REMINERALIZATION OF SELF-ASSEMBLING PEPTIDE P11-4 AND CASEIN PHOSPHOPEPTIDE-AMORPHOUS CALCIUM PHOSPHATE FLUORIDE ON CARIES-LIKE LESIONS IN PRIMARY TEETH (IN-VITRO STUDY)

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ABSTRACT

INTRODUCTION: The early stages of dental caries involve enamel demineralization which can be reversed. Attempts have been made to develop new materials to remineralize enamel.

OBJECTIVES: To assess remineralizing effect of Self-assembling Peptide P11-4 and Casein Phosphopeptide-Amorphous Calcium Phosphate Fluoride (CPP-ACPF) in comparison to Sodium Fluoride varnish on early carious lesions in primary teeth.

MATERIALS AND METHODS: Thirty-six sound-extracted primary anterior teeth were used. A 4x4mm window was made in the middle of the labial surface of each tooth. Teeth were subjected to artificial demineralization and then randomly allocated into 3 groups: Group I(5%NaF varnish), Group II(CPP-ACPF varnish) and Group III(P11-4). Teeth were sectioned longitudinally into two halves. One half was treated by the remineralizing agent, and the other was left untreated as a negative control. Samples were subjected to elemental analysis using Energy Dispersive X-ray spectrometer (EDX) where the Calcium and phosphorus contents were estimated and the surface topography was assessed by Scanning-Electron-Microscope (SEM). Comparison of Calcium/Phosphorus ratio was done using One Way ANOVA and the differences in percent change were analyzed using the Kruskal Wallis test.

RESULTS: There was a significant difference in the mineral content within each group before and after treatment indicating remineralization in all groups. The percent increase in Ca/P ratio after treatment was higher in P11-4 group with no significant difference between groups. Surface topography showed improvement in all groups with better surface quality in self-assembling peptide P11-4 group than other groups.

CONCLUSION: The CPP-ACPF varnish, Self-assembling peptide P11-4 and 5% Sodium fluoride varnish are effective in restoring the mineral content and improving surface morphology of early carious lesions in primary teeth.

KEYWORDS: Self-assembling peptide P11-4, casein phosphopeptide, amorphous calcium phosphate, Sodium Fluoride.

INTRODUCTION

Dental caries is one of the most prevalent diseases around the world. The process of caries development involves acid production by bacteria as a by-product of fermentable carbohydrates. (1) The acids produced affect enamel by dissolving its mineral content. The caries process involves continuous cycles of demineralization and remineralization, so it’s a dynamic rather than a static one. When periods of demineralization exceed those of remineralization, caries progress. In the early stages, caries appears clinically as white spot lesions and can be reversed at this stage. By progression of the caries process, cavitation occurs. (2) Changes in restorative and preventive dentistry have taken place in the past decades where minimal invasive approaches have been introduced to the field of dentistry. This includes early detection and
intervention of the disease before irreversible damage occurs. (3) Fluoride has been considered a “gold standard” for remineralization. Fluoride has not only been as a preventive measure against caries but, to arrest active carious lesions as well. (4) Calcium fluoride that forms on the tooth surface after therapy can act as fluoride reservoir. It also can lower the critical pH value of enamel demineralization from 5.5 to 4.5 in the oral cavity. Fluoride is incorporated in the formation of fluorapatite crystals which makes the surface more resistant to acid dissolution. It inhibits demineralization, enhances remineralization, inhibits plaque metabolism and, affects plaque formation. (5) In recent times, calcium phosphate-based remineralization systems have been developed and are now commercially available. One of these systems is casein phosphopeptide-amorphous calcium phosphate (CPP-ACP). Casein phosphopeptide (CPP) has the ability to stabilize calcium phosphate by binding amorphous calcium phosphate (ACP) and thus forming CPP-ACP clusters. (6) These CPP-ACP clusters act as a calcium and phosphate reservoir that attaches itself to dental plaque and tooth surfaces. On acid challenge, the attached CPP-ACP releases calcium and phosphate ions, thus maintaining a supersaturated mineral environment, thereby reducing demineralization and enhancing remineralization. (7) Casein Phosphopeptide-Amorphous Calcium Phosphate Fluoride (CPP-ACPF) varnish is a unique technology that includes CPP-ACP incorporated with 5% sodium fluoride. (8)

