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ORIGINAL ARTICLE

Ultrasonic assessment of the effects of self-assembling peptide scaffolds on preventing enamel demineralization

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Abstract

Objectives. This study evaluates the effect of self-assembling peptide P_{11}-4 (Curodont Repair, CDR) on bovine enamel remineralization by measuring changes in ultrasonic propagation velocity. ***Methods.*** Six specimens per group were prepared by sectioning bovine teeth into enamel blocks. These blocks were then immersed in lactic acid buffer solution (pH = 4.75) for 10 min twice a day and stored in artificial saliva. Other specimens were first treated with CDR, followed by a 10-min immersion in the lactic acid buffer solution twice a day, before storage in artificial saliva. The propagation time of longitudinal ultrasonic waves was measured using a pulser/receiver. Six specimens were used for each treatment protocol. The obtained data were statistically analyzed using ANOVA followed by Tukey's honestly significant difference tests (α = 0.05). Specimens were observed using laser scanning microscopy and scanning electron microscopy. ***Results.*** Sonic velocity was found to decrease with time for specimens stored in the demineralizing solution. On the other hand, increases in sonic velocity were found for specimens treated with CDR. These specimens also exhibited signs of mineral deposition. ***Conclusions.*** By measuring the ultrasonic propagation velocity, it can be concluded that CDR application has an ability to promote bovine enamel remineralization.

Key Words: *Enamel, peptide scaffolds, ultrasonic velocity, remineralization*

Introduction

The nature of tooth erosion is related to the presence of non-bacterial acids in the oral environment. The consumption of acidic food and beverages leads to tooth decay, which is defined as dental erosion. The development of erosion involves a chemical process in which the inorganic phase of the tooth substrate undergoes demineralization, thereby decreasing enamel hardness [1]. Subsequent abfraction and abrasion through brushing further increases wear of the tooth substrate [2]. In the past, erosive lesions in the cervical area were mostly seen in aging individuals, whose teeth had worn out over a number of years. Recently, however, the consumption of acidic food and carbonated beverages, along with insufficient oral hygiene, has led to increasing frequency of dental erosion among younger generations [3]. These lesions are predominantly caused by physical and chemical factors that act on the tooth surface, resulting in enamel loss, dentin exposure and dentin erosion. The prevention of erosion is important because tooth wear is a universal condition affecting all populations, including those with a decreased caries prevalence [4].

According to the principles of modern dentistry, optimal strategies should be favored in the treatment of early erosive lesions [5]. Efforts have been focused on decreasing the risk of tooth demineralization and these efforts have highlighted the importance of approaches to ensure success in the control of remineralization and demineralization. Nanotechnological developments for the repair of enamel demineralization have been reported previously [6]. Biomimetic strategies for artificial enamel formation may potentially repair enamel surface damage and increase the tooth longevity [7]. Amelogenin has been used to control calcium and phosphate crystallization, resulting in the growth of nanosized rod-like apatite crystals [8]. The effects of application of an anionic

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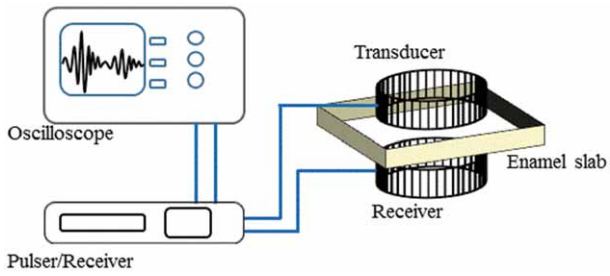

Figure 1. Experimental set-up of the ultrasonic device for detecting enamel remineralization.

self-assembling peptide P_{11}-4 on remineralization and demineralization of carious lesions has been investigated and it was concluded that P_{11}-4 treatment significantly promotes mineral gain because it exerted a combined effect by increasing remineralization and inhibiting demineralization [9]. P11-4 would be useful in the modulation to induce hydroxyapatite nucleation, leading to the remineralization of early carious lesion and erosive lesions through its diffusion into mineral loss and enhancement of hydroxyapatite precipitation.

