

THE EFFECT OF SELF ASSEMBLING PEPTIDES VERSUS SODIUM FLUORIDE VARNISH ON ARTIFICIALLY INDUCED ENAMEL LESIONS IN HUMAN PREMOLARS, IN VITRO STUDY. (SCANNING ELECTRON MICROSCOPE AND ENERGY DISPERSIVE X-RAY ANALYSIS)

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ABSTRACT

Background: Dental caries is a global health concern, with white spot lesions being a critical stage that can be reversed with preventive measures. Topical fluoride is commonly used, but researchers are exploring bioactive materials like self-assembling peptide P11-4 for biomimetic enamel remineralization.

Aim: Assessment of biomimetic self assembling peptides P11-4 effect versus sodium fluoride varnish on enamel remineralization.

Materials and methods: Thirty-five lower first premolars were alienated for five groups of seven specimens each. Group I (control): no treatment was applied. The remaining samples were exposed to demineralization. After initiation of lesions, samples were subdivided into four equal groups: Group II: examined immediately. Group III: No treatment was applied. Group IV: (fluoride group). Group V: (Curodont Group). Specimens were preserved in artificial saliva. Examination was done after one month using SEM, EDXA and polarized light microscope.

Results: EDXA results of Calcium values showed control and Curodont groups had significantly higher values than artificial saliva and demineralized groups. The phosphorous values showed control, artificial saliva, fluoride and Curodont groups had significantly higher values when compared to demineralized group. The Calcium/Phosphorous ratio showed Curodont to have significantly higher value than demineralized and artificial saliva groups. Polarized light microscope results confirmed the SEM-EDXA findings.

Conclusion: Self assembling peptide P11-4 was able to remineralize enamel carious lesions more efficiently than sodium fluoride varnish.

KEY WORDS: Self assembling peptides; Fluoride varnish; Remineralization; SEM; EDXA.

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INTRODUCTION

Dental caries is a global health issue that impacts a large population¹. While the treatment for dental diseases can be costly, prevention methods are relatively straightforward and highly effective². The demineralization of the tooth's crystalline mineral structure occurs due to the action of acids formed by dental biofilm bacteria. These bacteria metabolize fermentable carbohydrates, particularly sugars, present in the diet. Dental caries initiates at and beneath surface of the enamel, with the first demineralization occurring below the surface. Due to mineral loss, the tooth experiences increased porosity, leading to the widening between crystals and the surface becomes softer. Thus acids penetrate into tooth, causing subsurface demineralization. The lesion looks as a white spot when enough minerals are lost. At this point, lesion could be arrested or even reversed by modifying the underlying causes or implementing preventive measures. However, repair primarily occurs within the surface layer of the lesion^{3,4}.

White spot lesions (WSLs) are initial signs of dental caries occurring beneath the intact surface of enamel⁵. There is a growing demand in dentistry, which promotes non-invasive management of lesions through a biological methodology called remineralization⁶.

Remineralization refers to a process in which calcium and phosphate are redeposited in the crystal spaces of demineralized enamel, resulting in minerals gain that enhances function and aesthetics⁷.

Various mechanisms can be utilized to facilitate this process, with topical fluoride treatment being the most commonly employed method⁸. Topical fluoride is a commonly preferred method for controlling dental caries, especially in individuals at high risk for tooth decay. Amongst various fluoride agents, sodium fluoride varnishes (NaF) have emerged as the favorite form⁹.

While fluoride is widely recognized as a non-invasive treatment for non-cavitated lesions, it does have limitations. One of these limitations is its reliance on the availability of calcium and phosphate ions for effective remineralization¹⁰. Another drawback of using fluoride varnishes is that higher concentrations of fluoride primarily promote remineralization on the outer surface of the enamel, leading to the formation of a superficial remineralization layer. However, this may not result in complete remineralization of the entire body of the lesion¹¹. Additionally, different dental products containing fluoride are active in enamel remineralization however they are unable to promote formation of organized apatite crystals¹².