Research efforts have been directed towards a true regenerative approach aiming at regenerating hydroxyapatite crystals within the subsurface carious lesion possibly using the natural remineralization process from saliva. (9) Self-assembling peptides have proved their ability to form 3-dimensional fiber networks supporting tissue regeneration. Self-assembling peptide P11-4 has shown potential to treat and prevent dental caries. Its application on early carious lesion can increase mineral gain and formation of new hydroxyapatite crystals. It diffuses into the subsurface lesion body and assembles into higher order fibrils facilitating mineralization by mimicking the natural biomineralization of enamel. (10) There are many studies evaluating the effect of each of the remineralizing agents (4, 6, 10), however; to the best of our knowledge, there hasn’t been enough studies comparing the remineralizing effect of these materials on the primary teeth.

The aim of this study was to assess the remineralizing effect of self-assembling peptide P11-4 and CPP-ACPF in comparison to sodium fluoride varnish on caries-like lesions in primary teeth. The null hypothesis was that there was no significant difference in the change of the mineral content and surface topography between self-assembling peptide P11-4, CPP-ACPF and sodium fluoride on the remineralization of caries-like lesions in primary teeth.

MATERIALS AND METHODS
The current study was an experimental in-vitro study that was performed in the departments of Pediatric Dentistry and Dental Public Health and Oral Biology, Faculty of Dentistry, as well as Faculty of Science and Faculty of Medicine, Alexandria University. The protocol of the study was approved by the Committee of Ethics, Faculty of Dentistry, Alexandria University, Egypt (IRB No. 001056 – IORG 0008839).

Sample Size Estimation
The sample size was based on a 5% alpha error, 80% power, and survival rate of 4.2% and 60% for the test and control, respectively (11). The minimum sample size was calculated to be 10 teeth per group, increased to 12 teeth to make up for processing errors. Total sample= number per group x number of groups= 12 x 3 = 36 teeth.

The sample size was based on Rosner’s method (12) calculated by G*Power 3.1.9.7. (13)

Preparation of the specimens
Thirty-six sound extracted anterior primary teeth due to orthodontic purposes were included after the approval and informed consent from the legal guardians. Teeth were checked visually for the absence of any cavitations, white-spot lesions, restorations, developmental defects, and discoloration. A magnifying lens was used to check for the absence of any cracks. The teeth were cleaned with a brush using fluoride free pumice and then stored in deionized (DI) water till usage. The teeth were dried and self-adhesive labels 4X4 mm were stuck on the center of the labial surface of each tooth. An acid-resistant nail varnish was used to coat all the surfaces of the teeth. After drying, the varnish coating was removed exposing a window of enamel for creation of caries-like lesions. (14)

Artificial Caries Formation
A demineralizing solution was prepared containing Calcium Chloride (CaCl2) 2.2 mM, Potassium Dihydrogen Phosphate (KH2PO4) 2.2 mM, Acetic Acid (CH3COOH) 0.05 M and Potassium Hydroxide (KOH) 1 M. The pH of the solution was adjusted to 4.4 using a pH meter (15) The teeth were immersed in demineralizing solution (10 ml for each specimen) in a separate container for each specimen. All the containers were incubated for 96 hours to form artificial enamel carious lesions. The solution was changed every 48 hours. The teeth were visually checked for the appearance of chalky white lesions on enamel surfaces. (16)

