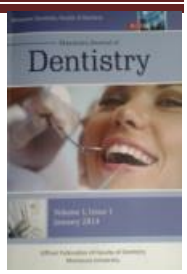

Biomimetic remineralization of artificial dentinal carious lesions using two different bioactive capping materials.

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Abstract:

Purpose: This study was conducted to evaluate the remineralization potential of two bioactive pulp capping materials (Biodentine ™ and Lime-lite ™) in the presence of biomimetic analogs (Poly-acrylic acid and Sodium trimetaphosphate).

Materials and Methods: Two standardized occlusal cavities (mesial and distal) were prepared within dentin surfaces with the dimensions of 2 mm in diameter & 0.5 mm in depth after removal of occlusal enamel. Artificial demineralized dentin was induced though pH cycling (8 hours in demineralizing solution & 16 hours in remineralizing solution). Whole occlusal dentin was protected from pH cycling except the cavities and a small rim (1 to 2 mm) surrounding them. Demineralized cavities were divided into four groups; the first two groups received capping materials. The other two were first treated with biomimetic analogs prior to material application. Teeth were sectioned buccolingually into two halves. Teeth halves that received treatment with analogs were stored in simulated body fluid containing poly-acrylic acid. Un-treated cavities were stored in simulated body fluid only. The specimens were examined after 1, 6 and 12 weeks storage using Energy dispersive x-ray spectroscopy (EDX) and Scanning electron microscope (SEM). Two-way ANOVA was used to analyze data statistically.

Results: Specimens received Biodentine with biomimetic analogs showed the highest statistically significant calcium and phosphorous Wt% after 12 weeks of storage. Scanning electron microscope images showed filling of intra-tubular dentin at the interface region in all tested groups.

Conclusion: Biodentine showed the best ability to enrich the artificial carious dentin with significantly higher Wt % of ions for remineralization especially in the presence of biomimetic analogs. Both tested materials have the ability to obliterate open dentinal tubules after total period of storage.

Introduction

The know how to manage and deal with the demineralized dentin is greatly important.[1] Most of the recent conservative approaches avoid unnecessary tooth structure removal and leave caries affected dentin as the clinical bonding substrate.[2] The remineralizing material used, on the other side, is crucially important. Variety of dental materials, with different bioactive abilities, are used nowadays. Some of them are calcium silicate, calcium hydroxide or hydroxyapatite based.[3,4] Most of these materials, like Biodentine, Theracal and MTA, have shown good signs of remineralization to tooth structure and in contact with cells.[5–8] However, the remineralization process, in accordance with such materials, will take place when they come in contact with simulated body fluids containing phosphate.[9] Residual mineral crystallites only will act as seed sites for remineralization and epitaxial growth of crystals apatite.[10] Other areas of the demineralized dentin depleted from these seeds will remain un-mineralized. In order to have reliable bottom-up dentine remineralization, evidence of minerals formation should be detected within intra-fibrillar and extra-fibrillar demineralized collagen fibrils.[11]

Accordingly, the presence of biomimetic analogs, to non-collagenous proteins (NCPs) in dentine matrix, has an important role in such situations even with the advancement of variable ion-releasing materials. These proteins play critical roles in controlling apatite nucleation and growth in collagenous tissues.[12] It was proved that addition of NCPs analogs to MTA formula significantly improved remineralization of demineralized dentine whether dentine adhesive was applied or not.[8] Despite of previous findings, no attempt to apply biomimetic analogs with recent formulations of capping materials was used. Thus the current study aimed to asses the remineralizing ability of these pulp capping materials and if it is still efficient enough if NCPs analogs are not applied. The null hypothesis is that the calcium silicate and calcium hydroxide based capping materials' remineralizing ability will not be affected with the presence of NCPs analogs.

