Preventing and arresting primary tooth enamel lesions using self-assembling peptide P11-4 in vitro

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Abstract

**Background:** Self-assembling peptides (SAP) may prevent and/or arrest caries lesions. The aim of the study was to evaluate SAP for caries prevention and arrest in primary tooth enamel in vitro.

**Methods:** 215 extracted primary teeth were used. In the prevention experiment, self-assembling peptide for prevention (SAPP), fluoride varnish/mouthwash (FV/FMW), casein-phosphopeptide amorphous-calcium phosphate (CPP-ACP), nanohydroxy-apatite (nHA) were applied. Samples were subjected to a demineralizing pH-cycling for 14-days. In the arrest experiment, 60 samples were pre-demineralized and induced lesions treated using self-assembling peptide for repair (SAPR), FV, CPP-ACP plus fluoride and resin infiltration (RI), and submitted to pH-cycling. Thirty-five samples were used as negative controls (NC). Mineral loss and its differences ($\Delta/\Delta Z$) were determined using transversal microradiography.

**Results:** FV ($\Delta Z$ median: -46 [interquartile range: 189] vol%×µm) and FMW (-28 [129] vol%×µm) prevented caries significantly more effective than all other groups (p<0.05), which did not show significant preventive effects compared with NC ($\Delta Z$=1446 [378] vol%×µm). RI ($\Delta\Delta Z$=1808 [2193] vol%×µm) and FV ($\Delta\Delta Z$=1494 [4274] vol%×µm) arrested lesions compared with NC ($\Delta\Delta Z$=5605 [1371] vol%×µm; p<0.05), while SAPR and CPP-ACPF did not show such arrest.

**Conclusions:** FV and FMW showed the largest caries-preventive effect, while RI and FV arrested lesion progression in primary tooth enamel in vitro.

**Clinical significance:** Preventing and arresting caries lesion allows decreasing invasive treatment needs and patient's morbidity and fosters the application of minimal invasive dentistry approach.

**Background**

Caries in the primary dentition is one of the most prevalent conditions of humankind [1] with over 500 million untreated cases and over 120 million incident cases each year. Conventional treatment of caries lesions in primary teeth using restorative approaches is challenging due to a combination of behavioral and micro- and macro-anatomic factors, and failure rates of most restorations in the primary dentition being high [2]. Dentists frequently refrain from restoring caries lesions in the primary dentition at all, even if cavitated. Caries in the primary dentition is a major reason for hospitalization for both routine treatments and emergencies [3, 4].

Hence, there is a great need for both preventing and arresting caries lesions in the primary dentition. The most accepted strategies for prevention are the delivery of fluoride, mainly via toothpaste or, in high-risk individuals, varnishes, gels or mouthwashes, as well as routine oral hygiene care and dietary control. The use of fluoridated toothpaste for caries prevention is supported by a large body of evidence [5]. Similarly, fluoride varnish (FV) application has been found highly efficacious for caries prevention [6] and, if applied risk-adjusted, cost-effective [7]. The use of fluoride mouthwash (FMW) also has been supported by a range of studies [8]. However, and especially in smaller children, the delivery of fluoride is limited by the risks of chronic and acute toxicity.
Alternative interventions to control the balance between de- and remineralization on the surface of primary teeth, specifically enamel, have been sought, among them casein-phosphopeptide amorphous calcium phosphate (CPP-ACP) with or without the addition of fluoride for home and in-office use, nanohydroxyapatite (nHA) or self-assembling peptides P_11-4 (SAP) [9]. SAP are oligomer β-sheet-forming peptides (Ace-Gln-Gln-Arg-Phe-Glu-Trp-Glu-Phe-Glu-Gln-Gln-NH₂), which when subjected to specific environmental conditions have the ability to self-assemble into fibrillar scaffolds thereby creating β-sheet called ‘nanotapes’ [10]. The process of self-assembly continues while the nanotapes connect by pairing and transform into ribbons, which further self-assemble to form fibrils and fibers [11], leading to scaffold-like structures attracting calcium and phosphate deposition [12–14]. SAP thereby is supposed to facilitate biomimetic remineralization of hard dental tissue [13] and has been found efficacious clinically, too [15].