Randomization and Grouping
All the teeth were randomly allocated using permuted block technique (17) to 3 groups (N=12) based on the
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remineralizing agent used. Each specimen was sectioned into 2 halves so one half of the tooth served as test and the other half as a negative control. Accordingly each group was subdivided into 2 subgroups as follows (figure 1)

Group I was subdivided into:
Subgroup Ia: 5% Sodium fluoride varnish (Duraphat® Varnish)
Subgroup Ib: Left in deionized water (Negative control)

Group II was subdivided into:
Subgroup IIa: CPP-ACPF varnish (MI Varnish®)
Subgroup IIb: Left in deionized water (Negative control)

Group III was subdivided into:
Subgroup IIIa: Self-assembling peptide P11-4 (CURODONT™ REPAIR)
Subgroup IIIb: Left in deionized water (Negative control)

Application of the Remineralizing Agents
Teeth were removed from the demineralizing solution, washed using deionized water and then sectioned longitudinally in a buccolingual direction through the center of the window into two equal halves a mesial half and a distal one using a microtome (Metkon®, MICRACUT®, PRECISION CUTTER). (14) One half was used as the test specimen and treated by the remineralizing agent, and the other half was left untreated as a negative control. All remineralizing agents were applied according to manufacturers’ instructions. For subgroup Ia, a thin, uniform topical coating of 5% NaF Varnish was applied with a disposable brush (18) (19) For subgroup IIa, each specimen was treated with a thin, uniform layer of CPP-ACPF varnish using a disposable brush (20) The varnishes then were removed gently with a cotton swab soaked with acetone without damaging the enamel surface. (19) For subgroup IIIa, the enamel surface was cleaned with 2% Sodium Hypochlorite for 20 seconds, then the enamel was etched with 35% Phosphoric Acid for 20 seconds, rinsed with distilled water, and dried. Self-assembling Peptide (P11-4) was then activated, removed from protective cover, applied on the tooth surface and allowed to diffuse for 5 minutes. (21)

pH cycling
A 10-day pH cycling model was applied in this study. During each day all the specimens (treated specimen with its untreated control) were immersed in the demineralizing solution for 3 hours, twice a day, with 2 hours of remineralization in between. Specimens were placed in the remineralizing solution overnight. The specimens were washed with deionized water between solutions and separate containers were used during pH cycling. Fresh new solutions were prepared and used for every cycle. The remineralizing solution
used contained calcium chloride (CaCl₂) 1.5 mM, sodium dihydrogen phosphate (NaH₂PO₄) 0.9 mM and potassium chloride (KCl) 0.15 M with a pH of 7.0. (15, 22)

Outcome assessment
The remineralizing effect of the different agents on the artificially created caries like lesions were assessed by the change in calcium (Ca), phosphorus (P) ions in mass % and Calcium/ Phosphorus ratio using the Energy Dispersive X-ray spectrometer (EDX) and the quality of surface enamel topography was assessed using scanning electron microscope.

Energy Dispersive X-ray spectrometer (EDX) provides quantitative information on the elemental distribution on the tooth surface. (23) It is incorporated into scanning electron microscope (JOEL,JSM-IT200 InTouchScope™ SEM, Faculty of Science, Alexandria University). For assessing surface topography using SEM, the specimens were dehydrated by ethyl alcohol 50%, 70% and 90% freshly prepared, vacuumed and sputter coated with a gold-palladium layer to be ready for examination with SEM. The result was presented as a three dimensional image with 5000x magnification providing information on surface topography. (24)

Statistical analysis
Normality was checked for all variables using Shapiro Wilk test and Q-Q plots. Percent change was calculated according to the following formula: [Values of remineralized enamel (after treatment)– Values of demineralized enamel (before treatment)] / Values of demineralized enamel x 100. Comparison of Ca/P ratio was done using One Way ANOVA. Comparison between demineralized and remineralized enamel surfaces (before and after treatment) within each group was done using paired t test. Differences in percent change were analyzed using the Kruskal Wallis test followed by Dunn’s test with Bonferroni correction. All tests were two tailed and the significance level was set at p value ≤ 0.05. Data were analyzed using IBM SPSS version 24, Armonk, NY, USA.