Materials and Methods

Two commonly used capping cements were used, one based on calcium silicate and resin-free (Biodentine™) and the other resin-based (Lime-lite™) with biomimetic analogs. Details of the used materials are mentioned in Table 1. Thirty-seven freshly extracted human third molars (due to periodontal or impaction reasons) were collected. All teeth were intact, non-carious and unrestored. Each tooth was thoroughly washed under distilled water. A sharp hand sickle scalar (Prima-Dent international, Frank. F Germany) was used to remove any soft tissue remnants. The teeth were stored in 0.1 wt. % Thymol in distilled water at 4°C till the time of cavity preparation and testing.[13] The occlusal enamel of the crowns was totally removed using a diamond wheel bur (Wr-13 high speed wheel shape

diamond dental bur, Shenzhen Dian Fong Abrasives Co, Guangdong, China)

fixed in water-cooled high-speed contra handpiece (Sirona T3 Racer, Dentsply, Germany). The dentin surfaces were thoroughly checked by eye inspection and diagnostic probe for absence of enamel and/or pulp tissue. After that, Two standard occluso-proximal circular cavities (2 mm diameter x 0.5 mm depth) were prepared within each dentin surface using a small diamond wheel bur (low speed wheel shape diamond dental bur, Shenzhen Dian Fong Abrasives Co, Guangdong, China) of diameter 2 mm and height 0.5 mm fixed in water cooled low speed contra hand-piece (External spray NSK Ex-203 low speed dental handpiece, China). All dentin surfaces were coated with two layers of acid-resistant varnish (nail polish, Revelon, Paris, France) except the internal cavities' surfaces and a small rim of dentin (1 to 2 mm) surrounding each cavity. The apices of the roots were closed with composite resin (Capo universal, Mani Schutz Dental, Rosbach, Germany).

Artificial caries induction and biomimetic analogs preparation

It was induced by pH cycling within the uncoated dentin surfaces. Phosphate containing simulated body fluid was prepared (SBF) with the same protocol previously utilized.[14] The teeth were cycled in 150 ml of both solutions for 8 hours in the demineralizing solution and 16 hours in the remineralizing solution. Both solutions were refreshed in each new cycle. This procedure was carried out for 14 days at room temperature without agitation.[15] After pH cycling, each tooth (specimen) was rinsed with distilled water and air dried and kept in distilled water for one day before testing. A solution of 2.5 wt. % Sodium trimetaphosphate (STMP) was prepared.[16] Since protein phosphorylation with STMP requires alkaline hydrolysis into linear form, it was first prepared at pH 12 for 5 hours then neutralized to pH 7.4 using HCl and NaOH buffer solutions.[17] 500 µg/ml (0.025 gram) of poly acrylic acid (PAA) powder was dissolved in 50 ml deionized water then the solution was buffered to pH 7.4 using NaOH and HCl buffer solutions. After pH cycling, one cavity in each tooth, with its demineralized surrounding rim, was then coated with two layers of the previously prepared STMP solution using micro applicator and left to dry for five minutes. After drying, the same cavity was coated with two layers of previously prepared PAA solution using another micro applicator then left for another five minutes to dry while the other cavity in each tooth was left uncoated. Sixteen teeth with coated and uncoated cavities were used for each of the tested materials (Biodentine and Lime-lite). Each tooth had two (coated and uncoated) cavities, which were filled with the same tested material according to manufacturer's instructions. A group of untreated teeth were left without any capping materials to be used as control. Afterwards, all teeth were sectioned vertically in bucco-lingual direction into two halves using microsaw (Isomet 4000 microsaw Buehler, USA) at 2500 rpm speed under copious water spray coolant. Each half contained area of sound protected dentin and one restored cavity surrounded by a rim of demineralized dentin. The teeth halves with restored untreated cavities with biomimetic analogs were stored in 150 ml of SBF. The other teeth halves with restored and coated cavities were stored in SBF with 500 µg/ml (0.5 g/l) PAA at ambient temperature. Both

simulated body fluids were refreshed weekly. The specimens were collected at intervals of 1, 6 and 12 weeks to monitor remineralization process.

X-ray Spectroscopy (EDX)

Eight specimens of each subgroup were collected after the three above intervals. They were dehydrated in graded ethanol series (70, 80, 90 for 30 minutes each and 99% for 1 hour) and stored in a dryer for 24 h.[18,19] The mineral content on demineralized rim around each cavity was evaluated using EDX apparatus (JEOL, Oxford X-Max 20, U.K) attached to JSM-6510LV Scanning Electrone Microscope (JEOL, Tokyo, Japan).