Similarly, for lesion arrest, the in-office application of FV or CPP-ACP, for example in higher concentrations [16], or SAP has been suggested [17]. Alternatively, resin infiltration (RI), where the lesion is infiltrated with lowly filled resins, which are light-cured and subsequently block any acid diffusion into the lesion body and hence mineral loss from it, can be applied to inhibit caries lesion progression [18]. There is a robust body of clinical data supporting RI, for example to arrest proximal lesions, mainly in the permanent dentition [19].

Overall, the majority of studies on preventing and/or arresting lesions using the described measures were conducted in the permanent, not the primary dentition. The body of evidence comparing fluoride applications, other mineral suppliers, RI or SAP is extremely limited. Therefore, we aimed to compare caries prevention and inhibition of lesion progression using SAP against those of other established measures in vitro, hypothesizing SAP to have significantly superior caries-preventive and arresting properties.

**Methods**

**Study design**

This study followed the CRIS (checklist for reporting *in-vitro* studies) guidelines [20] and was based on the fundamentals of ethical research practice. An informed consent was obtained from all patients’ legal guardians in order to include her/his extracted teeth in the experiments. This study assessed the caries-preventive and arresting effect of SAP and various alternatives, namely FV, FMW, CPP-ACP, CPP-ACP with fluoride (CPP-ACPF) and nHA for caries prevention, and FV, CPP-ACPF and RI for lesion arrest in primary tooth enamel in vitro (Table 1).
A combined study design was chosen, with samples being either prepared, the preventive strategies applied (experiment 1), and then challenged with demineralization (using pH cycling), or the samples being prepared, pre-demineralized using acetic acid for 21 days (to induce an artificial caries lesion), the application of arresting interventions (experiment 2), and then challenged with demineralization (using pH cycling). For both study parts, mineral loss $\Delta Z$ was assessed using transverse microradiography (TMR). The study flow is summarized in Fig. 1.

**Sample size estimation**

A sample size estimation was performed to have adequate power to apply a 2-sided statistical test of the research hypothesis (null hypothesis) that there is no difference SAP and FV in preventing and inhibiting carious lesions progression of primary teeth. According to the results of Sindhura, Vemulapalli, et al. [21]. Assuming an alpha ($\alpha$) level of 0.05 (5%), a Beta ($\beta$) level of 0.20 (20%) i.e. power = 80%, and an effect size (d) of (1.24), the required sample size (n) was 12 samples per group. Sample size calculation was performed using G*Power version 3.1.9.2.

**Specimens Preparation**

Two hundred fifteen sound primary anterior teeth (incisors and canines) obtained from Egyptian patients after exfoliation under an ethically approved protocol (ethical committee of the Ain Shams University, FDASURecIR022024) were collected and stored in 0.5% Chloramine T solution for maximum two months. Teeth were cleaned and those with stains, cracks, carious or developmental defects excluded. The root was separated from the crowns at the cemento-enamel junction using a water-cooled diamond coated band saw (Band Saw Exakt 300 cl; Exakt Apparatebau, Norderstedt, Germany). The samples were embedded in epoxy...
resin (Technovit 4071, Heraeus Kulzer, Hanau, Germany), with the labial surfaces of incisors and the lingual surfaces of canines facing upward, ground flat and polished sequentially (Mikroschleifsystem; Abrasive Paper WS flex 18C, SiC 1200–4000, Exakt Apparatebau, Norderstedt, Germany) until a surface of approximately 2 mm x 2 mm enamel was exposed.

**Interventions**

The 215 samples were divided into the two experimental arms; 120 were used to assess the caries preventive effect, 60 to assess lesion arrest, and 35 served as negative control group (NC), where 20 were used for the 1st experiment and 15 for the 2nd experiment. One third of the exposed surfaces of all samples was protected against the subsequent demineralization challenge using a nail varnish (Maybelline New York Express Finish 40, New York, USA), serving as sound control.

