International Journal of Applied Pharmaceutics

ISSN - 0975 - 7058 Vol 12, Special Issue 2, 2020

Research Article

CONCENTRATION DEPENDENT EFFECTS OF CARBOXYMETHYL CHITOSAN ON DENTIN REMINERALIZATION WITH AMORPHOUS CALCIUM PHOSPHATE

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Received: 21 February 2020, Revised and Accepted: 01 June 2020

ABSTRACT

Objective: Carboxymethyl chitosan (CMC) is a non-collagenous protein analog which has a similar role as dentin matrix protein 1. CMC stabilizes amorphous calcium phosphate (ACP); hence forming nanocomplexes of CMC-ACP. The purpose of this study was to evaluate the effect of CMC concentration in CMC-ACP on dentin remineralization.

Methods: Cavities were formed on the occlusal surfaces of freshly extracted premolar teeth. All samples were demineralized and immersed in phosphate-buffered saline and stored in a shaking incubator at 37°C. The teeth were randomly divided into five groups. Group 1 was control group (no treatment), whereas Groups 2, 3, 4, and 5 were treated with CMC-ACP containing 1%, 2.5%, 5%, and 10% CMC. The remineralized layer on the dentin surface was evaluated using scanning electron microscopy and energy-dispersive X-ray analysis.

Results: The highest dentin remineralization capacity was achieved in Group 5 (10% CMC), whereas diminishing effects were observed in Group 4 (5% CMC), Group 3 (2.5% CMC), and Group 2 (1% CMC). Although no significant differences in calcium levels were observed between 2.5%, 5%, and 10% CMC groups, phosphate levels differed significantly in all treatment groups.

Conclusion: Optimal dentin remineralization was achieved by the application of CMC–ACP containing 2.5% CMC.

Keywords: Carboxymethyl chitosan, Amorphous calcium phosphate, Demineralized dentin, Non-collagenous protein analog.

 DOI: http://dx.doi.org/10.22159/ijap.2020.v12s2.OP-42

INTRODUCTION

Dental caries causes destruction of the dentin network, resulting in collagen fibrils degradation and decreases dentin mechanical properties [1]. However, the inner layer (affected dentin) of carious dentin is reversibly denatured and not infected; therefore, it is capable of remineralization [2].

Dentin matrix protein 1, a non-collagenous protein (NCP) found in the extracellular matrix of dentin and bone, has an important role in regulating dentin mineralization and stabilizing amorphous calcium phosphate (ACP) [1-3]. Hence, facilitating ACP infiltration into the 40 nm-long gap zones of the collagen fibrils resulting in intrafibrillar remineralization [2,4].

However, extracting and purifying NCPs from dentin present a major challenge, which led researchers to focus on the development of analogs of NCPs, such as polyaspartic acid, polyacrylic acid, and carboxymethyl chitosan (CMC) to induce biomimetic remineralization of demineralized dentin collagen matrix, [1,3]. Finding analogs of NCPs that can stabilize ACP is fundamental in the development of biomimetic remineralizing agents.

Over the last decade, CMC has been developed for various biomedical applications due to its biocompatibility, biodegradability, non-toxicity, and antibacterial activity [2]. CMC, a derivative of chitosan, obtained from shells of crustaceans, such as crabs and shrimps, has been proven to stabilize ACP as a result of the abundant negatively charged carboxyl groups in CMC that can bind to calcium ions [2,3].

Furthermore, the concentration of NCPs also plays an important role in the stability of ACP. The previous study reported that lower concentration of polyaspartic acid would lead to the formation of larger ACP nanoparticle aggregates, reflecting higher rates of spontaneous calcium phosphate precipitation [5]. Hence, preventing ACP from entering the intrafibrillar collagen gap zones.

However, the influence of CMC concentration in CMC-ACP to dentin remineralization has not been extensively studied. In this study, we investigated the influence of CMC concentration in CMC–ACP that resulted in optimal dentin remineralization. The hypothesis for this study was that CMC concentration in CMC-ACP would influence the extent of dentin remineralization.

METHODS

This study was approved by the Ethical Committee of the University of Indonesia, Faculty of Dentistry (Ethics No. 16/Ethical Exempted/FKGUI/2018/Protocol no. 051171218). CMC was obtained from the Research Laboratory of Sumatera Utara University.

CMC-ACP preparation

CMC–ACP was prepared, as described by Chen *et al.* (2015) [2]. CMC–ACP treatments containing 1%, 2.5%, 5%, and 10% CMC (treatment group 1–4, respectively) were prepared by adding 0.5, 1.25, 2.5, and 5 g of CMC, respectively, to 50 mL water by mixing with a vortex stirrer (1000 rpm) until the CMC powder was dissolved. Subsequently, 0.498 g of K_2HPO_4 (Merck, Darmstadt, Germany) was added and mixed into the CMC gels at 500 rpm. In a separate glass reaction vial, 0.555 g of $CaCl_2$ (Merck, Darmstadt, Germany) was dissolved in 10 mL of deionized water (Waterone™ Onelab, Sidoarjo, East Java, Indonesia) and the resulting solution was added dropwise into the CMC gel by stirring for 5 min until CMC–ACP gel was formed. CMC–ACP gel was frozen at –80°C for 2 h and then lyophilized by freeze-drying for 6 h to form CMC–ACP scaffolds.

