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Application of the Self-Assembling Peptide P11-4 for Prevention of Acidic Erosion

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Clinical Relevance

The use of peptide P11-4 may be considered for preventing enamel acid erosion.

SUMMARY

The aim of this study was to use ultrasonography to evaluate the effect of the self-assembling peptide P11-4 on acid erosion prevention. Curodont Repair (CR), which includes peptide P11-4, was used. Rectangular prisms of bovine enamel (4×1×1 mm) were immersed in pure orange juice for a period of 5 minutes six times per day for 28 days. These samples were divided into four groups of six specimens each and treated differently for an additional period of 28 days: 1) baseline group specimens were stored in artificial saliva; 2) CR group specimens were exposed to curodont without acid challenge; 3) NCRA (no curodont+acid challenge) specimens were treated with orange juice without curodont exposure; and 4) CRA (CR+acid challenge) specimens were treated with curodont before treatment with orange juice. The propagation time of longitudinal ultrasonic velocity (UV) was measured. Ultrastructural observation of each tested enamel surface was carried out using field-emission scanning electron microscopy (SEM). The UV data were analyzed using two-way analysis of variance with time and treatment as confounding factors. Post hoc pairwise tests among groups were performed using the Tukey honestly significant difference test. The average UV in intact bovine enamel for the baseline group ranged from 4,483 to 4,549 m/s and did not vary significantly within the test period. The average ultrasonic velocity (UV) in all samples decreased after the initial erosion. The UV in NCRA decreased further over time. Increased UVs were found for CR and CRA. For CR and

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CRA, there was no significant difference in UV at the end of the experiment from the initial value before erosion. In the results of SEM observation, the CR and CRA groups had similar morphologic features in that etching patterns were not clearly due to precipitation between the enamel rods. From the results of this *in vitro* study, it might be concluded that applying enamel matrix derivatives and self-assembling peptides on erosive lesions can improve remineralization.

INTRODUCTION

Tooth wear is becoming increasingly significant due to the wide availability and frequent consumption of acidic soft drinks, sports drinks, and fruit juices.[1] Erosive tooth wear involves a chemical process in which the inorganic phase of a tooth is demineralized, thereby allowing for surface dissolution of hard dental tissues. It is known that acidic food and drinks that contain citric, malic, phosphoric, and other acids can soften hard dental tissues.[2] In clinical situations, the initial sign of enamel erosion is the appearance of a smooth glazed surface. Moreover, reduced hardness may induce further enamel loss with the appearance of shallow concavities, rounding of the cusps, and eruption of restorations that seem to rise above the adjacent tooth level.

Prevention of the erosive process can be influenced by many factors, including tooth exposure to fluoride and the use of remineralization agents and protective materials. Many strategies have been developed to this end, including the use of materials containing fluoride compounds, casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), and resin-based materials.[3-8] In particular, although fluoride products are extensively recommended and widely used in oral health, fluoride formulations alone provide only limited protection against erosive tooth wear.[9] There are few nonoperative treatments for remineralization or repair of erosive lesions at present.[10]

According to the principles of modern dentistry, optimal strategies should be favored for the treatment of early erosive lesions.[11] An early erosive lesion in this sense is one in which there has been no loss of bulk in the enamel, but demineralization has started, so that the enamel is softer. In clinical situations, pure early erosive lesions are very unusual, as some loss of bulk is almost inevitable, but they still represent a condition that is present, and which can be treated independently of bulk restorations. Amelogenin use to control calcium and phosphate crystallization resulted in the growth of nano-sized rod-like apatite crystals.[12] A new product containing the peptide P11-4 was recently introduced to enhance remineralization and inhibit demineralization of the tooth substrate.[13] Peptide P11-4 self-assembles into a three-dimensional (3D) scaffold on the surface of the tooth, where it promotes hydroxyapatite precipitation while inhibiting diffusive mineral loss. Therefore, due to its mineralization effect, this material is potentially useful not only for early carious lesions but also for enamel defects caused by erosion and tooth wear.[14] A previous report indicated that the application of peptide P11-4 had effectively prevented demineralization and enhanced remineralization of enamel, which is considered an effective microinvasive approach for preventing enamel demineralization.[15] The self-assembling peptide P11-4 is designed to form fibrils at a low pH and to be monomeric in solutions with higher pH, and dissolved Ca^{2+} is attracted by the negative charged surfaces and phosphorylated serine residues in the peptide. The presence of peptide P11-4 might permit a rapid return to normal mineral concentrations and allow ions to be reused in crystal growth, in addition to controlling the orientation and elongation of the hydroxyapatite crystals.[16] As demineralization is an equilibrium process, an elevated local concentration of Ca^{2+} can be expected to inhibit it. However, these potential mechanisms have yet to be directly confirmed.