Enamel matrix proteins which guide enamel formation are almost lost during enamel maturation^{13,14}. As the research uncovers more enamel matrix components, several important questions arise regarding their origin, significance, and role in enamel development and formation. These questions include determining which proteins are endogenous and which ones are exogenous, as well as identifying the essential proteins and understanding their specific contributions to enamel development and formation. Answering these questions is crucial for developing clinical advancements such as improved diagnosis and repair techniques. Furthermore, this knowledge is vital for supporting biomimetic enamel regeneration or develops novel materials that possess remarkable criteria found in enamel^{15,16}.

Regenerative approach aims to enamel matrix replacement by a biomimetic matrix that might increase lesions remineralization. Remineralizing products currently available on the market fail to regenerate enamel. The development of biomimetic enamel remineralizing agents like self-assembling peptides P11 -4 was made possible by understanding the fundamentals of mediated mineralization^{17,18}. Biocompatible small molecules that possess the ability to self-assemble and penetrate into the sub-

surface carious lesion hold great potential as perfect blocks guiding enamel regeneration. These molecules can act as scaffolds, providing a framework for the regeneration process^{19,20}. These scaffold-like structures simulate the function of matrix proteins which regulate deposition and growth of crystals during natural mineralization process²¹.

As a result, this study aimed to evaluate the efficacy of biomimetic self-assembling peptides P11-4 and sodium fluoride varnish on enamel remineralization.

MATERIALS AND METHODS

Materials

1. Artificial saliva was prepared as said by Ten Cate and Duijsters, it was formed of 1.5mM CaCl₂ (calcium chloride) , 0.9mM NaH₂ PO₄ (Sodium dihydrogen phosphate), 0.15M KCl (potassium chloride) at pH 7.0²².
2. Demineralizing solution The solution contained 2.2 mM KH₂ PO₄ (potassium dihydrogen orthophosphate dehydrate), 2.2 mM CaCl₂ (calcium chloride), 0.05 M acetic acid; pH was adjusted to 4.4 with 1 M KOH (potassium hydroxide)²². Both were prepared at Faculty of Pharmacy Ain Shams University.
3. Fluoride based varnish containing 5% sodium fluoride (Enamelast, Ultradent, US). It is a 5% sodium fluoride in a resin carrier, which is flavored and xylitol-sweetened. It is prepared with an adhesion-promoting agent for better retention and fluoride release as reported by manufacturer²³.
4. CURODONT Repair™ (Credentis AG, Windisch, Switzerland) which incorporates the self-assembling peptide (P11-4) based Curolox™ technology. The peptide P11-4 was supplied as Curodont powder (Curodont Repair, Credentis AG, Windisch, Switzerland) in glass vials.

Ethical regulation

The present study was carried out after exemption from Research Ethics Committee of Faculty of Dentistry, Ain Shams university since it was conducted on unidentified extracted lower first premolars. The exemption number is FDASU-RecEM012154.

Sample selection and preparation

Thirty-five freshly extracted lower first premolars were collected from Oral and Maxillofacial Surgery Department, faculty of dentistry, Badr university in Cairo. Teeth extracted for orthodontic, prosthodontic, and periodontal reasons were collected. Teeth were examined under stereomicroscope (40x) to avoid teeth with morphological abnormalities, developmental abnormalities, caries, enamel defects and cracks. Teeth were preserved in distilled water until beginning of investigation²⁴. Roots were removed 2 mm below cemento - enamel junction. Teeth were cleaned using scalers and rubber cup/pumice prophylaxis²⁵. Buccal surfaces were polished using disks in gradually finer grits²⁶. Buccal surfaces were coated nail varnish leaving a window of exposed enamel 2 × 2 mms in middle third of the buccal surface²⁷.

Teeth grouping

Specimens were randomly divided into five groups (n=7) according to treatment employed.

Group I: (control group): Intact enamel, without any treatment applied.