**Experiment 1:** To test the caries preventive effect, the remaining two thirds of the exposed surfaces of the 120 samples were treated using one of six interventions (n = 20/group, Table 1) before being challenged for demineralization: (1) SAPP (Curodont Protect, Credentis, Windisch, Switzerland), (2) FV (5% NaF Profliuorid, Voco, Cuxhaven, Germany), (3) CPP-ACP (Tooth Mousse, GC, Tokyo, Japan), (4) CPP-ACP plus fluoride (CPP-ACPF, MI Paste Plus, GC, Tokyo, Japan), (5) 500 ppm sodium Fluoride Mouthwash (FMW) (pharmacy of the Charité - Universitätsmedizin Berlin), (6) nHA mouthwash (Biorepair Mouth Wash, Dr. Kurt Wolff, Bielefeld, Germany). Curodont Protect gel was applied on a semi-dry surface with a microbrush, rubbed in and left for a couple of minutes to dry. It was then washed away as instructed by the manufacturer. FV, CPP-ACP and CPP-ACPF were applied on a dry surface with a microbrush and left 30 min to set, then rinsed off with water to mimic the conditions of the oral cavity. Both mouthwashes were utilized once daily after the demineralization cycle for 15 minutes by storing the samples in them, while the other samples were stored in distilled water during that time.

**Experiment 2:** To test lesion arrest, the remaining two thirds of the exposed surfaces of the 60 samples were pre-demineralized using 3 mM CaCl₂, 3 mM KH₂PO₄, 0,006 mM methylhydroxydiphosphonate (MHDP), 50 mM CH₃COOH, 10M KOH. The pH was adjusted to 4.95 using KOH for 21 days (Carl Roth, Karlsruhe, Germany) [22]. Half of the demineralized surface was covered with a nail varnish to allow the assessment of the mineral loss of the lesions after the first and prior to the second demineralization challenge. The remaining one third of the exposed surface of the sample received one of four interventions (n = 15/group, Table 1) prior to being challenged again for demineralization: (1) SAPR (Curodont Repair, Credentis, Windisch, Switzerland), (2) 5% FV (5% NaF Profliuorid, Voco, Cuxhaven, Germany), (3) CPP-ACPF (MI Paste Plus, GC), (4) RI (Icon DMG, Hamburg, Germany). For SAPR, samples were etched with 37% phosphoric acid (Fine Etch 37, Spident, Korea) for five s and rinsed with tap water. After drying the surface, Curodont Repair (In Vitro Vial, Credentis, Windisch, Switzerland) was dissolved without any further purification in 50 µL distilled water applied on each sample and left 5 min for setting. FV and CPP-ACPF were applied as described. Before RI, samples were etched using 37% phosphoric acid (FineEtch 37) for five s. The specimens were thereafter washed and dried using Icon Dry for 30 s and infiltrated using Icon Infiltrant for 3 min. After removing the excess material, light-curing was performed using a LED curing light (Valo, Ultradent, Salt Lake City, USA) with an intensity of 1400 mW/cm² for 40 s from < 1 mm distance. The procedure was repeated, with the infiltrant being applied for only 1 min, as recommended by the manufacturer. For both groups, an untreated negative
control (NC) (n = 35) was carried along the experiments, where 20 samples were utilized for experiment no.1 and 15 for experiment no.2.

**Demineralization challenge using pH cycling**

All samples were subsequently subjected to a pH cycling using a demineralization solution containing 2.2 mM CaCl$_2$, 2.2 mM NaH$_2$PO$_4$ and 50 mM acetic acid adjusted to a pH of 4.8 by NaOH (Carl Roth, Karlsruhe, Germany). The remineralizing solution contained 1.5 mM CaCl$_2$, 0.9 mM NaH$_2$PO$_4$ and 0.15 M KCl adjusted to a pH of 7.0 by KOH (Carl Roth, Karlsruhe, Germany). Each group was cycled separately for 8 h in 100 ml demineralizing solution and 16 h in 100 ml remineralizing solution for 14 days at room temperature without agitation. Between the de- and remineralizing cycles, the samples were washed with distilled water. The mouthwashes in the prevention groups were renewed daily [23].