Sample preparation – artificial caries lesion model

Extracted non-carious human premolars (n=15) were immediately immersed in phosphate-buffered saline (PBS; BR0014G; Oxoid, Basingstoke, Hampshire, UK). Two cavities were formed on the occlusal surfaces of each tooth using a round diamond bur to a depth of 3 mm. The bottom of the cavity was exposed to 17% EDTA solution (MD-Cleanser™, Meta Biomed Co. Ltd., Cheongju City, Chungbuk, Korea) to completely demineralize the dentin for 1 week in a shaking incubator at 37°C. Afterward, the cavities were rinsed with deionized water for 30 min, and the teeth were immersed in 20 mL of 1 M NaCl at 25°C for 8 h.

Remineralization procedures

The samples were randomly divided into four treatment groups and one control group of five cavities each: One control group that was not treated and treatment Groups 1, 2, 3, and 4 were treated with CMC–ACP containing 0.5, 1.25, 2.5, and 5 g CMC, respectively. All samples were temporarily filled with light-curing restoration (Quicks® Yellow, Dentkist, Inc., Gyeonggi, Korea). The roots were immersed in PBS solution and stored in a shaking incubator at 37°C. All the samples were observed after 14 days of remineralization using scanning electron microscopy (SEM; TESCAN VEGA3, Czech Republic) and energy-dispersive X-ray (EDX) silicone detector (X-act) of 10-mm^2 connected to the SEM. The instrument was operated using INCA software (Oxford Instruments).

SEM and EDX analysis

Dentin samples were obtained by cutting the enamel and dentine portions down to the bottom of the cavity. The samples were then rinsed with deionized water and dehydrated using ethanol at a gradient concentration of 50%, 70%, 80%, and 90% for 20 min and 100% ethanol for 2 h. The samples were then coated with gold nanoparticles for SEM, and quantitative analysis of calcium and phosphate contents was carried out using EDX.

Statistical analysis

All statistical analysis was conducted using IBM SPSS Statistics v22.0 (IBM Corporation, NY, USA). Data normality was assessed using Shapiro-Wilk tests. Differences between groups were identified using One-way ANOVA was considered significant when $p<0.05$.

RESULTS AND DISCUSSION

SEM analysis was conducted to evaluate the changes in dentin surface morphology whereas EDX examination revealed the calcium and phosphate contents on dentin surfaces after remineralization.

Fig. 1 shows SEM images of demineralized dentine surface morphology with and without CMC–ACP treatments at various CMC concentrations. In Fig. 1a and b, no significant differences in surface morphology of dentin tubules are apparent. In contrast, Fig. 1c-e show mineral deposition (remineralization) on dentin surfaces, as indicated by whiter colors and irregularities on the edges of dentin tubules.

The data in Table 1 show that the highest mean calcium and phosphate contents were found in the 10% CMC group.

No significant differences in calcium levels were identified between control and 1% CMC ($p>0.05$) groups, but significant effects were identified in comparisons of 2.5%, 5%, and 10% CMC groups with the control group. Calcium levels also differed significantly between 1%, 5%, and 10% CMC groups (Table 2), whereas the phosphate levels of all treatment groups differed from the control group and significant differences were identified between 1%, 2.5%, 5%, and 10% CMC groups.

This study presents demineralized dentin of tooth models to mimic dental caries by drilling cavities into extracted teeth and exposing them to EDTA for 7 days [2]. The chelating agent EDTA binds to metal ions such as calcium, resulting in dentin demineralization; however, EDTA has no effect on collagen [6]. We immersed samples in PBS, which contains sodium chloride, phosphate disodium hydrogen, and phosphate dihydrogen potassium and represents body fluids. In our

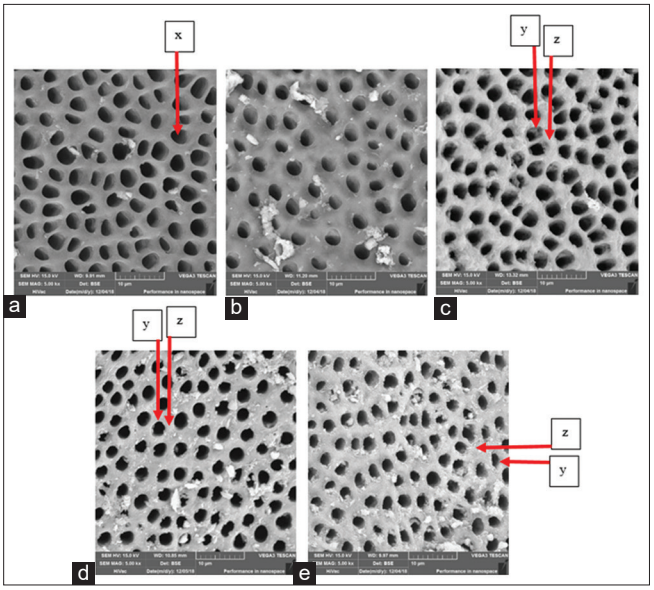

Fig. 1: Surface morphologies of dentin surfaces at ×5000; control group (a) demineralized dentin; carboxymethyl chitosan (CMC)-amorphous calcium phosphate treatment groups with 1% (b) 2.5% (c), 5% (d), and 10% (e) CMC; arrows labeled with x indicate exposed dentin tubules. Arrows labeled with y arrow indicate mineral deposits on peritubular dentin, and arrows labeled with z indicate mineral deposits on inter-tubular dentin surfaces