The remaining samples were exposed to demineralization to initiate caries like lesion. Each specimen was submerged in a daily renewed demineralizing solution for 4 days (96 hours)^{27,28}. After initiation of caries like lesions, samples were subdivided into four equal groups.

Group II: (demineralized group): No treatment was applied after creation of the lesion and teeth were examined immediately and used as a base line of the carious lesion.

Group III: (artificial saliva group): No treatment was applied after lesions formation and teeth were stored in daily renewed artificial saliva for one month till the time of investigation²⁷.

Group IV: (fluoride group): Teeth were lightly dried and painted with the varnish in a single stroke painting motion for one time²³. Teeth were stored in daily renewed artificial saliva for one month till the time of investigation²⁷.

Group V: (Self assembling peptide group, Curodont Repair): The Curodont Repair solution was prepared according to the manufacturer's instructions by reconstitution of each vial with 50 µl of distilled water that equals 0.05 milliliter. Then surfaces were dried and one drop was immediately applied onto enamel surfaces. Then, samples were allowed to stand for 5 min (till disappearance) to allow for diffusion and self-assembly²⁷. Curodont was used only once in the study²⁹. Teeth were stored in daily renewed artificial saliva for one month till investigation²⁷.

SEM examination, Energy Dispersive X ray Analysis (EDXA) and Statistical analysis:

The middle thirds of buccal surfaces of enamel were examined x1000 and x 4000. Scanning electron microscope was used to examine and compare the variations between the different groups. EDXA was performed to measure calcium (Ca) and phosphorus (P) weight % and Ca/P ratio was calculated. EDXA values were calculated analyzed using ANOVA and post-hoc test.

Polarized light microscope

Representative samples from each of the five groups were taken examining microstructural changes. The lesion depth were measured quantitatively using Image J Software. Three measuring sites per lesion were selected and average lesion depth was obtained.

RESULTS

Scanning Electron Microscopic results

The middle third of buccal surface enamel of control group showed typical morphology of the enamel surface layer. Areas of rodless enamel were seen on the ridges, microcracks were also detected. Interprismatic regions and the enamel surface showed the typical morphology of enamel rods (prism pattern) with fish scale pattern (**Fig1A**). With higher magnification enamel rod ends (EREs) showed fish scale pattern while other areas showed keyhole pattern. The prism pattern is well defined with regular margins (**Fig1B**).

Group II showed irregular enamel surface with scattered porosity, and microcracks (**Fig2A**). Enamel rods integrity was greatly affected. Demineralization (defects) of the Rods core were observed (**Fig2B**). Enamel rod boundaries were irregular and ill defined (**Fig2C**).

Group III showed less pronounced porosity and irregularity of enamel surface in some areas of enamel. Demineralization of rod core was still detected in some areas of enamel surface (**Fig3A**). Some areas still show irregular ill-defined boundaries of the enamel rods (**Fig 3B**).

Fluoride group showed aggregation of scattered mineralization deposits (globular masses) of variable sizes over the enamel surface (**Fig 4A**). Some of these masses were encountered along the periphery or in the concavities of the EREs, which lead to the partial obliteration of some of the EREs, some areas still show irregular ill-defined borders of enamel rods (**Fig 4B**).

Curodont group showed restoration of normal enamel architecture with the normal appearance of the fish scale of prism pattern in some areas (**Fig5A**), with no destruction of the rod core, other areas still show irregular ill-defined borders of enamel rods (**Fig 5B**).

