THE EFFECT OF GYPSUM EXTENSION ON A MIXTURE OF CARBOXYMETHYL CHITOSAN AND AMORPHOUS CALCIUM PHOSPHATE IN DENTAL REMINERALIZATION

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ABSTRACT

INTRODUCTION: Carboxymethyl chitosan/amorphous calcium phosphate (CMC/ACP) is a non-collagenous protein analog material with dentin remineralization ability, and gypsum is a mixing material that facilitates the clinical application. Gypsum is a natural material used to release the regenerative calcium ion and has a good biological response, with osteoconductive, resorbable, and compatible properties.

OBJECTIVES: The aim of this study was to examine the effect of the addition of gypsum to the non-protein analog material CMC/ACP in dentin remineralization.

MATERIAL AND METHODS: Tooth samples for this study were taken from patients with tooth imprints at the Maxillofacial Surgery Clinic. Twenty-seven cavities were divided into three groups: dentin demineralization without any material applied, application of dentin demineralization with CMC/ACP, and application of dentin demineralization with gypsum + CMC/ACP. The results were elucidated on Day 14, using scanning electron microscopy with energy-dispersive X-ray. The data were analyzed using Statistical Package for the Social Sciences version 22 software (IBM Corp., Armonk, NY, USA), analysis of variance (ANOVA) one-way statistic test, and Kruskal-Wallis test.

RESULTS: Gypsum showed a positive effect on the ability of CMC/ACP in dentin remineralization. The edge of the dentin tubules appeared to be irregular and smaller, with some covered up. This shows remineralization occurrence after the bio-active material of the CMC/ACP and gypsum application.

CONCLUSIONS: The addition of gypsum to the CMC/ACP has a positive effect in dentin remineralization.

KEY WORDS: chitosan, amorphous calcium phosphate, gypsum, calcium, phosphate.

J Stoma 2020; 73, 2: 69-73
DOI: https://doi.org/10.5114/jos.2020.96021

INTRODUCTION

The caries process causes dental collagen matrix degredation by proteolytic enzyme application. The outer layer of caries is the caries-infected dentin (CID) band and cannot be remineralized, while below the CID, the caries-affected dentin is located, which has a collagen matrix structure. This is an intact layer with vanishing minerals, despite its remineralization [1].

The remineralization process can occur in two ways, conventional remineralization, and guided tissue remineralization (GTR). Conventional remineralization can occur by utilizing the remaining apatite crystals, while GTR is a biomimetic mineralization process. The formation of apatite crystals in GTR can arise even though there are no residual apatite crystals but requires non-collagen proteins to occur. Non-collagen proteins are generally damaged due to caries, so there is a need...
for substitute non-collagen protein analog material to trigger GTR [2].

Amorphous calcium phosphate (ACP) is a solvent consisting of dense phosphate calcium particles that form in an amorphous arrangement. A significant amount of carboxyl group material is required to stabilize the ACP and fasten the calcium ion [3]. Carboxymethyl chitosan (CMC) acts as the protein analog of non-collagen, since it has several carboxyl groups and can fasten the solvent of ACP nano precursor to become CMC/ACP [4-6]. A previous study proved the ability of CMC/ACP in the process of GTR with transmission electron microscope (TEM) to analyze the intrafibrillar remineralization at gap zone collagen [7, 8]. The gel is a good form of CMC/ACP, although it is difficult to apply to dental tissues, it requires an addition of extension material to ease its use.

Gypsum is a natural material used to release the regenerative calcium ion and has a good biological response, with osteoconductive, resorbable, and compatible properties [9, 10]. According to Subhi et al., chitosan and BMP2 are essential materials in direct pulp capping for promoting the physical and mechanical characteristics as well as the cellular effects on dental pulp stem cells [11]. Its pH is nearly neutral, with a setting time and compressive strength that are better than those of Dycal treatment. It produces good viability and increases the phosphoric alkaline activity on the third day in the cell [12]. The hypothesis is that gypsum extension does not have a negative effect on the ability of CMC/ACP non-collagen protein analog material in dentin remineralization.