**Transversal Microradiography (TMR)**

Samples were cut along their longitudinal axes (Band Saw Exakt, Exakt Apparatebau, Norderstedt, Germany) and thereafter, thin plano-parallel slices with a thickness of 100 ± 10 µm prepared (Mikroschleifsystem, Exakt Apparatebau, Norderstedt, Germany). During the sample preparation, five samples were lost. The samples were placed on film holders and exposed to a nickel-filtered copper radiation source operating at 20 kV and 20 mA with an exposure time of 10 s. Films (Fine 71337, Fujifilm, Tokyo, Japan) were developed according to the manufacturer's instructions under standardized conditions. The microradiographs were analyzed with a digital image-analyzing system (XC 77 CE, Sony, Tokyo, Japan) interfaced with a universal microscope (Axioskop 60318, Zeiss, Oberkochen, Germany) and a personal computer (TMR for Windows 2.0.27.2, Inspector, Research, Amsterdam, Netherland). Calibration standardization was done using an aluminum step-wedge with different aluminum thicknesses and a calibration curve between aluminum thickness and grey levels was constructed.

**Statistical Analysis**

Statistical analysis was performed using SPSS 20 (IBM, Armonk, NY, USA). Data were controlled for normal distribution using Shapiro-Wilk-test. Mineral loss $\Delta Z$ was calculated for each group as the median $\Delta Z$ together with interquartile deviations (ID) and expressed as vol.%/µm. In addition, the lesion depth (LD) was determined. Analyses of variance and post-hoc Tukey’s honestly significant difference (HSD) test were used to compare $\Delta / \Delta \Delta Z$ between groups. The level of significance was set at p < 0.05.

**Results**

When preventing the development of caries lesions, $\Delta Z$ was significantly lower for FV (median: -46 [interquartile range: 189] vol.%×µm) and FMW (-28 [129] vol.%×µm) than all other groups (p < 0.05). SAPP (1956 [11826] vol.%×µm) and CPP-ACPF ($\Delta Z = 1606 [935]$ vol.%×µm), CPP-ACP ($\Delta Z = 2099 [2042]$ vol.%×µm) and nHA ($\Delta Z = 1074 [971]$ vol.%×µm) did not show significantly different $\Delta Z$ than untreated controls ($\Delta Z = 1446 [378]$ vol.%×µm; p < 0.001, Fig. 2). Exemplary microradiographs are shown in Fig. 2.

When arresting caries lesions, $\Delta \Delta Z$ was significantly lower in RI ($\Delta \Delta Z = 1808 [2193]$ vol.%×µm) than SAPR ($\Delta \Delta Z = 5293 [7804]$ vol.%×µm) and NC ($\Delta \Delta Z = 5605 [1371]$ vol.%×µm); FV ($\Delta \Delta Z = 1494 [4274]$ vol.%×µm) also
showed ΔΔZ lower than NC. No significant differences were found between the other groups (p > 0.05; Fig. 3). Exemplary microradiographs are shown in Fig. 3.

FV and FMW resulted in significantly reduced LD when preventing lesions compared with all other preventive strategies, which did not differ in LD compared with NC (Table 2). For lesion arrest, LD did not differ between any treatment groups; all treatment groups showed significantly lower LD than NC (p < 0.05).

**Table 2**

<table>
<thead>
<tr>
<th>Preventive strategies</th>
<th>Inhibition of Lesion Progression strategies</th>
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<tbody>
<tr>
<td>Intervention</td>
<td>SAPP</td>
</tr>
<tr>
<td>Median</td>
<td>62</td>
</tr>
<tr>
<td>25th Percentile</td>
<td>58</td>
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<tr>
<td>75th Percentile</td>
<td>73</td>
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<tr>
<td>Significance</td>
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SAPP (Self-assembling peptide for prevention); FV (fluoride varnish); CPP-ACP/CPP-ACPF (casein-phosphopeptide amorphous-calcium-phosphate without/with fluoride); FMW (fluoride Mouthwash); nHA (Nanohydroxyapatite); NC (negative control). SAPR (self-assembling peptide for repair); RI (resin infiltration).