Ultrasonic imaging is a useful technique that shows considerable potential as a non-invasive diagnostic and research tool [10]. These devices can be used to detect carious lesions and to measure the elastic modulus of enamel and dentin. The enamel substrate is mainly composed of hydroxyapatite. Differences in ultrasonic velocity may be related to variations in the degree of mineralization, because the ultrasonic velocity increases proportionally with the volumetric concentration of minerals [11]. Ultrasonic velocity has also been shown to be an index of remineralization and demineralization because of its relation to the mineral content of the tooth substrate [12,13]. When enamel remineralization occurs, both mineral concentration and ultrasonic velocity increase.

The present study evaluated the effects of application of the self-assembling peptide P_{11}-4 on enamel remineralization by the measurement of changes in ultrasonic velocity as well as observations with laser scanning microscopy (LSM) and scanning electron microscopy (SEM). The null hypothesis to be tested was that the self-assembling peptide P_{11}-4 could not prevent demineralization of the enamel substrate.

Materials and methods

Twenty-four freshly extracted bovine incisors without cracks or erosions were cleaned and stored in physiological saline for 2 weeks. The teeth were sectioned longitudinally at a 1-mm thickness and then sectioned in the buccolingual direction with a low-speed diamond saw (Buehler Ltd., Lake Bluff, IL). Each slab was carefully shaped into a rectangular form ($4 \times 4 \times 1$ mm) using a super-fine diamond finishing point (ISO #021; Shofu Inc., Kyoto, Japan) attached to an air-turbine (TwinPower Turbine, J. Morita Mfg. Corp., Kyoto, Japan). Specimen surfaces were successively ground using wet silicon carbide (SiC) paper with grit sizes of #600, #1200, and #2000. The thickness and size of the specimens were measured using a dial gauge micrometer (CPM15-25DM; Mitutoyo, Tokyo, Japan).

The resource equation method was used for a sample size calculation [14] and the sample was divided into four groups of six samples each.

(1) *Control group*: Stored in artificial saliva for the experimental period of time.
(2) *DE group*: Treated with undersaturated 0.1-M lactic acid buffer solution (pH 4.75, 0.75-mM $CaCl_2{\cdot}2H_2O$, and 0.45-mM KH_2PO_4) for 10 min and then placed in artificial saliva (pH 7.0, 14.4-mM NaCl, 16.1-mM KCl, 0.3-mM $MgCl_2{\bullet}6H_2O$, 2.0-mM K_2HPO_4, 1.0-mM $CaCl_2{\bullet}2H_2O$ and 0.10-g% sodium carboxymethyl cellulose [CMC-Na]). This was repeated twice daily over the 4-week test period and the specimens were stored in between treatments in artificial saliva at 37°C.
(3) *CD-De group*: The self-assembling peptide used in this study was Curodont Repair (CDR, Credentis AG, Windisch, Switzerland). The tooth surfaces were cleaned with 2% sodium hypochlorite for 20 s, followed by 35% phosphoric acid etching for 20 s. After rinsing with a three-way syringe and air drying, CDR was applied on the etched enamel surface, followed by treatment with undersaturated 0.1-M lactic acid buffer solution for 10 min. The specimens were then placed in artificial saliva. Demineralization procedures were repeated twice daily over the 4-week test period.
(4) *CD group*: The specimens exposed to CDR treatment without acidic challenge were stored in artificial saliva for the same period of time.

Ultrasonic velocity was measured using a pulser/receiver (Model 5900PR; Panametrics, Waltham, MA), a transducer for longitudinal waves (V112; Panametrics) and an oscilloscope (Wave Runner LT584; LeCroy Corp., Chestnut Ridge, NY) (Figure 1). Measurements were taken before the test and then again on days 1–7, 14, 21 and 28. The equipment was initially calibrated using a standard procedure with 304 stainless steel calibration blocks (2211M; Panametrics). The transducer was oriented perpendicular to the contact surface of each specimen so as to obtain the echo signal. The ultrasonic waves were propagated from the transducer to the tooth, transmitted through the tooth and then detected by the transmitter on the opposite side. Each measurement was made at 23°C ± 1°C, and 50% ± 5% relative humidity.