Table 1: Percentage calcium and phosphate contents in control and experimental groups; data are presented as means±SD with p-values

Materials	n	Mean±SD	p
Calcium			
Control	5	12.40±2.30	0.000*
1% CMC	5	16.26±0.86	
2.5% CMC	5	25.40±5.17	
5% CMC	5	25.94±3.31	
10% CMC	5	28.42±3.58	
Phosphate			
Control	5	4.32±1.94	0.000*
1% CMC	5	6.56±0.55	
2.5% CMC	5	9.98±0.60	
5% CMC	5	11.88±0.42	
10% CMC	5	14.20±0.78	

experiments, we also applied physiological conditions of temperature and the oral cavity using a shaking incubator [7,8].

CMC is synthesized by introducing carboxymethyl groups to the structure of chitosan [9]. During preparation, carboxymethylation of the hydroxyl and amine groups of chitosan enhances the solubility of the polymers in the water at near-neutral pH, and improves calcium chelating properties, antimicrobial activity, toxicity, and biocompatibility [9,10], ensuring its wide range of applications in biomedical and pharmaceutical fields [10]. In pharmaceutical studies, CMC has been used in the development of controlled-release drug delivery systems and pH-responsive drug delivery systems [10]. In regenerative medicine, CMC polymers have been used as scaffolds for tissue repair [10].

During hard tissue formation, ACP is initially present in the solid phase [2] and has high cell adhesion and excellent bioactivity and osteoconduction properties [2]. ACP can be converted, however, to stable crystalline phases such as octacalcium phosphate and hydroxyapatite (HAP). However, ACP has to be stabilized in its amorphous nanoparticle

Table 2: Differences in calcium and phosphate levels in control and experimental groups

Materials	Calcium level[a]				Phosphate level[b]			
	1% CMC	2.5% CMC	5% CMC	10% CMC	1% CMC	2.5% CMC	5% CMC	10% CMC
Control	0.160	0.027*	0.001*	0.001*	0.025*	0.000*	0.000*	0.000*
1% CMC		0.148	0.021*	0.011*		0.000*	0.000*	0.000*
2.5% CMC			1.000	0.978			0.083	0.000*
5% CMC				0.967				0.019*

[a]Significant differences in calcium levels as identified using *post hoc* Tamhane's T2 tests; p<0.05 (*). [b]Significant differences in phosphate levels as identified using *post hoc* Bonferroni tests; p<0.05 (*)

form to participate in intrafibrillar remineralization [11]. Nonetheless, ACP has been widely applied in orthopedic and dental fields [2].

The present SEM images and EDX data indicate the effect of CMC concentration in CMC–ACP on the extent of dentin remineralization. Optimal dentin remineralization was observed after the application of CMC–ACP containing 2.5% CMC, reflecting optimal ACP stability, as indicated by the formation of HAP. Our data also suggest that CMC concentrations of more than 1% resulted in constant dentin remineralization.

CMC stabilizes ACP by forming CMC–ACP nanocomplexes that inhibit spontaneous calcium phosphate precipitation. These CMC–ACP nanocomplexes have diameters of <40 nm; therefore, they can penetrate into collagen fibrils through the gap zones. Due to the presence of numerous chelating carboxyl groups, CMC becomes positively charged with calcium ions, and thereby sequesters phosphate ions [2].

This study revealed CMC-ACP has a promising potential as a dentin remineralizing agent for deep caries. CMC–ACP could be used before permanent restoration or as an indirect pulp capping material that can increase the mechanical properties of dentin. However, due to its viciousness and the absence of self-curing components, CMC can only be used as a temporary material.

Our study is limited to five samples in each study group. A larger sample size would improve precision and strengthen our conclusions. Moreover, the present *in vitro* conditions are limited in their representation of clinical conditions. In particular, *in vivo* calcium and phosphate levels may be different than in the present study.

Further studies are required to improve material consistency, to evaluate antibacterial properties and cytotoxic effects of CMC-ACP on dental pulp cells and to determine the potential for induction of restorative dentinogenesis.

CONCLUSION

Higher CMC concentrations in CMC–ACP resulted in higher calcium and phosphate levels on demineralized dentin, and optimal remineralization was achieved with CMC–ACP containing 2.5% CMC.

ACKNOWLEDGMENT

This research was supported by the Directorate of Research and Community Engagement of Universitas Indonesia, funded by PITTA grant funds. We thank our colleagues from the Department of Dental Material and the Environmental Sciences Laboratory in Universitas Indonesia.

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