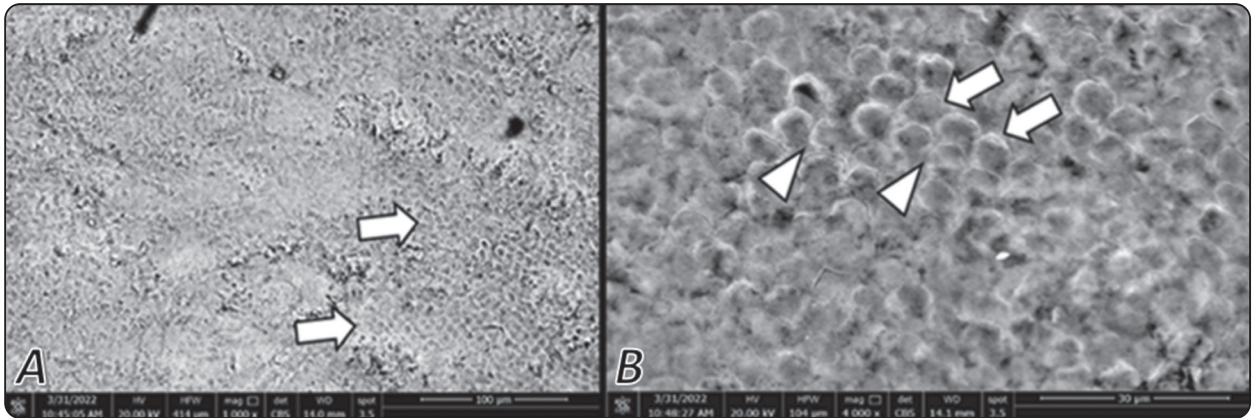


Fig. (1) Group I: (A): Scanning Electron micrograph showing smooth surface with EREs appearing in some areas with fish scale pattern (arrows). (B): Enamel rod ends appearing in some areas with fish scale pattern (arrows) and other areas as keyhole pattern (arrow heads). Prism pattern is well defined with regular margins. Magnification: A (x1000), B (x4000).

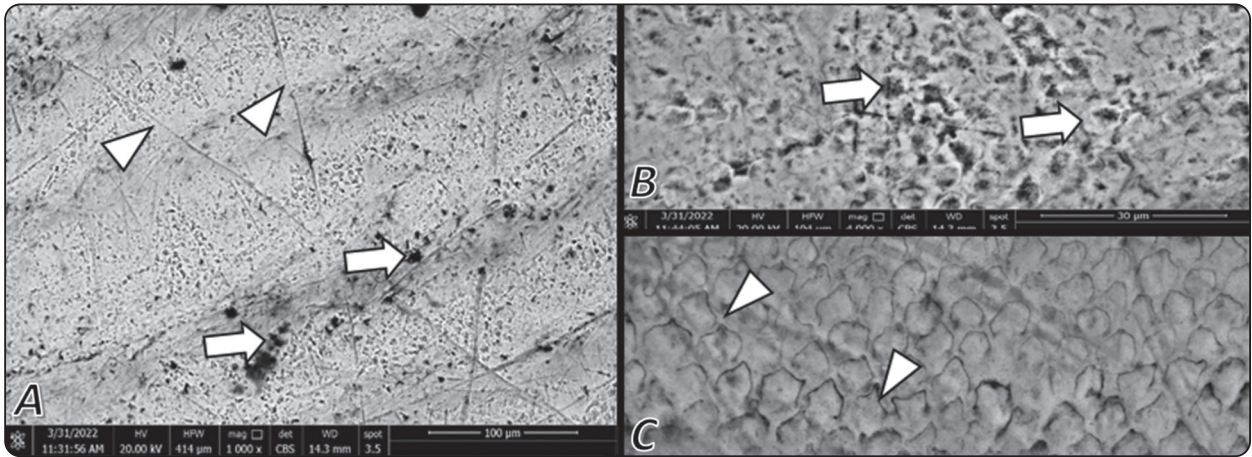


Fig. (2) Group II: (A): irregular enamel surface with scattered porosity (arrows) and microcracks (arrow heads). (B): The integrity of enamel rods is severely affected, demineralization of rods core are observed (arrows). (C): Irregular ill-defined boundaries of enamel rods (arrow heads). Magnification: A (x1000), B (x4000), C (x4000).