Specimens from each group were treated using the same methods and observed under three-dimensional

Table I. Average ultrasonic velocities (m/s) of bovine enamel specimens by treatment.

Group	Treatment time (days)								
	0	1	2	3	4	5	6	7	14	21	28
Control	$4{,}927^{a,A}$ (56)	$4{,}876^{a,A}$ (55)	$4{,}868^{a,b,A}$ (63)	$4.864^{b,A}$ (97)	$4{,}861^{c,A}$ (91)	$4{,}868^{c,A}$ (79)	$4{,}869^{b,A}$ (92)	$4{,}878^{c,A}$ (60)	$4{,}857^{c,A}$ (92)	$4{,}855^{c,A}$ (82)	$4{,}832^{c,A}$ (80)
De	$4{,}868^{a,A}$ (80)	$4{,}729^{a,B}$ (81)	$4{,}707^{b,B}$ (95)	$4{,}677^{c,B,C}$ (93)	$4{,}652^{d,C}$ (86)	$4{,}644^{d,C}$ (85)	$4{,}631^{c,C}$ (84)	$4{,}612^{d,C,DE}$ (88)	$4{,}575^{d,D,E}$ (69)	$4{,}562^{d,D,E}$ (68)	$4{,}519^{d,E}$ (67)
CD	$4{,}987^{a,B,C}$ (67)	$4{,}759^{a,D}$ (175)	$4{,}948^{a,B}$ (133)	$5{,}010^{a,b,A,B}$ (157)	$5{,}059^{b,A,B}$ (174)	$5{,}105^{b,A,B}$ (163)	$5{,}171^{a,A}$ (103)	$5{,}173^{b,A}$ (87)	$5{,}163^{b,A}$ (114)	$5{,}136^{b,A}$ (111)	$5{,}111^{b,A,B}$ (95)
CD-De	$4{,}922^{a,B,C}$ (116)	$4{,}815^{a,C}$ (164)	$4{,}965^{a,B,C}$ (179)	$5{,}166^{a,A,B}$ (82)	$5{,}307^{a,A}$ (118)	$5{,}345^{a,A}$ (136)	$5{,}338^{a,A}$ (152)	$5{,}367^{a,A}$ (126)	$5{,}347^{a,A}$ (118)	$5{,}325^{a,A}$ (108)	$5{,}327^{a,A}$ (137)

De, demineralization group; CD, Curodont Repair application group; CD-De, demineralization after Curodont Repair application group. Data are shown as mean (standard deviation). $n = 6$ per group.
Between groups at the same treatment times, values with the same lowercase superscript letter are not significantly different ($p > 0.05$).
Within groups, values with the same uppercase superscript letter are not significantly different ($p > 0.05$)

LSM (LSM; VK-8700; Keyence Corp., Osaka, Japan). The excitation light had a maximum wavelength of 658 nm. The intensity of the excitation light and amplification of the photomultiplier remained constant throughout the analysis. The size of the image recorded was 81.5 × 71.5 μm^2 and the resolution was 1024 × 768 pixels. Images were obtained for four different sites on each specimen.

Ultrastructural observation of enamel surfaces was carried out using field-emission SEM. Specimens were first dehydrated in ascending concentrations of tert-butanol (50% for 20 min, 75% for 20 min, 95% for 20 min and 100% for 2 h) and then transferred to a critical-point dryer for 30 min. The surfaces were then coated with a thin Au film in a vacuum evaporator (Quick Coater Type SC-701; Sanyu Denshi Inc., Tokyo, Japan) and observed by SEM (ERA 8800FE; Elionix Ltd., Tokyo, Japan) at an accelerating voltage of 10 kV.

The ultrasonic velocity data were analyzed using two-way analysis of variance, with time and treatment as factors. Time was treated as a repeated measure. *Post hoc* pairwise tests among groups were performed using Tukey's honestly significant difference test. The level of significance (p value) was 0.05 and all calculations were carried out using Sigma Stat software, version 3.1 (SPSS Inc., Chicago, IL).

