available, the CPP-ACP technology has the most evidence to support its use. The clinical benefits of using Ceramir are still being investigated. Well-designed random clinical trials are needed to improve the level of evidence in this area.

Conflict of interest:

The authors received no financial support and declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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