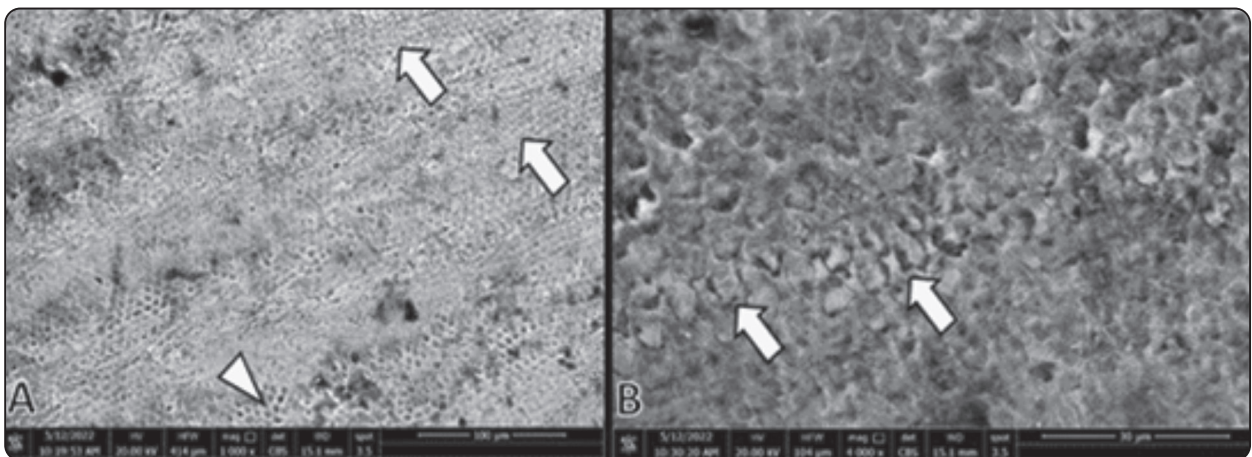


Fig. (3) Group III: (A): less pronounced porosity, destruction of prism pattern and irregularity of enamel surface (arrows). Demineralization of rod core is still detected in some areas of enamel surface (arrow head). (B): Some areas still show irregular ill-defined boundaries of enamel rods (arrows). Magnification: A (x1000), B (x4000).

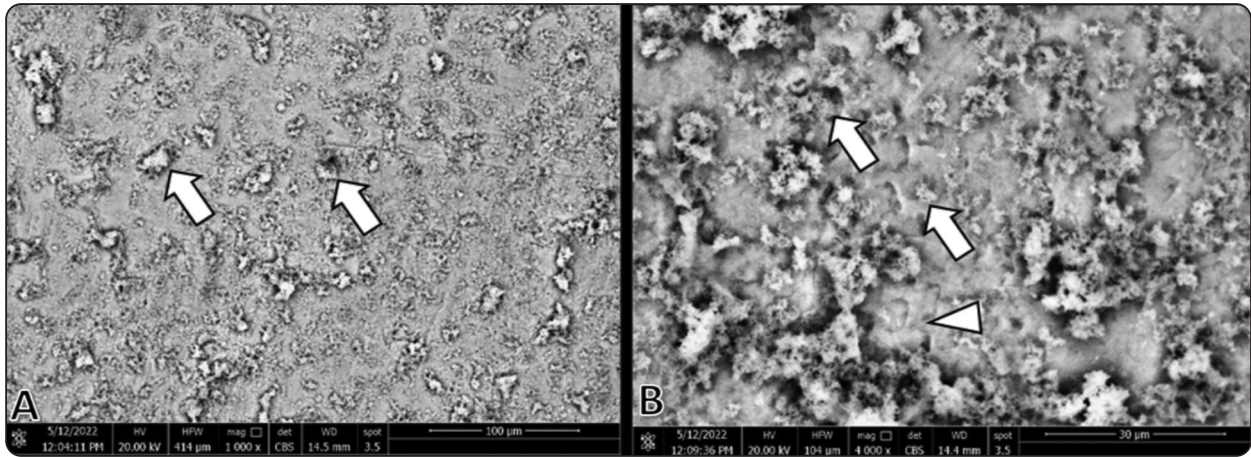


Fig. (4) Fluoride group: (A): scattered deposits of variable sizes over enamel surface (arrows). (B): Partially occluded EREs (arrow head), other areas still show irregular ill-defined borders of enamel rods (arrows). Magnification: A (x1000), B (x4000).