Figure (1): Flow diagram of the study design.
RESULTS

Regarding the Ca ion content, there was a significant difference in Ca ion content before and after treatment in all groups (P < 0.0001, P < 0.0001 and P < 0.0001 respectively). There was not a significant difference in Ca ion content between the three study groups before treatment (P = 0.378) nor after treatment (P = 0.227).

Casein Phosphopeptide-Amorphous Calcium Phosphate Fluoride (CPP-ACPF) group showed the highest percent increase in Ca ion content [Median (IQR) = 49.86 (35.73)] followed by Self-assembling peptide P11-4 group [Median (IQR) = 37.83 (16.04)] followed by NaF group [Median (IQR) = 19.98 (12.47)]. The difference in percent increase in Ca ion content was not significant between the three groups (P = 0.273). (Table 1 and Table 2)

Referring to the P ion content, there was a significant difference in P ion content before and after treatment in all groups (P < 0.0001, P < 0.0001 and P < 0.0001 respectively). There was not a significant difference in P ion content between study groups before treatment (P = 0.096) nor after treatment (P = 0.383). Self-assembling peptide P11-4 group showed the highest percent reduction in P ion content [Median (IQR) = 37.83 (16.04)] followed by CPP-ACPF group [Median (IQR) = 28.11 (34.87)] and NaF group [Median (IQR) = 23.05 (16.39)]. The difference in percent decrease in P ion content was not significant between groups (P = 0.686). (Table 1 and Table 2)

There was a significant difference in Ca/P ratio before and after treatment in all groups (P < 0.0001). There was not a significant difference in Ca/P ratio between study groups before treatment (P = 0.538) nor after treatment (P = 0.936). Self-assembling peptide P11-4 group showed the highest percent increase in Ca/P ratio [Median (IQR) = 12.38 (8.09)] followed by CPP-ACP group [Median (IQR) = 10.98 (7.95)] and NaF group [Median (IQR) = 9.00 (8.09)]. The difference in percent increase in Ca/P ratio was not significant between groups (P = 0.386). (Table 1 and Table 2)

Evaluation of the specimens of scanning electron microscope (SEM) in the untreated enamel (negative control subgroups) revealed severe porosity of the demineralized enamel surface. Loss of surface enamel was noted with dominant erosions in the rod and interrod substance. (Figure 2a) Subgroup Ia treated with fluoride varnish showed a smooth remineralized enamel surface with minor focal patches of shallow irregularities. (Figure 2b) Subgroup Ila treated with CPP-ACPF displayed a remineralized surface of enamel with area of evident globules of calcification. (Figure 2c) Subgroup IIIa treated with self-assembling peptide P11-4 revealed a remineralized enamel surface with notable calcified deposits filling the demineralized pores. (Figure 2d)

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<th>Table (1): Comparison of Ca, P, and Ca/P ratio content before and after treatment among the study groups</th>
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<td><strong>Ca/P Ratio</strong></td>
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<td><strong>Before treatment</strong> (Demineralized enamel) Me an ± SD</td>
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<td><strong>After treatment</strong> (Remineralized enamel) Me an ± SD</td>
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**Calcium**

| | **Before treatment** (Demineralized enamel) Me an ± SD | 23.05 ± 3.80 | 21.32 ± 2.32 | 22.72 ± 3.24 | 1.003 (0.37 8) |
| | **After treatment** (Remineralized enamel) Me an ± SD | 29.78 ± 32.50 | 30.48 ± 32.82 | 30.53 ± 35.72 | 1.550 (0.22 7) |