OBJECTIVES

The aim of this study was to examine the effect of the addition of gypsum to the non-protein analog material CMC/ACP in dentin remineralization.

MATERIAL AND METHODS

The Ethics Dental Research Commission of Faculty of Dentistry, Universitas Indonesia approved the research, protocol number 051171218. Tooth samples for this study were taken from patients with tooth impactions at the Maxillofacial Surgery Clinic. Patient consents were obtained prior to the study. Nine intact teeth free from caries were extracted and then maintained at 40°C temperature in phosphate-buffered saline solvent. A total of 27 cavities with depth and diameter of about 3 mm, using a cylindrical diamond size no. 16 (Meisinger, Neuss, Germany) were produced. All samples were filled with 17% ethylene diamine tetra acetic acid (Merck, Kenilworth, NJ, USA) in the cavity for forced demineralization and stored in a shaker incubator (100 RPM) at 37°C temperature for seven days. After seven days of demineralization, all tooth cavities were rinsed with aquabidest (WaterOne, Jakarta, Indonesia), and then soaked using 1M of NaCl, pH 7.0 (Merck, Kenilworth, NJ, USA) for eight hours at 25°C temperature. This process is meant to remove all soluble parts and maintain non-collagen protein in dentin [7, 8].

According to Chen et al. [7], CMC/ACP mixture was made using 2.5 g of CMC (Chimultiguna, Jakarta, Indonesia) mixed with 40 ml of water to produce a gel, followed by addition of 0.498 g of K$_2$HPO$_4$ (Merck, Kenilworth, NJ, USA). Next, CMC gel formed from 0.555 g of CaCl$_2$ (Merck, Kenilworth, NJ, USA) in 10 ml of water was added to form the CMC/ACP gel. This gel was subsequently frozen at −80°C temperature for two hours and lyophilized by freeze-drying for six hours in a freezer dryer (Buchi, Flawil, Switzerland).

In an electric oven (Memmert, Schwabach, Germany), the gypsum (Merck, Kenilworth, NJ, USA) was heated at 110°C temperature for three hours to change the calcium sulfate dihydrate to hemihydrate [12]. To obtain the mixture of gypsum and CMC/ACP 1 g of gypsum with 0.6 ml of the CMC/ACP gel was heated and stirred until putty consistency was reached. It was then ready to be used.

All demineralized cavity samples were divided into three groups. The first group included nine samples without treatment immediately filled with a temporary filling (control), while the second group was filled with CMC/ACP at the base of cavity and then closed with a temporary filling. The samples from third group was filled with a mixture of gypsum and CMC/ACP at the base of cavity and then closed with a temporary filling. Subsequently, all samples were soaked in phosphate-buffered saline and stored in a shaker incubator (Stuart SI500, T-Equipment, Long Branch, NJ, USA) set to 100 RPM at 37°C temperature for 14 days [7, 8]. Once the samples were removed from the incubator, the crown areas were cut off down to the base of cavity and fixed with gradual dehydration [7, 8]. Each sample was analyzed for dentin morphology using scanning electron microscopy (SEM) and the amount of calcium and phosphate was analyzed by energy-dispersive X-ray (SEM-EDX VEGA 3 TESCAN; Tescan Analytics, Fuveau, France). The data obtained were tested for normality using Schapiro-Wilk test because the number of samples was less than 50.

The data of phosphate and calcium were analyzed using Statistical Package for the Social Sciences version 22 software (IBM Corp., Armonk, NY, USA), analysis of variance (ANOVA) one-way statistic test, and Kruskal-Wallis test, with a rating of significance of less than 0.05.