Ultrasonic imaging is a useful technique that shows considerable potential as a noninvasive diagnostic and research tool.[17] This modality can be used to detect carious lesions and to measure the elastic modulus of the tooth substrate.[18] The enamel substrate is mainly composed of hydroxyapatite, and differences in ultrasonic velocity (UV) are related to variations in the degree of mineralization, as UV increases proportionally with the volumetric concentration of minerals.[19] UV has also been shown to be an index of remineralization and demineralization because of this correlation to the mineral content of the tooth substrate.[20] The level of mineralization of dental enamel is closely linked to its stiffness and/or elastic response.[21] When remineralization of enamel occurs, mineral concentration and UV will increase.[22] In this experiment, ultrasonic measurements were used to determine the density of internal structure, and thus degree of mineralization, of the enamel and not to measure any loss of enamel bulk.

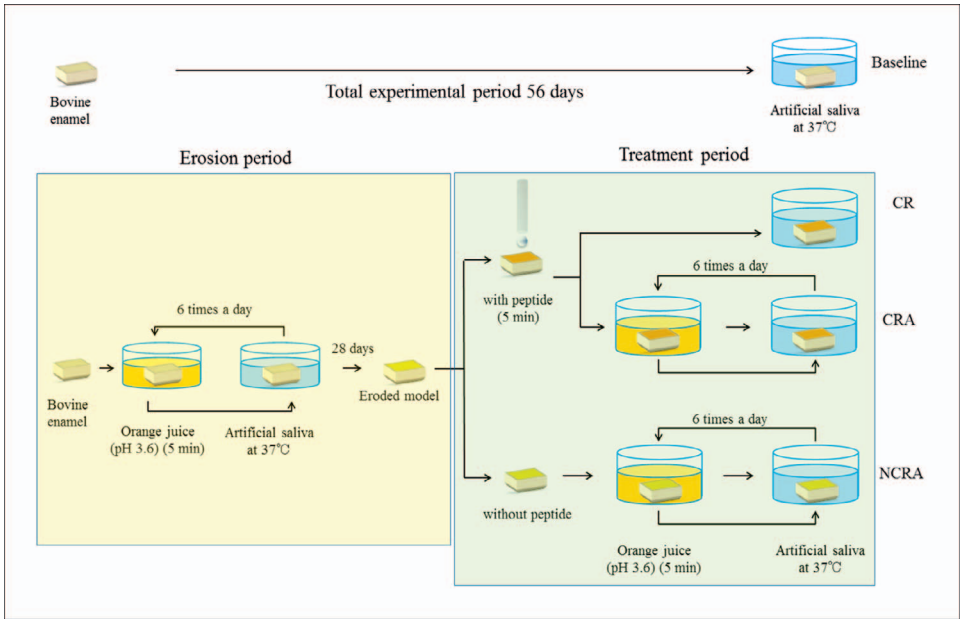

Figure 1. *Experimental treatment steps of the enamel specimens.*

Considering the potential of the self-assembly peptide P11-4 to enhance enamel remineralization, the purpose of this study was to use ultrasonic measurements to evaluate the preventive effects of peptide P11-4 on acidic erosion of enamel substrates. The null hypothesis was that use of the self-assembling peptide P11-4 would not result in a protective effect against enamel erosion.

METHODS AND MATERIALS

Specimen Preparation

A total of 26 freshly extracted bovine incisors without cracks or erosive lesions were cleaned and stored in physiological saline. The teeth were sliced into 1 mm sections in different directions with a low-speed diamond saw (Buehler–Illinois Tool Works Inc., Lake Bluff, IL, USA). Each enamel slab was carefully shaped into a rectangular form (4×4×1 mm) using a super-fine diamond finishing point (ISO #021; Shofu Inc, Kyoto, Japan) attached to an air-turbine (Twin-Power Turbine; J. Morita Mfg Corp, Kyoto, Japan). Specimen surfaces were successively ground using wet silicon carbide 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; Mitsutoyo, Tokyo, Japan). The resource equation method was used for sample-size calculation[23] and the samples were divided into four groups of six samples each. Two eroded samples were used for scanning electron microscopy (SEM) observations and the remaining 24 were divided into three groups of six specimens each.