**Phosphorus**

| | **Before treatment** (Demineralized enamel) Me an ± SD | 16.39 ± 1.34 | 16.58 ± 0.87 | 17.33 ± 0.98 | 2.515 (0.09 6) |
| | **After treatment** (Remineralized enamel) Me an ± SD | 15.54 ± 1.72 | 16.02 ± 1.73 | 16.70 ± 1.75 | 0.961 (0.38 3) |

**Figure (2):** SEM images of enamel surfaces using 5000x magnification; (a) negative control subgroups showing a porous enamel surface with erosions of enamel rods and partial loss of interprismatic substance; (b) subgroup Ia (5%NaF) showing a relatively smooth surface with focal patches of mild shallow irregularities; (c) subgroup Ila (CPP-ACPF) showing a smooth surface of remineralized enamel with evident areas of globular calcification; (d) subgroup IIIa (P11-4) showing a remineralized enamel surface with notable calcified deposits filling the demineralized pores.
This study to evaluate the difference in surface morphology when using three different strategies of remineralization.

In the current study, the teeth were divided into halves, one half received the remineralizing agent and the other half served as negative control, this rules out any variation in the tooth structure as results depend on the change in mineral content and surface morphology on the same tooth other than using a separate negative control group. (14) This study used a pH cycling model to simulate oral conditions through mineral loss and mineral gain dynamics, which can approximate pH balance in daily life. (15) Throughout the 10 days of pH cycling, the solutions were renewed on a daily basis to prevent oversaturation and separate containers were used to avoid cross-reactions. (22)

All three remineralizing agents were able to achieve successful remineralization to the demineralized enamel surface. Casein Phosphopeptide-Amorphous Calcium Phosphate Fluoride (CPP-ACPF) showed the highest Ca ions percent increase due to the ability of casein phosphopeptide to stabilize amorphous calcium phosphate and maintaining a supersaturated mineral state. (6, 7) However, it showed no statistically significant difference than that of 5%NaF group. This could be attributed to the release of calcium and phosphate ions from enamel substrate to the surface during pH cycling, which can make the mineral content comparable in both groups. (26)

The non-significant difference between self-assembling peptide P11-4 group and other groups in mineral content can be related to the time needed for self-assembling peptide P11-4 to get minerals and restore mineral content of enamel. (27) Manufacturer’s instructions for application of self-assembling peptide P11-4 include acid etching step, which can initially cause further mineral loss. This can be compensated by time when fibrillar network forms and minerals deposit to form enamel like structure with high mineral content. (28) These findings agree with the results of Sindura et al 2018 (27) who used EDX to compare Ca/P ratio between self-assembling peptide P11-4 and CPP-ACP. CPP-ACP had a significantly higher Ca/P ratio after 1-week and 1-month but after 3 months self-assembling peptide P11-4 was significantly higher due to gradual increase in Ca/P ratio over time. In contrast to our results, Shetty et al (29) found a statistically significant higher calcium content in P11-4 group than fluoride varnish group. This could be attributed to the longer remineralization period used in this study which is 21 days in contrast to the 10-days pH cycling used in our study, which allows more calcium mineral gain to enamel surface after P11-4 application.

Within group comparison showed a statistically significant change in Ca ion content and Ca/P ratio before and after treatment in all groups.

| Table (2): Comparison of percent change in Ca, P, and Ca/P ratio content after treatment among the study groups |
|---|---|---|---|---|
| After treatment (Remineralized enamel) | Group I (5%NaF varnish) (n=12) | Group II (CPP-ACPF varnish) (n=12) | Group III (Self-assembling peptide P11-4) (n=12) | Paired t test (p value) |
| Ca | 33.72 (49.29) | 49.86 (35.73) | 46.95 (32.09) | 1.03 (0.273) |
| P | 10.17 – 78.07 | 15.51 – 82.25 | 4.94 – 91.59 | 0.163 (0.686) |
| Ca/P ratio | 2.76 – 43.08 | 28.11 (34.87) | 37.83 (16.04) | 0.750 (0.386) |

