

The Potency of Carboxymethyl Chitosan/Amorphous Calcium Phosphate (CMC/ACP) as a Noncollagen Protein Analog to Dentine Remineralization

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Abstract

Carboxymethyl chitosan/amorphous calcium phosphate (CMC/ACP) is a noncollagen protein analog material that may serve as a regulator of the intrafibrillar remineralization mechanism through the guided tissue remineralization (GTR) approach. **Methods:** Eight 3-mm depth demineralized cavities were divided into two groups: group 1 cavities were immediately filled with temporary restoration; while group 2 received CMC/ACP first and only then were filled with temporary restoration. All samples were immersed in phosphate buffered saline (PBS) and were stored in a 37 °C shaking incubator. Remineralization evaluation with scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX) was done on day seven. **Results:** In group 2, SEM images showed the presence of irregularity in dentinal tubules, and EDX analysis showed significantly increased calcium and phosphate levels in dentinal tubules. **Conclusion:** CMC/ACP has the potency to remineralize dentine.

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Introduction

Dentine collagen is composed of tropocollagen, which is three chains of unified polypeptide or triple helix. This tropocollagen forms fibrils with a gap at the end called a gap zone. Tropocollagen is approximately 300 nm long and the gap zone is 67 nm wide. Minerals in collagen is divided into two types; namely intrafibrillary minerals found in the gap zone, and extrafibrillary minerals contained in the interstitial space of collagen fibrils.¹ Some researchers claim that intrafibrillary mineralization contributes greatly to elasticity and hardness of dentine.²

Dentine can remineralize in two ways; conventional/top-down/classical ion-based remineralization, and guided-tissue (GTR)/nonclassical remineralization.³ Conventional remineralization occurs epitaxially; that is, mineral deposition over the underlying mineral is

present and occurs in an extrafibrillary manner.⁴ Conventional remineralization cannot occur spontaneously in organic matrix. Instead it occurs in residual apatite crystals which are present in demineralized dentine carious lesions.⁴ Guided Tissue Remineralization is remineralization through a bottom-up method, that is, remineralization occurs through the formation of nano crystals that may enter into the gap zone between collagens and build a larger apatite mineral structure in the collagen.^{4,5} Histopathologically, dentine caries can be divided into two types; infected, and affected dentine. In affected dentine, intrafibrillar remineralization can occur because the collagen crosslinks remain intact.⁶

In the process of dentine remineralization, two components have an important role, these being collagen and noncollagen protein. Collagen protein acts as a scaffold, while noncollagen protein acts as a regulator and mineral stabilizer for apatite minerals. The noncollagen protein in dentine is dentine matrix proteinase 1 (DMP1), which acts as an inhibitor, promoter, and/or nanocluster stabilizer for ACP.⁷⁻¹⁰

Noncollagen protein analog material can substitutes DMP1 role, which prevent transformation of ACP nanoparticles into apatite

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crystals before entering the intrafibrillary gap zone. In GTR, intrafibrillary and extrafibrillary remineralization can occur even if apatite crystals are absent in collagen.¹¹

Carboxymethyl chitosan (CMC) is a chitosan derived from crustacean shells that is biodegradable, biocompatible, and nontoxic.^{9,12,13} Carboxymethyl chitosan can inhibit the calcium phosphate precipitation rate so that mineralization may occur in the gap zone.⁹ In addition, when compared with phi-chitosan, CMC has a higher affinity for calcium ions due to its abundant carboxyl groups.^{9,14} In their study, Chen et al.⁹ studied artificial remineralization of caries using CMC/amorphous calcium phosphate (CMC/ACP). The results showed that CMC can stabilize the ACP nanocomplex and prevent it from breaking up into larger particles until the ACP nanocomplex reaches the gap zone.⁹

We analyzed the potency of a noncollagen protein analog of CMC/ACP to dentine remineralization using scanning electron microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDX) devices.

Materials and methods

This study was approved by the ethics committee of the Universitas Indonesia, Faculty of Dentistry (Ethics No. 132/Ethical Approval/FKGUI/XI/2017 – Protocol: 051311017).