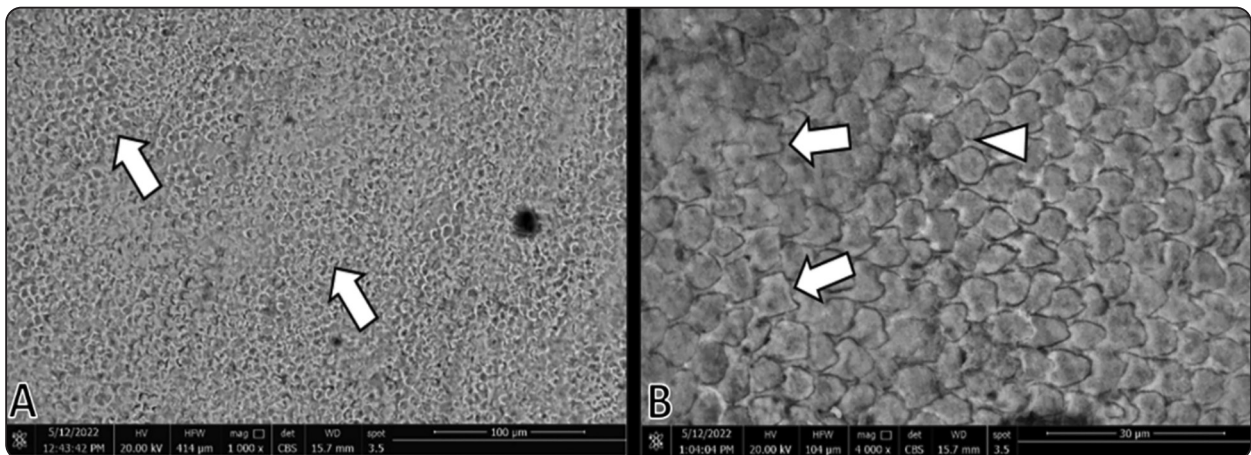


Fig. (5) Curodont group: (A): Re-establishment of normal enamel architecture with normal appearance of fish scale of prism pattern (arrows). (B): normal appearance of fish scale in some areas with no destruction of rod core (arrow head), other areas still show irregular ill-defined borders of enamel rods (arrows). Magnification: A (x1000), B (x4000).

Energy Dispersive X ray Analysis results:

A- Calcium: Using one-way ANOVA test there was a significant difference between different groups ($p=0.004$). Post hoc pairwise comparisons revealed control and Curodont groups have significantly higher values than artificial saliva and demineralized groups (**Table 1 and Fig 6A**).

B- Phosphorus: Using one-way ANOVA test there was a significant difference between different groups ($p=0.005$). Post hoc pairwise

comparisons showed control, artificial saliva, fluoride and Curodont groups have significantly higher values when compared to the demineralized group (**Table 2 and Fig 6B**).

C- Ca/P: The one-way ANOVA test showed there was a significant difference between different groups ($p=0.006$). Post hoc pairwise comparisons showed Curodont to have significantly higher value than demineralized and artificial saliva groups (**Table 3 and Fig 6C**).

TABLE (1) Mean and standard deviation (SD) values of calcium weight percentage (%) for different groups.

Calcium weight percentage (%) (mean±SD)					p-value
Control group	Demineralized group	Artificial saliva	Fluoride	Curodont	
41.10±1.48 ^{AB}	37.36±3.25 ^B	37.82±1.22 ^B	39.68±2.92 ^{AB}	41.98±2.55 ^A	0.004*

*Different superscript letters indicate a statistically significant difference. *: significant ($p \leq 0.05$), ns: non-significant ($p > 0.05$).*

TABLE (2) Mean and standard deviation (SD) values of phosphorus weight percentage (%) for different groups.