Experimental Protocol

To create a model of eroded enamel, the prepared samples were immersed in pure orange juice (pH 3.6) for 5 minutes six times per day for 28 days. For the first group, Curodont Repair (CR group), the eroded specimens were treated with peptide P11-4 and stored in artificial saliva (pH 7.0, 14.4 mM NaCl, 16.1 mM KCl, 0.3 mM $MgCl_2{\cdot}6\ H_2O$, 2.0 mM K_2HPO_4, 1.0 mM $CaCl_2{\cdot}2\ H_2O$, and 0.10 g% sodium carboxymethyl cellulose) without acid challenge for a further 28 days. For the second group (curodont + acid challenge; CRA group), the eroded specimens were treated with peptide P11-4 and then with orange juice in the same way for a further 28 days. For the CR and CRA groups, treatment with peptide P11-4 was only carried out once, after the initial erosion period finished (28 days) and before the treatment period began (the further 28 days). In the last group (no curodont + acid challenge; NCRA group), the eroded specimens were not treated with peptide P11-4 and then were treated in the same way as the CRA group. Baseline group specimens were stored in artificial saliva for the experimental period (erosion period + treatment period, 56 days). (Figure 1).

Ultrasonic Measurement

Ultrasonic velocity (UV) was measured using a pulser/receiver (Model 5900PR; Panametrics, Inc, Waltham, MA, USA), a transducer for longitudinal waves (V112; Panametrics, Inc), and an oscilloscope (Wave RunnerLT584; Teledyne LeCroy, Chestnut

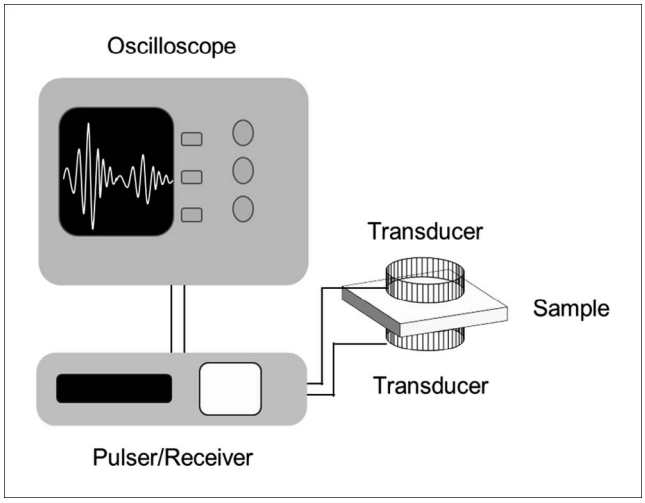

Figure 2. *Set-up of the ultrasonic device to detect changes in enamel remineralization and demineralization.*

Ridge, NY, USA). The equipment was initially calibrated using a standard procedure with 304 stainless steel calibration blocks (2211M; Panametrics, Inc). The transducer was oriented perpendicular to the contact surface of each specimen to obtain an echo signal (Figure 2). Ultrasonic waves were propagated from the transducer to the tooth, transmitted through the tooth, and then detected by the receiver on the opposite side. The transducers were connected to the pulser-receiver in through-transmission mode, and 16 μJ pulses were applied at a pulse repetition frequency of 500 Hz. The signal was captured using an oscilloscope. Heat generation was not observed from the transducers during measurement, and the system was calibrated following standard procedures before each specimen was measured. Each measurement was made at 23°C ± 1°C and 50% ± 5% relative humidity. Measurements were taken before the test and on days 1, 7, 14, 21, and 28 during both the preparatory erosion period and the treatment period. In addition, every sample thickness was measured using a dial gauge micrometer (CPM15-25DM, Mitsutoyo) before each set of UV measurements. Measurements were made before peptide application on day 28 of the preparation period and then on day 1 of the test period, which was the day after peptide application. The propagation velocity of ultrasound (UV) was calculated from sample thickness and transmission time.

SEM Observations

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 minutes, 75% for 20 minutes, 95% for 20 minutes, and 100% for 2 hours) and then transferred to a critical-point dryer for 30 minutes. The surfaces were then coated with a thin gold 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.

Statistical Analysis

The UV data were analyzed using two-way analysis of variance with time and treatment as confounding factors. Time was treated as a repeated measure. Post hoc pairwise tests among groups were performed using the Tukey honestly significant difference test. The level of significance was set at 0.05, and all calculations were performed using SigmaStat software (Systat Software, Inc, San Jose, CA, USA).