Scanning Electrone Microscope

Three randomly selected specimens were taken from each subgroup (n=3 per each subgroup and storage period). Vertical sections for each specimen were obtained through cutting them using a microsaw. The sections were first coated with gold prior to examination. The surface of the dentinal axial wall of each cavity in each coated section was examined using SEM (Quanta FEG 250, FEI company, Czech

the least mean values of Ca levels especially after 1st and 6th week of storage (14.913±1.709, 11.903±0.982,

Table 1. Materials used in the current study.

Materials	Specification	Composition	Manufacturer	Lot No
Lime-lite ™	Light cured resin modified calcium hydroxide.	Paste: hydroxyapatite in a urethane dimethacrylate resin.	Pulpdent Corporation, Watertown, MA, USA.	160601
Biodentine	Bioactive calcium-silicate-based cement	Powder: tri- calcium silicate (C_3S), di-calcium silicate (C_2S), calcium-carbonate, iron oxide and zirconium oxide. Liquid: calcium chloride and hydrosoluble polymer (poly-carboxylate).	Septodont Corporation, PA, France	B17297
Poly acrylic acid (PAA).	Biomimetic analog.		Sigma-Aldrich, St-Louis, MO, USA.	SLBQ2339V
Sodium trimetaphosphate (STMP).	Biomimetic analog (Na3P3O9)		Sigma-Aldrich, St-Louis, MO, USA.	SLBQ2339V

Statistical analysis.

Data were fed to the computer and analyzed using **SAS (2004)** software version 9.1.3 using the general linear models (GLM). Quantitative data were described using means and standard deviations. *Two Way ANOVA test was used* for parametric data, to compare between studied groups. When the *F*-statistic was significant ($P < 0.05$), a mean separation was performed using the least significant difference (LSD) test.

Results

X-ray Spectroscopy (EDX)

Biodentine + BAs groups showed the highest mean values of Ca levels, except for sound dentin groups, especially after 6th and 12th week of storage (23.653 ± 3.071, 23.117 ± 1.124, respectively). On the other hand, Lime-lite groups showed

respectively). There was a statistically significant difference among all tested materials groups ($p < 0.05$) within the three different storage periods (1, 6 & 12 weeks). Similarly, there was a statistically significant difference of Ca levels ($p < 0.05$) among the three tested Lime-lite + BAs groups of different storage periods. Lime-lite + BAs group after 12 weeks of storage showed the highest mean Ca value (20.967 ± 1.984) among Lime-lite groups, while Lime-lite group

after 6 weeks of storage showed the least mean Ca value (11.903 ± 0.982). Mean values and standard deviations

(SDs) of Ca levels (wt %) of all tested groups at different storage periods are shown in Table 2 and Figure 1.

Table 2: Mean values and standard deviations (SDs) of calcium levels (wt %) of all tested groups at different storage periods.

Groups / Periods	Sound dentin	Biodentine + biomimetic analogs	Biodentine	Lime-lite +biomimetic analogs	Lime-lite	P value
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	
1st week	24.333±4.107^{Aa}	23.011±4.987^{Aa}	19.143±1.339^{ABa}	15.283±0.805^{Bb}	14.913±1.709^{Ba}	0.0043*
6th week	24.580±4.012^{Aa}	23.653±3.071^{Aa}	20.733±3.237^{Aa}	14.083±1.076^{Bb}	11.903±0.982^{Ba}	0.0006*
12th week	23.158±1.427^{Aa}	23.117±1.124^{Aa}	21.543±1.502^{Aa}	20.967±1.984^{Aa}	15.270±3.282^{Ba}	0.0013*
P value	0.7986	0.9787	0.4470	0.0019*	0.2035	

*: statistically significant.
A-b = Means with the same capital letter in each row are not statistically significant different at $p \leq 0.05$.
a-b = Means with the same small letter in each column are not statistically significant different at $p \leq 0.05$.