Results

The average ultrasonic velocities of the enamel specimens are shown in Table I. Because the differences between storage periods were greater than expected, multiple comparisons were performed on the data, after factoring in the effects of storage conditions. The average ultrasonic velocity in intact bovine enamel (Control group) ranged from 4832–4927 m/s, and it did not vary significantly with treatment time. The ultrasonic velocities in the De group decreased over time and were significantly lower than those in the Control group after 2 days (4519~4707 m/s). On the other hand, increases in the sonic velocities were found for CD and CD-De groups with treatment time. Compared with the Control group, there were significant increases in the ultrasonic velocity with treatment time after 4 days in the CD group (5059~5173 m/s), and after 3 days in the Cd-De group (5166~5367 m/s).

Representative LEM and SEM images of enamel specimens are shown in Figure 2 and these images revealed morphological changes in different treatment groups. From the SEM observations, demineralization of the enamel surfaces was more pronounced in the De group that presented with enamel-etching patterns. Compared with the De group, the smear layer was completely removed from the ground enamel surfaces and relatively smoother surfaces were observed for the CD and De-CD groups. The

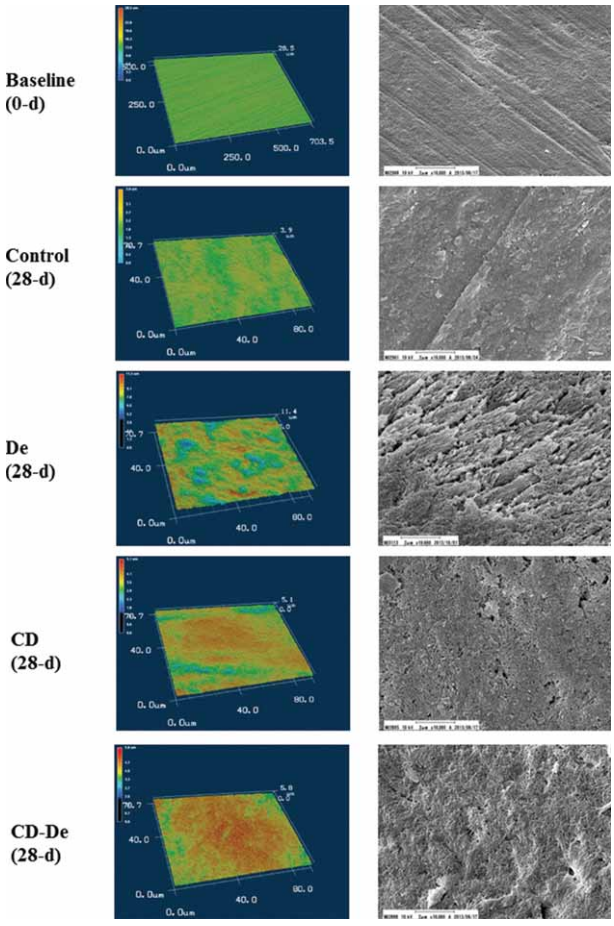

Figure 2. Representative 3D-LSM and SEM images of enamel surfaces after 28 days of the study period. SEM observations (original magnification = ×10 000) revealed differences in morphological features among storage conditions. Demineralization of the enamel surfaces was more pronounced in the De group, whereas enamel surfaces of the CDR group exhibited homogeneous features covered by precipitants. In the LSM images, different colors indicated different depths, shown in the vertical burs. Surface roughness of each group was corresponded to the SEM images of the same groups.

CD and De-CD groups exhibited homogeneous surfaces on all surfaces covered by needle-like deposits. In the LSM images, different colors indicated different depths, which was shown in the vertical bur. LSM images of each group exhibited the corresponding surface to the SEM images. Compared to the Control group, the De group exhibited rough and irregular surfaces. On the other hand, the CD and De-CD groups showed relatively smooth surfaces and were covered with an amorphous layer.