Four healthy premolar teeth extracted for orthodontic purposes were stored at 4 °C in a phosphate buffered saline (PBS) solution for up to 14 days. Each tooth had two cavities made on the occlusal aspect using a No. 16 cylindrical diamond bur and with a depth of 3 mm each. All teeth were then covered with nail polish, except for their cavity walls and base. Then the cavities were demineralized for seven days in 37 °C incubators using a 17% ethylenediaminetetraacetic acid (EDTA) solution. The teeth with cavities were divided into two groups; demineralized dentine, and CMC/ACP groups. The cavities in the demineralized dentine group

were directly subjected to light-cured temporary restoration, while in the CMC/ACP group, the cavity base was first treated with CMC/ACP and then was subjected to light-cured temporary restoration. The entire tooth was placed on an incubator shaker at 37 °C and the tooth root was immersed in foam in a container containing PBS solution for seven days. The sample was cut to the bottom of the cavity and fixed with multilevel dehydration. Each cavity then was analyzed with SEM and EDX.

Analysis

Data were analyzed using one-way ANOVA with a significance value of $P < 0.05$.

Results

In our study of the remineralization of demineralized dentine using CMC/ACP, SEM was used to evaluate changes in dentine structure, and EDX was used to analyze the minerals contained in dentine remineralization by calculating calcium and phosphate levels.

Figure 1A shows SEM imaging of the surface morphology of the demineralized dentine group. Visible open dentinal tubules and collagen around the tubules can be seen regularly; this indicates loss of appetite minerals in the demineralized dentine group. Figure 1B shows SEM imaging of the demineralized dentine surface after seven days, while Fig. 1C shows around of orifice tubular edges after CMC/ACP application, where irregular orifice tubular edges are present. This irregular white form signifies the occurrence of remineralization.

In Table 1, calcium and phosphate levels were 16.757 and 7.380, respectively, in the CMC/ACP group and 1.967 and 0.930, respectively, in the demineralization group. When both groups were compared, the differences in calcium and phosphate levels were significant ($P < 0.004$ and $P < 0.001$, respectively).

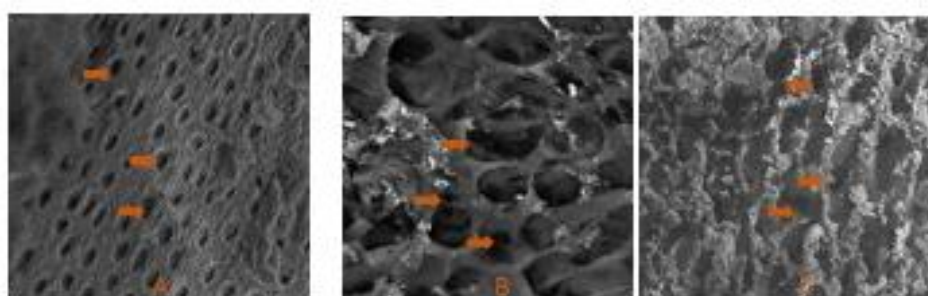


Figure 1 Dentine SEM images:(A) Dentine surface morphology after demineralization, open orifices of the tubular dentine and visible regular edge can be seen (yellow arrow); (B) Dentine surface morphology of the control group at day 7. There is little change in tubular dentine irregularity (blue arrow); (C) CMC/ACP group at day 7 shows white irregularity around dentinal tubule orifice (red arrow).

	<i>n</i>	Mean ± SD	<i>P</i> Value
Calcium			
Demineralized dentine	4	1.967 ± 1.799	0.004*
CMC/ACP	4	16.757 ± 7.636	
Phosphate			
Demineralized dentine	4	0.930 ± 0.695	0,001*
CMC/ACP	4	7.380 ± 2.745	

Table 1 Mean value, standard deviation (SD), and significance of calcium and phosphate levels in dentine demineralization and CMC/ACP groups after 7 days (%)