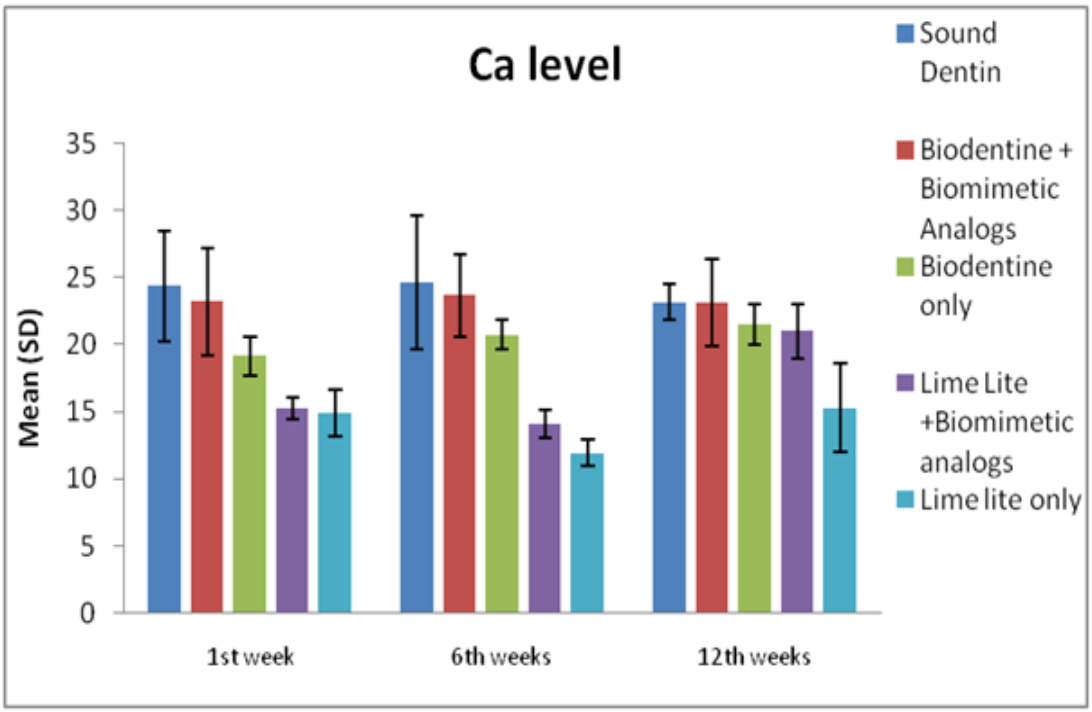

Figure 1: Bar chart showing mean values and standard deviations (SD) of calcium levels of all tested groups at different storage periods

Biodentine + BAs groups showed the highest mean values of P levels, except for sound dentin groups, especially after 6th and 12th week of storage (11.213 ± 1.349, 13.237 ± 0.508, respectively). On the other hand, Lime-lite groups showed the least mean values of P levels especially after 1st and 6th week of storage (9.547 ± 0.885, 8.107 ± 0.590, respectively). There was a statistically significant difference among all tested materials groups ($p < 0.05$) after one week of storage. Lime-lite + BAs group after 12 weeks of storage showed the highest mean P value (11.383 ± 2.585) among Lime-lite groups, while Lime-lite group after 6 weeks of storage showed the least mean P value (8.107 ± 0.590). Mean values and standard deviations (SDs) of P levels (wt %) of all tested groups at different storage periods are shown in Table 3 and Figure 2.

Scanning Electrone Microscope

Figure 3 shows SEM image of horizontal section of negative control group (demineralized dentin) with open dentinal tubules due to artificial caries induction process.

SEM images of vertical sections of demineralized dentin adjacent to tested materials show obliteration of open dentinal tubules with more homogenous and smooth surface either in presence or absence of BAs (Fig. 4A, B, C & D).

Table 3: Mean values and SDs of phosphorus levels (wt %) of all tested groups at different storage periods.

Groups / Periods	Sound dentin	Biodentine + biomimetic analogs	Biodentine	Lime-lite +biomimetic analogs	Lime-lite	P value
	Mean±SD	Mean±SD	Mean±SD	Mean±SD	Mean±SD	
1st week	13.675±1.285^{Aa}	12.890±3.008^{Aa}	10.123±0.707^{Ba}	10.027±0.465^{Aa}	9.547±0.885^{Ba}	0.0046*
6th week	11.825±2.740^{Aa}	11.213±1.349^{ABa}	9.580±1.077^{ABa}	8.393±0.597^{Ba}	8.107±0.590^{Ba}	0.0531
12th week	13.633±0.948^{Aa}	13.237±0.508^{Aa}	11.597±1.735^{Aa}	11.383±2.585^{Aa}	9.863±1.786^{Aa}	0.1111
P value	0.2558	0.4390	0.2037	0.1397	0.2402	

*: statistically significant.