Table 1: *Mean (Standard Deviation) Ultrasonic Velocities (m/s) of Bovine Enamel Specimens by Treatment (n=6)*[a]

Group	Erosion Period (Days)					Treatment (Days)					
	0	7	14	21	28	Peptide P_{11-4}	0	7	14	21	28
Baseline	4549 (90) aA	4538 (90) aA	4584 (61) aA	4547 (52.4) aA	4547 (44.2) aA	Not applied (without acid challenge)	4483 (61.1) aA	4485 (97.9) aA	4538 (88.6) aA	4520 (95.8) aA	4536 (87.1) aA
CR	4545 (90) aA	4308 (90) bB	4184 (61) cB	4147 (52) cB	4047 (44) dB	Applied (without acid challenge)	4083 (61) dC	4285 (98) bcBCCD	4338 (89) bB	4320 (96) bC	4336 (87) bC
CRA	4555 (53) aA	4341 (54) bB	4222 (90) bcB	4138 (35) cB	4092 (42) cB	Applied (with acid challenge)	4167 (22) cB	4341 (64) bB	4362 (71) bB	4416 (77) abB	4415 (62) abB
NCRA	4539 (54) aA	4231 (67) bB	4155 (78) cC	4113 (81) cC	4069 (41) dB	No applied (with acid challenge)	4088 (42) cC	4080 (26) cC	4062 (20) dC	4032 (10) dcD	3968 (11) cD

Abbreviations: CR, curodent repair; CRA, curodont + acid challenge; NCRA, no curodont + acid challenge.
[a] *Within groups, means with the same lowercase letter are not significantly different (Dunnett test, $p>0.05$). Between groups at the same storage times, means with the same uppercase letter are not significantly different (Tukey-Kramer post hoc test, $p>0.05$).*

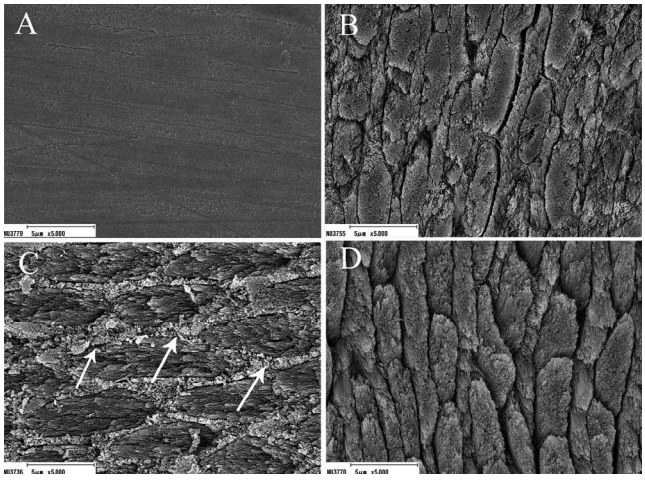

Figure 3. *Representative SEM images of the enamel surface of each specimen. SEM observations revealed differences in morphologic features among storage conditions. Demineralization of the enamel surfaces was more pronounced in the NCRA group, whereas the enamel surfaces of the CRA groups exhibited unclear etching patterns due to precipitation between the enamel rods. (A): Untreated enamel ground with #2,000 SiC paper (×5,000). (B): After initial treatment with pure orange juice (pH 3.6) for 5 minutes six times per day for 28 days (×5,000). (C): Representative CRA sample at 28 days (×5,000). Arrows indicate precipitation between the enamel rods. (3): Representative NCRA sample at 28 days (×5,000).*

RESULTS

Ultrasonic Measurement

Changes in the average UVs of each group are shown in Table 1. During the erosion period, the UVs in all groups decreased with increased erosion period. 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 UV in intact bovine enamel for the baseline group ranged from 4,483 to 4,549 m/s and did not vary significantly within the test period. The UVs in the NCRA group continued to decrease over time and were significantly lower than those of the other groups after 7 days. On the other hand, the velocities of the CR and CRA groups increased. In addition, there were no significant differences in UV after 28 days from the initial value before erosion between the CR and CRA groups. The UV changes in the CR and CRA groups are thought to be due to the influence of the peptide.

SEM Observations

Representative SEM images of specimens are shown in Figure 3. After initial treatment, the smear layer on the surface dissolved and etching patterns were observed. For the NCRA group, etching patterns and enamel rods were clearly observed. On the other hand, the CR and CRA groups had similar morphologic features in that etching patterns were not clearly due to precipitation between the enamel rods (indicated by arrows).