**Discussion**

A range of novel caries-preventive and arresting strategies based on both inhibition of demineralization and facilitation of remineralization are available. Especially in younger children and primary teeth, the availability of efficacious alternatives to fluoride are of relevance given concerns of acute and chronic toxicity of fluoride and the associated negative public sense [24]. SAP have been proposed as one such alternative. We compared a range of novel strategies suggested to prevent caries and inhibit lesion progression against each other and tested SAP for their effects on primary tooth enamel in vitro. For preventing caries lesions, we found established means like FV and FMW to be effective to reduce mineral loss, while novel strategies like SAP, CPP-ACP, CPP-ACPF and nHA did not have any significant preventive effect in vitro. For inhibition of lesion progression, we found RI to most effectively inhibit the lesions from progressing, followed by FV, while again novel strategies like CPP-ACPF and SAP did not have any significant inhibition of lesion progression effect in vitro. We hence reject our hypotheses.

This study has a number of strengths and limitations. First, data on novel strategies, especially SAP, are scarce, and so far, no study tested SAP to prevent caries or inhibit lesion progression in primary teeth. Previous studies on SAP focused on human enamel from permanent teeth or bovine enamel. Both show
different mineralization and maturation potential than human primary tooth enamel. Second, SAP have largely been tested for lesion arrest (i.e. facilitation of remineralization), not prevention [25], and this study is one of few testing this novel strategy for preventive applications as well as caries arrest. Third, the assessment of mineral loss using transverse microradiography is highly sensitive and a valid method that was not employed in previous studies on SAP. Instead, scanning electron microscopy (SEM), surface microhardness or laser fluorescence, which are all only determining proxies for mineral loss [26, 27] had been used. Fourth, and as a limitation, the employed in vitro protocol may have biased our findings to some degree. Notably, grinding and polishing the enamel surface has removed the aprismatic enamel surface layer which is hypothesized to be required for SAP and SAPR action: On prismatic enamel, columnar calcium of the hydroxyapatite crystal is not available any longer; it is assumed that peptide matrix development may be impeded to some degree in prismatic compared with aprismatic enamel. We nevertheless used the described setup, as only then valid mineral loss measurements in TMR are possible. Moreover, and notable, the aprismatic outermost layer of enamel is usually gradually worn off in a clinical setting, too, at least occlusally, and any clinical efficacy of self-assembling peptides might be reduced if strongly relying on this aprismatic enamel being available. In addition, previous studies had by large employed polished specimens, which is why we aimed to retain this concept for reasons of comparability [26, 27, 25]. Fifth, we applied SAP only once, i.e. before the 14d pH cycling period for reasons for standardization (other materials, e.g. FV, were also only applied once). The manufacturer recommends applying it 1–2 times per week. Also, we neither employed human nor artificial saliva, which has been suggested to be required for full action of SAP, as the 3-dimensional matrix formed by the peptides increases the surface area for calcium and phosphate deposition, present in the saliva, thereby allowing the formation of de novo hydroxyapatite crystals [13, 14, 12]. Application frequency and the availability of saliva might explain the differences between our findings and those yielded in situ and in vivo (8), with saliva also being a relevant source of mineralization related to fluoride applications, for instance. Furthermore, we employed an etching step when testing lesion arrest using SAP to mimic its clinical application. Etching of our polished samples might have removed the remaining pseudo-intact surface layer of the lesion claimed to be needed for SAP to effectively remineralize the enamel. The etching step was suggested in vivo to clean the pseudo-intact surface of enamel from pellicle and remove mineral debris; both are not present in artificially induced enamel lesions.

A range of findings needs to be discussed. SAP were not effective to prevent caries or inhibit lesion progression. A limited body of evidence on SAP is available, as discussed, and our findings align with some, but not all of the reported studies. This might be partially due to the mentioned methodological reasons (application time and frequency, absence of aprismatic enamel, lack of saliva and pellicle formation). However, especially for SAP, there remain a number of questions towards its hypothesized preventive mechanism: SAP are designed to assemble in the acidic environment of an active caries lesion and then attract minerals present in human saliva. It is unclear how this mechanism should apply to prevent lesions. In our study, it cannot be excluded that the material was washed away during the first demineralization cycles, or that only very thin peptide layers formed on the sound enamel surfaces, possibly insufficient to protect the enamel from subsequent demineralization.