A-b = Means with the same capital letter in each row are not statistically significant different at $p \leq 0.05$

a-b = Means with the same small letter in each column are not statistically significant different at $p \leq 0.05$.

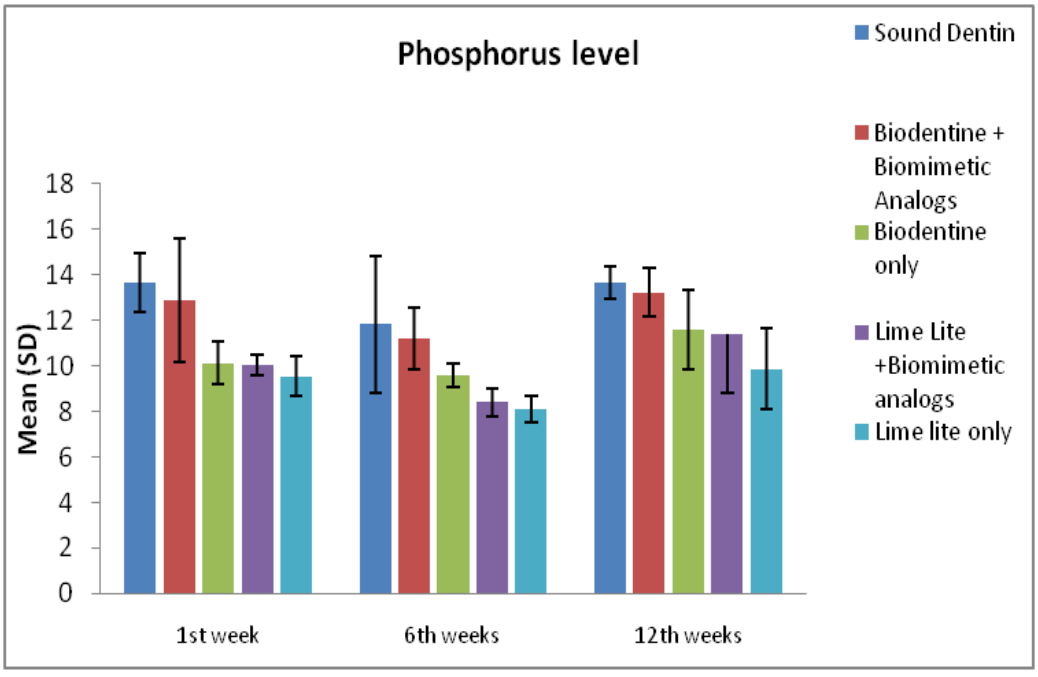

Figure 2: Bar chart showing mean values and SD of phosphorus levels of all tested groups at different storage periods.