DISCUSSION

This study assessed the potential of the self-assembling peptide P11-4 to prevent acidic erosion. Although we acknowledge that bovine and human enamel are not identical due to genetic, environmental, and dietary differences,[24] the samples in this study were prepared from bovine lower incisors as bovine teeth are easier to obtain in large quantities, are in better general condition, and have a more uniform composition when extracted for study purposes than human teeth.[25] Another reason for the use of bovine teeth is the larger size, which allows preparation of more samples from the same tooth, resulting in fewer differences between bovine and human enamel, although the mineral distribution characteristics of the resulting lesions were almost indistinguishable.[26] Since sonic velocity data were only compared within the same sample and only interpreted within the different groups of the present study, the advantages of using bovine teeth might outbalance the disadvantages of not using human teeth.

Demineralization processes in enamel from erosive tooth wear are difficult to detect by visual inspection at early stages of formation. If the development of such demineralization processes can be identified and evaluated at an early stage, reparative approaches can be applied to halt the erosion process or even repair the affected tooth structure. Current diagnostic techniques are relatively ineffective for quantifying early enamel erosion, which is an important parameter when determining the severity of demineralization and assessing the efficacy of preventive measures.

Most techniques to quantify the elastic modulus of teeth involve destructive contact probing, which renders repetitive measurements unreliable.[27] In this regard, a nondestructive technique with the potential for localized evaluation of changes to the mechanical properties of enamel is desirable. Sonic velocity related to the mineral content of the cavity of an enamel lesion has been reported.[28] Since the mineralization content of enamel is reported to be closely linked to its stiffness and elasticity,[29] during demineralization of the tooth surface, the mineral content of the enamel and the specific sonic velocity should decrease in response to changes in enamel stiffness. The sonic velocity increases with the

volumetric concentration of mineral components and, thus, is a suitable index of the degree of mineralization. Therefore, we were inspired to use an ultrasonic device to detect enamel structure altered by acid erosion. When viewing the plane of the tooth surface, the enamel prisms are at different angles to the direction of acoustic images.[30] In this study, the enamel specimens were obtained from the labial surfaces of bovine teeth and the orientation of the specimens was precisely determined to avoid this kind of negative effect on sonic velocity.

The present study supports the forward-looking objective to manage acidic erosive lesions noninvasively by biomimetic remineralization.[31] The self-assembling peptide P11-4 is a rationally designed small molecule that undergoes hierarchical self-assembly into fibrillar scaffolds in response to specific environmental triggers.[32] The peptide undergoes one-dimensional self-assembly, forming micrometer-long nanotapes, preceding the formation of fibrils and edge-to-edge fibers.[33] The design criteria for peptides enabling self-assembly are well understood and have led to the development of a class of self-assembling peptides with a number of candidates showing potential for hard tissue regeneration.[34] The surface of fibers of the self-assembling peptide P11-4 has been shown to support hydroxyapatite formation and the remineralization of early carious lesions in an *in vitro* pH-cycling model, resulting in surface remineralization with formation of needle-shaped crystals[15] and increased microhardness after remineralization of subsurface lesions,[27] The penetration depth of the P11-4 peptide into subsurface lesions was addressed by confocal microscopy using a mixture of the P11-4 peptide and a fluorescent-labeled fusion peptide.[35]

The results of this study showed no significant difference in UV between the start of the treatment period and the end of the treatment period in CR and CRA groups. SEM images revealed that after initial treatment, the smear layer on the surface dissolved and etching patterns were observed. For the NCRA group, etching patterns and enamel rods were clearly observed. On the other hand, the CR and CRA groups had similar morphologic appearances in that etching patterns were not clearly due to precipitation between the enamel rods. This precipitation might be related to the formation of the 3D scaffold structure, which, in turn, may play an important role in retaining various dissolved ions as a reservoir.[36] That is, the surface is negatively charged and attracts Ca^{2+} and other related ions, which may initiate crystal formation and control the orientation and elongation of the hydroxyapatite crystals. In the results of this *in vitro* study, it might be concluded that the application of enamel matrix derivatives and self-assembling peptides on erosive lesions can prevent the progress of erosion and encourage remineralization, although further studies are needed to verify this approach under *in vivo* conditions.

CONCLUSION

Within the limitations of this *in vitro* study, application of the self-assembling peptide P11-4 may be able to prevent the progress of erosion from acid challenge and even to promote remineralization.

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Regulatory Statement

This study was conducted in accordance with all the provisions of the local oversight committee guidelines and policies of the Nihon University School of Dentistry.

Conflict of Interest

The authors of this manuscript certify that they have no proprietary, financial, or other personal interest of any nature or kind in any product, service, and/or company that is mentioned in this article.

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