RESULTS

The distribution results of the Schapiro-Wilk test were normal and homogeneous; therefore, they were tested further using one-way ANOVA with post-hoc Tamhane test. Table 1 shows different amount of calcium mineral level in the three groups. Evidently, group 3 presented...
TABLE 1. Mean value, standard deviation, and significance between calcium groups (%)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Mean (SD)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9</td>
<td>15.8% (1.0320)</td>
<td>≤ 0.001*</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>23.2% (4.1394)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>9</td>
<td>27.5% (5.3018)</td>
<td></td>
</tr>
</tbody>
</table>

*Descriptive statistic test of one-way ANOVA, p ≤ 0.05. Group 1: dentine demineralized without material content. Group 2: dentine demineralized with CMC/ACP application. Group 3: dentine demineralized with CMC/ACP + gypsum application

TABLE 2. Calcium level significance between groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.001*</td>
<td>0.000*</td>
</tr>
<tr>
<td>2</td>
<td>0.097</td>
<td></td>
</tr>
</tbody>
</table>

*Post-hoc paired wise comparison with Tamhane test, p ≤ 0.00. Group 1: dentine demineralized without material content. Group 2: dentine demineralized with CMC/ACP application. Group 3: dentine demineralized with CMC/ACP + gypsum application

TABLE 3. The mean, median, minimum, and maximum values of phosphate level (%)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Median (Min-Max)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9</td>
<td>7.2% (5.8-8.9)</td>
<td>≤ 0.001*</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>11.0% (10.0-15.1)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>9</td>
<td>13.9% (10.8-18.6)</td>
<td></td>
</tr>
</tbody>
</table>

*Descriptive statistic of Kruskal-Wallis test, p ≤ 0.05. Group 1: dentine demineralized without material content. Group 2: dentine demineralized with CMC/ACP application. Group 3: dentine demineralized with CMC/ACP + gypsum application

TABLE 4. The significance value of phosphate level between groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Group 2</th>
<th>Group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.001*</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.000*</td>
<td></td>
</tr>
</tbody>
</table>

*Post-hoc test Mann-Whitney, p ≤ 0.05. Group 1: dentine demineralized without material content. Group 2: dentine demineralized with CMC/ACP application. Group 3: dentine demineralized with CMC/ACP + gypsum application

the highest mean. Therefore, CMC/ACP and gypsum increased the dentin calcium level.

In Table 2, the calcium level significance between groups was calculated using post-hoc paired wise comparison with Tamhane test (p ≤ 0.05), and there were significant differences between groups 1 and 2 and groups 1 and 3, whereas discrepancies between groups 3 and 2 were not significant. This suggests that CMC/ACP and gypsum improved the dentin calcium level significantly.

Phosphate data distribution was not normal, so non-parametric statistic testing was done using Kruskal-Wallis with post-hoc Mann-Whitney U testing. In Table 3, the highest phosphate level was in group 3, followed by

FIGURE 1. Scanning electron microscopy result with 10x magnification. A) Group 1, B) group 2, C) group 3. Red arrow: debris, yellow arrow: regular dentin tubule, green arrow: irregular dentin tubule
group 2 and group 1. The value of \( p \leq 0.001 \) was found when compared with the three groups. Therefore, CMC/ACP and gypsum improved the dentin phosphate level.

The significance of the phosphate level between groups was calculated using post-hoc Mann-Whitney \( U \) test (Table 4), which revealed significantly different results in group 1, group 2, and group 3, with \( p \leq 0.05 \) for the content of phosphate mineral level.

In group 1, the dentin morphology surface indicated an opened dentin tubule with regular edge form, which means that the affected teeth would lose mineral and collagen matrix because of undergoing demineralization process (Figure 1A). In group 2, irregular dentin of the tubule edge can be seen (Figure 1B), which means that a remineralization process occurred after demineralized samples in group 2 received CMC/ACP. While in group 3, the edge of dentin tubules appeared to be irregular and smaller, with some covered up (Figure 1C). This shows that the remineralization in group 3 had occurred more than in group 2 after the bio-active material of CMC/ACP and gypsum was applied.