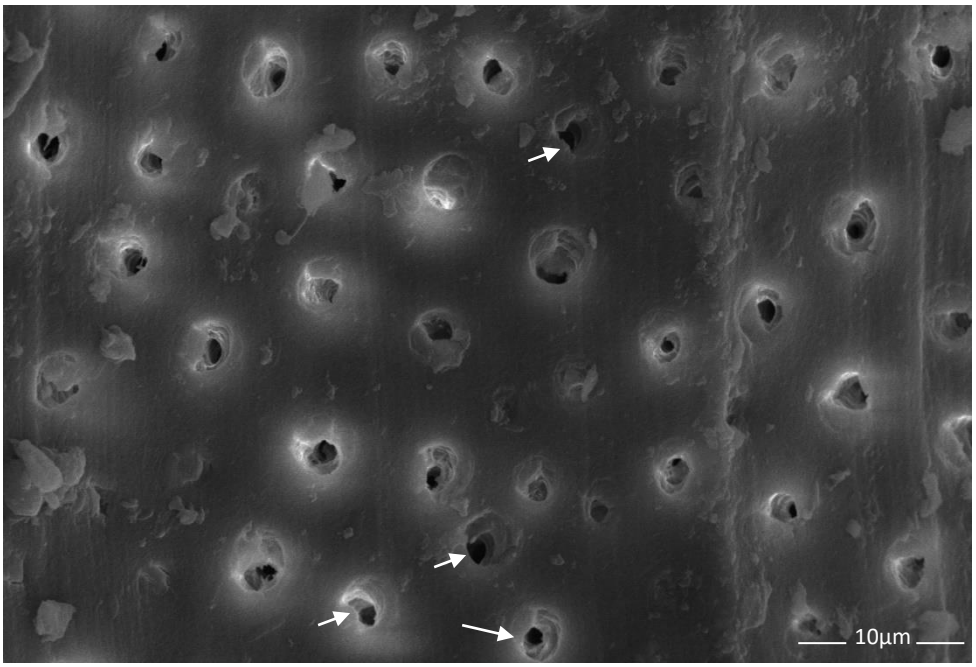

Figure 3. SEM image of horizontal section of negative control specimen (demineralized dentin) showing open dentinal tubules (arrows) (Magnification: 2000x).