Topical fluoride is considered the gold standard for caries prevention and lesion arrest (9). This was confirmed by our study. There is evidence suggesting that SAP should be combined with fluoride to harness
their complementary mechanism and location of action when it comes to inhibiting lesion progression (fluoride mainly acts on the pseudo-intact surface of initial lesions (9), while SAP is suggested to diffuse into the subsurface body of the lesion (1)). In our study, a possible advantage of the used FV was its consistency and stickiness. It is possible that FV not only had a chemical but also mechanical effect by “sealing” the surface, thereby protecting it from demineralization. Such sealing effect has been described for FV in studies on root caries prevention [28]. Given that we also found FMW to be efficacious, this explanation may not fully apply, though (notably, however, FMW was provided daily in contrast to most other alternatives).

RI is well known for its lesion progression inhibition in non-cavitated lesions by infiltrating carious enamel porosities and thereby occluding the diffusion pathways leading to caries arrest [29]. RI has been demonstrated to be superior to fluoride-based alternatives for lesion arrest by a range of clinical studies [19], also in the primary dentition [30], and our data also point into this direction. Notably, the application of RI is technique-sensitive, something which may be relevant especially in the primary dentition and in children.

CPP-ACP and CPP-ACPF did not show any caries preventive or lesion progression inhibiting effect in our study, which may be attributed to the fact that their effect is thought to be enhanced by the presence of a biofilm, which acts as a reservoir for the delivered calcium and phosphate ions and hence prevents mineral loss in intermittent periods of demineralization. Moreover, and as discussed for SAP, the application frequency of CPP-ACP and CPP-ACPF might have been insufficient.

Based on our results, a range of future directions can be derived. First, future in vitro studies assessing SAP should aim to mimic the aprismatic enamel layer even when using ground specimens. Second, the application of saliva prior to remineralization may be recommended in an in vitro setting to allow pellicle formation and simulate clinical conditions as far as possible. Alternative, in situ designs may be employed. Third, mineral loss measurement should not focus on artifact-prone methods (like laser-fluorescence) or unsuitable proxies (like SEM evaluation), but strive to truly determine the mineralization effects, for example using TMR, transverse wavelength-independent microradiography or micro-CT. Last, clinical studies should be employed before translating our findings into any clinical recommendations; so far, clinical data largely focused on lesion remineralization, as described.

**Conclusion**

In conclusion, and within the described limitations, FV and FMW showed consistent and significant caries preventive effects on human primary teeth enamel in vitro, while RI and FV were shown to be effective to inhibit caries lesion progression in this set-up. SAP, CPP-ACP and CPP-ACPF and nHA did not show any significant caries preventive or progression inhibition effects.

**Abbreviations**

SAP  
Self-assembling peptides

SAPP  
Self-assembling peptides for prevention
FV
Fluoride varnish

FMW
Fluoride mouthwash

CPP-ACP
Casein-phosphopeptide amorphous-calcium phosphate

nHA
Nanohydroxy-apatite

RI
Resin infiltration

NC
Untreated controls

SAPR
Self-assembling peptides for repair

Ace-Gln-Gln-Arg-Phe-Glu-Trp-Glu-Phe-Glu-Gln-Gln-NH2
oligomer β-sheet-forming peptides

CRIS
Checklist for reporting in-vitro studies

CPP-ACP F
Casein-phosphopeptide amorphous-calcium phosphate with fluoride

TMR
Transverse microradiography

ID
Interquartile deviations

LD
Lesion depth

HSD
Post-hoc Tukey’s honestly significant difference

SEM
Scanning electron microscopy

**Declarations**

**Ethics approval:**

The study was approved by the ethical committee of the Ain Shams University, FDASUReleIR022024. This article does not contain any studies with human participants or animals performed by any of the authors.

**Consent to participate:**

An informed consent was obtained from the patients’ guardians in order to include her/his extracted teeth in the experiments.
Consent for publication:

Not applicable

Availability of data and materials:

All data generated or analyzed during this study are included in this published article.

Competing Interests:

All authors declare that they have no competing interests.

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Authors’ contributions:

The study was conceived by KE, FS, SP, NW, NK, GA and SP, FS, KE, PG JB, MK planned the analysis. NW and KE collected the data. FS, PG JB und KE performed the analysis. NW, KE, FS, NK and PG JB wrote the manuscript. All authors read and approved the manuscript.

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