*Statistically significant difference at p value≤0.05, H test: Kruskal Wallis test

**DISCUSSION**

The results of the present study showed no statistically significant difference in the change of mineral content between the three study groups. However, a difference was detected in surface topography between the study groups, so the proposed null hypothesis is partially rejected. Fluoride varnish was used in the study as a positive control as it is the material that proved its ability to remineralize enamel surface after P11-4 application. Chloride et al (21) found that fluoride varnish is effective in remineralizing the surface of primary teeth. (21) Jackson et al (22) stated that fluoride varnish is widely used in dental practice. Casein phosphopeptide is effective in remineralizing the enamel surface after P11-4 application. (23) Scanning Electron Microscope (SEM) is a qualitative method combined with EDX that can evaluate surface morphology. (24) SEM was used in...
Although the three remineralizing agents used different techniques, all of them were able to achieve enamel remineralization. Self-assembling peptide P11-4 has the ability to promote de novo nucleation of hydroxyapatite nanocrystals. (10) Fibers form through the body of the lesion enabling regeneration of deeper subsurface lesion and improvement of mineral content of the lesion. (30) This comes in accordance with previous studies. (10, 30) Regarding CPP-ACPF, CPP stabilizes ACP preventing phase transformations making ions available to diffuse into mineral-deficient lesions allowing remineralization of demineralized enamel. (31, 32) On the other hand, calcium fluoride deposition after applying fluoride varnish results in chemical stability and remineralization by making changes in enamel hydroxyapatite. (33, 34) Fluoride varnish when applied on enamel surface results in the formation of calcium fluoride reservoir on the tooth surface. It aids in the formation of fluorapatite crystals which are more resistant to acid dissolution than hydroxyapatite. (35) The effect of fluoride varnish on enamel remineralization was supported by previous studies. (33, 34)

The scanning electron microscope revealed difference in surface topography between study groups. Non treated enamel showed increased porosity with erosion of enamel rods due to dissolved minerals. These results come in agreement with previous studies. (27, 36) All surfaces that were treated with remineralizing agent showed smaller pore size and smoother surface than negative control group. CPP-ACPF group showed globular arrangement of the minerals, a finding that was reported in other studies. (27, 28) Self-assembling peptide group showed homogenous enamel surface with dominant masses of calcification indicating a best surface quality among all study groups. Previous studies found that, although P11-4 supports enamel tissue regeneration, it doesn’t necessarily regenerate the same original enamel structure and that lesions treated with P11-4 don’t necessarily have the same prismatic alignment of HAP crystals. (37, 38)

Self-assembling peptide P11-4 converts from low viscosity liquid that is able to penetrate the pores of white spot lesions into an elastomeric gel which acts as scaffold for nucleation of hydroxyapatite crystal formation. (39-41) Also CPP-ACPF is found to have a remineralizing effect on the subsurface lesions. (6) So one of the limitations of this study is that it only studies the change in mineral content and surface morphology and didn’t assess the depth of remineralization. An evaluation method that can measure the penetration of remineralizing agent and evaluating lesion depth is recommended in further studies.

Another limitation was that the controlled pH cycling used in in-vitro studies does not necessarily mimic the uncontrolled pH fluctuations in the oral cavity. Also, in the oral cavity there are some factors that can affect the effect of using a remineralizing agent and there are individual variations like diet, oral hygiene, fluoride use and the composition of saliva and plaque.

CONCLUSION
Within the limitations of the current study, it can be concluded that,
Self-assembling Peptide P11-4, CPP-ACPF varnish and 5% Sodium fluoride varnish are effective in restoring the mineral content of early carious lesions in primary teeth.
Self-assembling peptide P11-4 showed the most favorable enamel surface topography among study groups.
CONFLICT OF INTEREST
There was no conflict of interest in the following study.

FUNDING STATEMENT
No institutional funding was provided.

REFERENCES