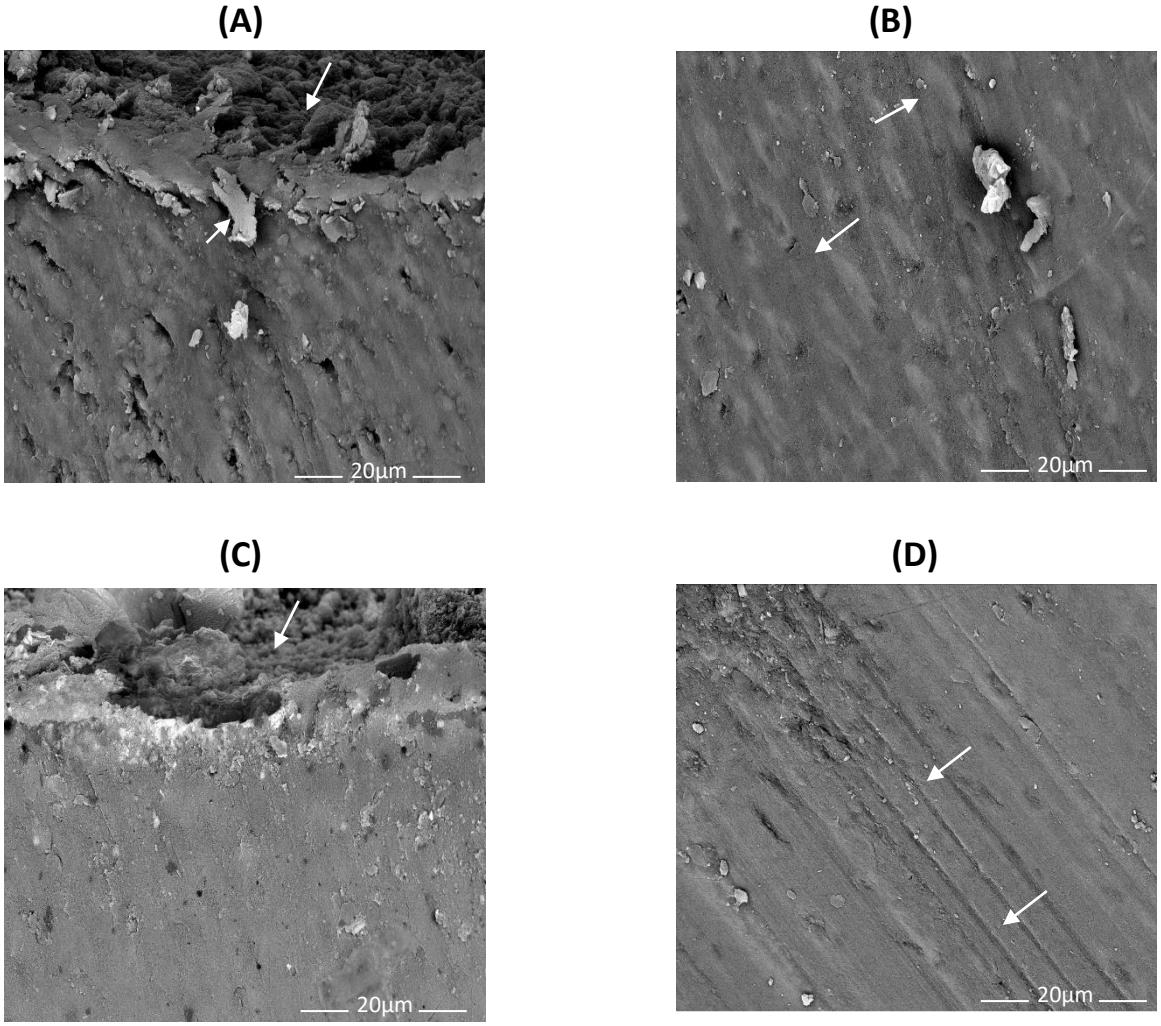

Figure 4. SEM images of vertical sections of two groups (sections parallel to tooth long axis). (A) Biodentine – dentin interface with remnants of the remineralizing material (arrows), (B) Longtidunal view of dentinal tubules (arrows) beneath a cavity filled with biodentine, (C) Lime-lite + BAs – dentin interface with remnants of the remineralizing material (arrow), (D) Longtidunal view of dentinal tubules (arrows) beneath a cavity filled with Lime-lite + BAs (Magnification: 5000x).

Discussion

Calcium silicate and calcium hydroxide based materials are considered to be the most commonly used remineralizing agents. Their ability to release different remineralizing ions such as: Ca, P and hydroxyl (OH) ions is considered to be the key factor for successful remineralization.[20] They create alkaline environment, which is a favorable condition for antibacterial/bacteriostatic action.[21] This alkalinity could encourage apatite formation in phosphate-rich fluids.[22]

Despite of their advantages, calcium silicate and calcium hydroxide based biomaterials still have some drawbacks and the remineralization process is still not complete.[10,23] Non-collagenous proteins that found in sound dentin play important role in dentin biomineralization especially intrafibrillar mineralization.[24] However, they are lost (denaturated) during caries process.[25] Thus, the use of NCPs analogs in conjugation with remineralizing biomaterials enhanced the remineralization process.

The present study was conducted to investigate the remineralization potential of two pulp capping materials with/without the use of NCPs biomimetic analogs. The first is calcium silicate based and resin free cement (Biodentine™ Septodont, France). It forms calcium hydroxide as a by-product of hydration.[26] The calcium silicate resin free cement is bioactive because it increases pulp cell proliferation and biomineralization.[27] After time, the formation of homogenous dentin bridge is observed at the injury site.[28] Tricalcium silicate based materials are also defined as a source of hydroxyapatite when they are in contact with synthetic tissue fluid.[29] In addition, it is biocompatible, easily handled product, has short setting time and high surface microhardness.[30,31] The second is resin based calcium hydroxide cement (Lime-lite™, Pulpdent, USA). According to the manufacturer, it has many advantages. It releases favorable remineralizing ions such as, Ca, OH, P and fluoride (F) ions. It stimulates secondary dentin formation and sets into extremely hard and insoluble mass in water & oral fluids. It chemically bonds to adhesives and composites. In addition, it is easy in manipulation as it is delivered in convenient syringe with dispensing tips.[32]

Biomimetic mineralization method used in this study, was based on the use of polyanionic molecules to mimic the functions of matrix proteins in biomineralization together with remineralizing capping materials.[10] Poly-acrylic acid {(PAA, $C_3H_4O_2)_n$} based biomimetic analog was used as a sequestration agent which has the ability to stabilize remineralizing ions derived from the set pulp capping materials and SBF in the form of liquid-like nanoparticles so they could penetrate the water compartments of collagen fibrils.[33] Sodium trimetaphosphate (STMP, $Na_3P_3O_9$) a phosphorus-based analog that mimics the collagen-binding function of matrix phosphoproteins was also used. The phosphate groups of STMP rendered immobile on the collagen fibrils to attract calcium ions and direct apatite crystals nucleation within the gap zones of collagen fibrils (intrafibrillar apatites).[11]

Artificial caries-like affected dentin instead of natural caries-affected dentin was employed in this study. A great variety of approaches had been developed to prepare artificially demineralized dentin, including the application of acidic

solutions,[34] the use of a pH-cycling protocol,[8] and the presence of cariogenic bacteria.[35] The latter microbiological caries-induction system intends to mimic caries development using either a single bacterial strain of S. mutans or the co-culture of S. mutans and Actinomyces viscosus as acid producers. However this microbial method resulted in a layer of degraded collagen, which was morphologically more similar to natural caries-infected lesions. Thus, pH-cycling model was used in this study as it mimics the natural dynamic process of caries formation that includes alternating de- and re-mineralization.