Discussion

Immersion of extracted tooth samples in a PBS solution with storage at 4 °C was done according to the Type I collagen storage protocol performed in the study by Doillon et al.¹⁵ This protocol also aims to keep the collagen structure in good condition. In the affected dentine layer, remineralization remains possible because the collagen crosslinks of this layer have only lost the apatite minerals. Crosslinked collagen acts as a scaffold for apatite mineral deposition.⁸ The teeth were immersed in a 17% EDTA solution for seven days to simulate dentine demineralization and stored at 37 °C. Seventeen percent EDTA has been shown to have good sustenance of chelates against calcium ions, which can lead to release of calcium minerals from dentine.¹⁶ In addition, 17% EDTA is able to keep the collagen crosslink intact.⁸ In this study, PBS was used as a medium for remineralization because it contained ionic concentrations that resembled those of bodily fluid and consisted of sodium chloride, sodium bicarbonate, potassium chloride, and potassium phosphate.¹⁷ Based on the

composition, PBS also can be used as an additional source for the remineralization process of calcium ions and phosphate.^{8,9}

In addition to the presence of collagen, the process of remineralization also required noncollagen protein to act as an ACP regulator and nucleation stabilizer.⁷⁻⁹ The noncollagen protein in dentine is DMP1, which serves to keep ACP stable and prevent nucleation until ACP reaches the remineralization site.¹⁸ In this study, the noncollagen protein analog used was CMC because it could act as a remineralization regulator and stabilizer of ACP structure.⁹ CMC has a large number of carboxyl groups and a high affinity for calcium ions.^{9,14} The carboxyl group of CMC attracts calcium and phosphate ions.

We investigated the occurrence of dentine remineralization on demineralized dentine surfaces after application of CMC/ACP, a noncollagen protein analog material. In 2015, Chen et al.⁹ in their study proved that CMC/ACP is capable of revitalizing intrafibrillar and extrafibrillar minerals in deep dentine caries. In this study, chitosan was processed into CMC/ACP with a particle size of 50 nm. The

CMC/ACP in our study was prepared according to the method of Chen et al.⁹, using a nano-sized CMC powder manufactured by the Chemical Laboratory University of Northern Sumatra.

Dentine remineralization results were evaluated by SEM; however, the results showed only morphologic changes in dentine surface structure. To complete and prove the occurrence of remineralization, EDX analysis was performed, which showed the increased calcium and phosphate mineral levels.

The SEM dentine images after day seven of CMC/ACP application showed a white irregularity around the dentinal tubular orifice, indicating remineralization (Fig. 1C). This result is in accordance with the results reported in the study by Chen et al.⁹ Evaluation using SEM and EDX on day seven refers to the study of Budiharjo et al.,¹⁹ who analyzed the bioactivity of carboxymethyl chitosan mixed with MTA which was then applied to the tooth model. Their results indicated that CMC is bioactive and is capable of remineralizing the tooth model. Remineralization was observed on day seven by SEM and EDX, even though remineralization could already be observed on day three. This was as a result of the newly-formed octacalcium phosphate, which was not by that stage a hydroxyapatite mineral. The hydroxyapatite mineral was seen only on day seven.

The remineralization process using CMC/ACP combination, which is as a result of formation of the CMC molecular chain together with the ACP nanocomplex, then breaks apart to form CMC/ACP nucleation, which will then bind and regenerate collagen.⁹ Calcium and phosphate ions have a vital role in remineralization. The major sources of calcium and phosphate in this remineralization process are from the ACP in the CMC/ACP nanocomplex.⁹ In addition, other calcium and phosphate source ions can also possibly be obtained from the PBS solutions.

Statistically, in Table 1, there was a significant difference in calcium and phosphate levels between the demineralized dentine and CMC/ACP groups. This result is consistent with the results in the study of Chen et al.,⁹ although the chitosan powder and sample storage method used are different. This result is also in accordance with the results of SEM examination, which describe morphologic changes of dentine structure in the CMC/ACP group after seven days.

Conclusions

CMC/ACP has the potential to remineralize dentine, as was proved by SEM images showing morphologic changes in dentine surface structure. These changes were noted by irregularity around the dentinal tubule orifice with a white color. This was supported by EDX analysis showing a significant increase in calcium and phosphate ion concentrations.

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Declaration of Interest

The authors declare that there are no conflicts of interest.

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