DISCUSSION

In this study, the results indicate that the levels of phosphate and calcium increased in the CMC/ACP and CMC/ACP and gypsum groups. The most notable results occurred in the group with added gypsum (Tables 1 and 2). The improvement of calcium and phosphate from control to CMC/ACP and CMC/ACP and gypsum indicates that there was a remineralization process that took place. This is in accordance with previous research on CMC/ACP by Chen et al. and Annisa et al., who concluded that dentin remineralization takes place in deep caries [7, 8]. In the present study, in the group with gypsum, the level of improvement of ionized calcium was not significant, but the level of phosphate was significant. The phosphate level improvement was likely caused by the ACP ingredient in CMC/ACP. According to Combes et al., remineralization is also influenced by the surrounding environment containing calcium mineral and phosphate [13].

The highest level of phosphate in group 3 was an indication of biomimetic remineralization in collagen [14]. However, there is a need for further research with an examination by TEM observation to find out whether the remineralization that occurred is intrafibrillar or guided tissue remineralization.

Our data show that there were different calcium levels than for phosphate. Evidently, the calcium level was much higher due to the addition of gypsum. The high number of calcium ion shaped the apatite crystal at the 14th examination day, leading to a non-significant distinction from the other groups, with the morphology alteration of dentin tubule observed and shown in SEM. There was a possibility of the phosphate and calcium levels being the same or different.

Calcium and phosphate ion can form a hydroxyapatite precursor before forming a hydroxyapatite crystal, so calcium and phosphate ion can reach narrow and complicated areas within the dentin structure, resulting in extrafibrillar and intrafibrillar remineralization.

However, the formed hydroxyapatite was not always realized through the apatite precursor. This precursor was usually in the form of hydrated calcium phosphate and phosphate octacalcium. There was precipitation, followed by the formation of hydroxyapatite. ACP was a hydroxyl apatite precursor [13].

As seen in group 3 of this study, the number of calcium ions and the phosphate on the 14th day were still high as compared to group 2. This may happen because the calcium ion and the phosphate are still in the forms of hydroxyapatite precursor and phosphate octacalcium; therefore, group 3 at the 14th day had only immature hydroxyapatite. According to Combes et al., there is phosphate octacalcium in the immature hydroxyapatite. The process of remineralization is longer when the hydroxyapatite forms through the phosphate octacalcium. Such was marked by the calcium mineral level and phosphate around the environment, which was relatively high. Therefore, the forming of hydroxyapatite and the ripeness was slower [13].

In group 2, there was a lower calcium mineral level than in group 3. However, there was also a different calcium and phosphate level improvement that was statistically significant as compared to group 1. Based on SEM analysis results, there was an alteration of the dentin edge tubule from regular to irregular, which means that a remineralization process occurred in group 2, in line with studies by Chen et al. and Annisa et al. [7, 8]. Due to high amount of phosphate and calcium in the remineralization process, the outcome was optimized, producing a stronger dentin structure. This was because of the occurrence of extrafibrillar and intrafibrillar remineralizations, which established dentin collagen fiber. As such, it generates a good dentin mechanical character in accepting the mastication pressure. The limitation of this study was the use of SEM only and not including TEM.

CONCLUSIONS

The results of our study showed that gypsum extension did not have a negative effect on the ability of CMC/ACP non-collagen protein analog material on dentin remineralization, but rather improves dentin remineralization by increasing the level of calcium and phosphate mineral.

ACKNOWLEDGEMENTS

I would like to express my deep gratitude to my research supervisors for their patient guidance, enthusiastic encouragement, and useful critiques of this study.
I would also like to extend my thanks to the technicians of the laboratory of Gestrindo Sakti Utama for their help in offering me the resources in running the SEM and EDX programs. Finally, I wish to thank my family for their support and encouragement throughout my study.

CONFLICT OF INTEREST

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

References