The pH-cycling model has some limitations. The duration of demineralization and remineralization periods is not known. Further, this method did not employ saliva and biofilm. The oral environment complexity is certainly the reason why pH-cycling model of caries lesions induction produced shallower caries lesions than naturally created ones.[35]

X-ray Spectroscopy (EDX) Results.

In this study, the effectiveness of dentin remineralization induced by remineralizing agents with/without the use of BAs was quantified by EDX elemental analysis. Generally, EDX displayed that Biodentine groups showed higher content of Ca and P surface ions on DMD than Lime-lite groups. This result is in agreement with the result of a previous study [3] that correlated the high Ca release of Biodentine with the presence of a calcium silicate component in powder and calcium chloride in liquid. In addition, its liquid contains a hydrosoluble polymer that serves as a water reducing agent and allows for Biodentine prolonged hydration.[30] Calcium carbonate component in powder could also act as Ca based matrix. It is one of the most abundant and important biominerals in nature. It is deposited by osteoblasts together with calcium phosphates during the mineralization process and its both natural and synthetic forms are now used as biomaterials for tissue engineering. On the other hand, Lime-lite showed less Ca ions content on DMD surface through the three periods of storage than Biodentine. It may be attributed to the presence of urethane dimethacrylate resin in its composition.[36] This resin-based heterogenous structure may impede enough moisture diffusion within the material which is necessary for complete material hydration and dissociation for ions release.[37,38] Un-like polymer-free commercial calcium hydroxide based capping materials (Calxyl® and Dycal®), Lime-lite showed lower Ca ions leaching.[3] However, Lime-lite in the presence of BAs showed statistically significant increase of remineralizing ions content, especially Ca and P, within adjacent DMD after total period of storage. That improvement within

remineralizing ability may be attributed to the ability of BAs to achieve intrafibrillar ions deposition.[11,34]

At different periods of storage, Biodentine with BAs groups showed the highest Ca and P content. While, Lime-lite groups showed the least Ca and P content. Significant increase in Ca & P ions which are essential for remineralization was detected from the first week. However up on 12 weeks storage, Ca and P ions content was higher for the remineralization zones induced by capping materials

with the presence of biomimetic analogs. This finding is in agreement with a previous study [39] that explained this time-

dependent beneficial effect of biomimetic analogs could be associated with their relatively slow release kinetics. An earlier study reported a continuous release of STMP from set MTA blocks during 6 weeks. This ion release may have been promoted in two ways: first, the dual biomimetic analogs ability to stabilize Ca and P as well as ordered deposition of intrafibrillar apatite platelets. Second, STMP can serve as a supplementary phosphate source that may promote P release as well.[8] This study also reported that the close and long-term proximity of the biomimetic analogs with the artificial caries-like lesions (as being incorporated within the ion releasing material itself not in the SBF) fastened the ion release kinetics. This also may give an acceptable explaination for the improved releasing ability induced by Lime-lite with BAs. The latter group had statistically higher remineralizing ions content for the longest period of storage (12 weeks) than the 6 weeks storage period.

Scanning Electrone Microscope Results.

Scanning electron microscope images of Biodentine-dentin interface showed obliteration of opened dentinal tubules that resulted in artificial carious dentin due to pH-cycling. These findings were previously reported for Biodentine-dentin interface even in absence of BAs.[40] Similar SEM images were obtained for Lime-lite-dentin interface. These results are in agreement with the results of a previous work which use ion releasing composite-resin material in conjugation with BAs.[41] Scanning electron microscope findings may be attributed to that all materials showed significant enrichment of demineralized dentin areas with Ca and P ions ready for remineralization process. In addition, SEM images are qualitative approach for these surfaces without ability to quantify the content of these ions.

Conclusions: 1- Calcium silicate and resin free cement (Biodentine) showed the best ability to enrich the artificial carious dentin with ions for remineralization. 2- Both tested materials have the ability to obliterate open dentinal tubules after total period of storage. 3- Prolongation in the storage period had more significant improvement in remineralizing process.

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