Discussion

The parameters that must be considered in this type of study are the experimental design, type of substrate and the method used to assess the mineral content. There are several diagnostic techniques for detecting remineralization and demineralization that occurs as a result of acidic challenges [15]. The measurement of sonic velocity has been reported to be related to the mineral content of the enamel substrate. The ultrasonic velocity increases proportionally to the concentration of mineral components in the enamel, thus acting as an index of the degree of mineralization [16,17]. The enamel is a mineralized tissue with a highly complex hierarchical structure composed of aligned enamel rods, arranged perpendicular to the tooth surface. Because the orientation of the enamel prisms might modify the ultrasonic velocity [18], we collected enamel specimens from the labial surfaces of bovine teeth to avoid any such effects.

The natural enamel protein amelogenin has been used *in vitro* to control calcium and phosphate crystallization, resulting in the growth of nanosized rod-like apatite crystals [19]. This method demonstrates the remineralization of the etched enamel surface by the formation of a mineral layer containing needle-like fluoridated hydroxyapatite crystals. Self-assembling peptides have been considered suitable for the formation of nanostructured scaffolds in a specific tissue-engineering approach [6]. The role of domains was examined using the corresponding peptides with dentin matrix protein 1 and it was found that the self-assembled β-sheet acidic domains serve as ideal templates for hydroxyapatite nucleation. The effect of β-sheet and carboxyl groups on hydroxyapatite formation using specifically designed polypeptides has also been examined and the results showed that polypeptides containing β-sheet structure in a solution can effectively induce hydroxyapatite deposition on the surface [20].

The results obtained in the present study demonstrated decreases in the ultrasonic velocity of enamel in the De group as a function of treatment time. In addition, increases in the ultrasonic velocities of specimens in the CD and De-CD groups were observed with test time periods. The present study also showed that CDR application resulted in an increase in sonic velocity, suggesting that CDR enhances enamel remineralization. The De group without CDR application showed no resistance to enamel demineralization. There were no signs of demineralization in the CD and De-CD groups, according to the LEM and SEM images. These results are consistent with previous studies that used CDR to promote enamel remineralization. The effects of application of the anionic self-assembling peptide P_{11}-4 on enamel remineralization has been examined before and it was concluded that the peptide had the ability to increase mineral gain and inhibit mineral loss [9]. The results of the present study were consistent with those of previous reports. CDR has an ability to form scaffold-like structures with negatively charged domains, which control initial mineral deposition and subsequent hydroxyapatite crystal growth [21]. Therefore, the presence of CDR might permit a rapid return to resting mineral concentrations and allow the immediate remineralization of the

enamel substrate. Application of fluoride or oral hygiene education are successful in impeding further progress of the lesion. The use of CDR also has the additional advantage of enhancing natural repair by regenerating minerals itself [22].

Self-assembling peptides of the P_{11}-family exhibit one-dimensional self-assembly by forming single-molecule-thick β-sheet nanotapes [23]. Several types of P_{11} peptides have been designed to self-assemble after various physical and chemical triggers. The self-assembling peptide CDR (P_{11}-4) was rationally designed to form fibrils at a low pH and to be monomeric in solution with higher pH. These peptides play an important role in the mineralization process, where negatively charged surfaces and phosphorylated serine residues attract Ca^{2+}, initiate crystal formation and control the orientation and elongation of the hydroxyapatite crystals [24]. Salivary factors such as salivary flow rate, composition and buffering capacity may exert protective actions on the enamel surfaces [25]. Enhancing the remineralization capability of saliva is important from a clinical point of view. CDR presents a new approach by inducing the formation of an enamel matrix to facilitate the incorporation of calcium and phosphate from saliva. Therefore, the influence of CDR on the promotion of enamel remineralization, as observed in the present study, would prove to be clinically beneficial.

Within the limitations of this *in vitro* study, it can be concluded that CDR application was effective in preventing enamel demineralization and had the ability to enhance enamel remineralization. Therefore, use of CDR may be considered as a micro-invasive approach for preventing enamel demineralization.

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