Phosphorus weight percentage (%) (mean±SD)					p-value
Control group	Demineralized group	Artificial saliva	Fluoride	Curodont	
18.57±0.51 ^A	17.05±1.27 ^B	17.38±0.48 ^{AB}	17.77±0.85 ^{AB}	18.34±0.54 ^A	0.005*

*Different superscript letters indicate a statistically significant difference. *: significant ($p \leq 0.05$), ns: non-significant ($p > 0.05$).*

TABLE (3) Mean and standard deviation (SD) values of Ca/P ratio for different groups.

Ca/P ratio (mean±SD)					p-value
Control group	Demineralized group	Artificial saliva	Fluoride	Curodont	
2.22±0.04 ^{AB}	2.18±0.03 ^B	2.19±0.04 ^B	2.23±0.07 ^{AB}	2.29±0.08 ^A	0.006*

*Different superscript letters indicate a statistically significant difference. *: significant ($p \leq 0.05$), ns: non-significant ($p > 0.05$).*

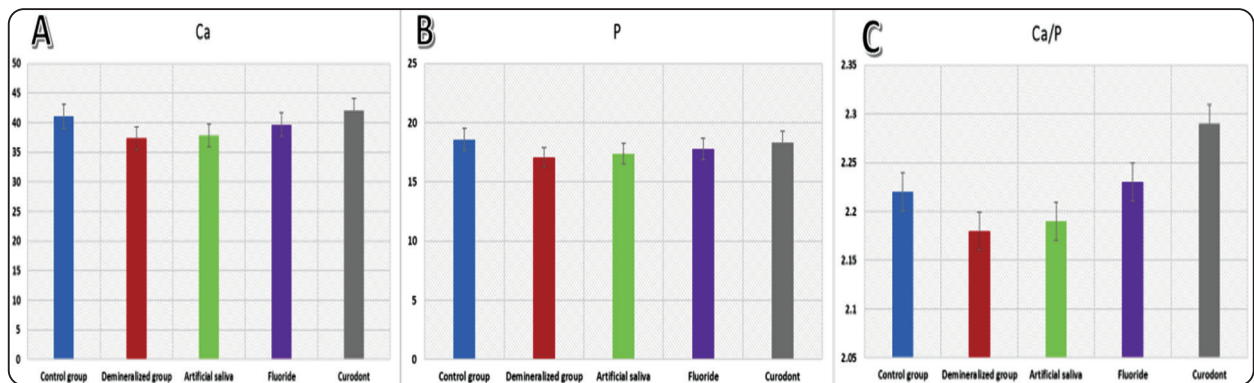


Fig. (6) Bar chart showing (A): average calcium weight percentage (%) for different groups, (B): average phosphorus weight percentage (%) for different groups, (C): average Ca/P ratio for different groups.

Polarized light microscope results:

Polarized light photomicrograph of longitudinal section of control group demonstrated normal course of enamel rods with Hunter-Schreger Bands (HSBs) denoting normal mineralization and birefringence (**Fig 7A**). Group II showed a quite high degree of positive birefringence with loss of typical enamel structure in lesion (**Fig 7B**). Using image J software lesion depth ranged from 60.23 μm to 64.77 μm with average lesion depth 62.88

μm . Group III revealed a slight decrease in body of the lesion extent (**Fig 7C**). The lesion depth ranged from 46.59 μm to 51.71 μm with average lesion depth 49.05 μm . Fluoride group showed noticeable decrease in extent of body of lesion and obvious negative birefringence compared with control (**Fig 7D**). Lesion depth ranged from 22.16 μm to 27.84 μm with average lesion depth 24.43 μm . Curodont group showed a disappearance of the lesion and re-establishment of typical enamel structure with noticeable negative birefringence (**Fig 7E**).

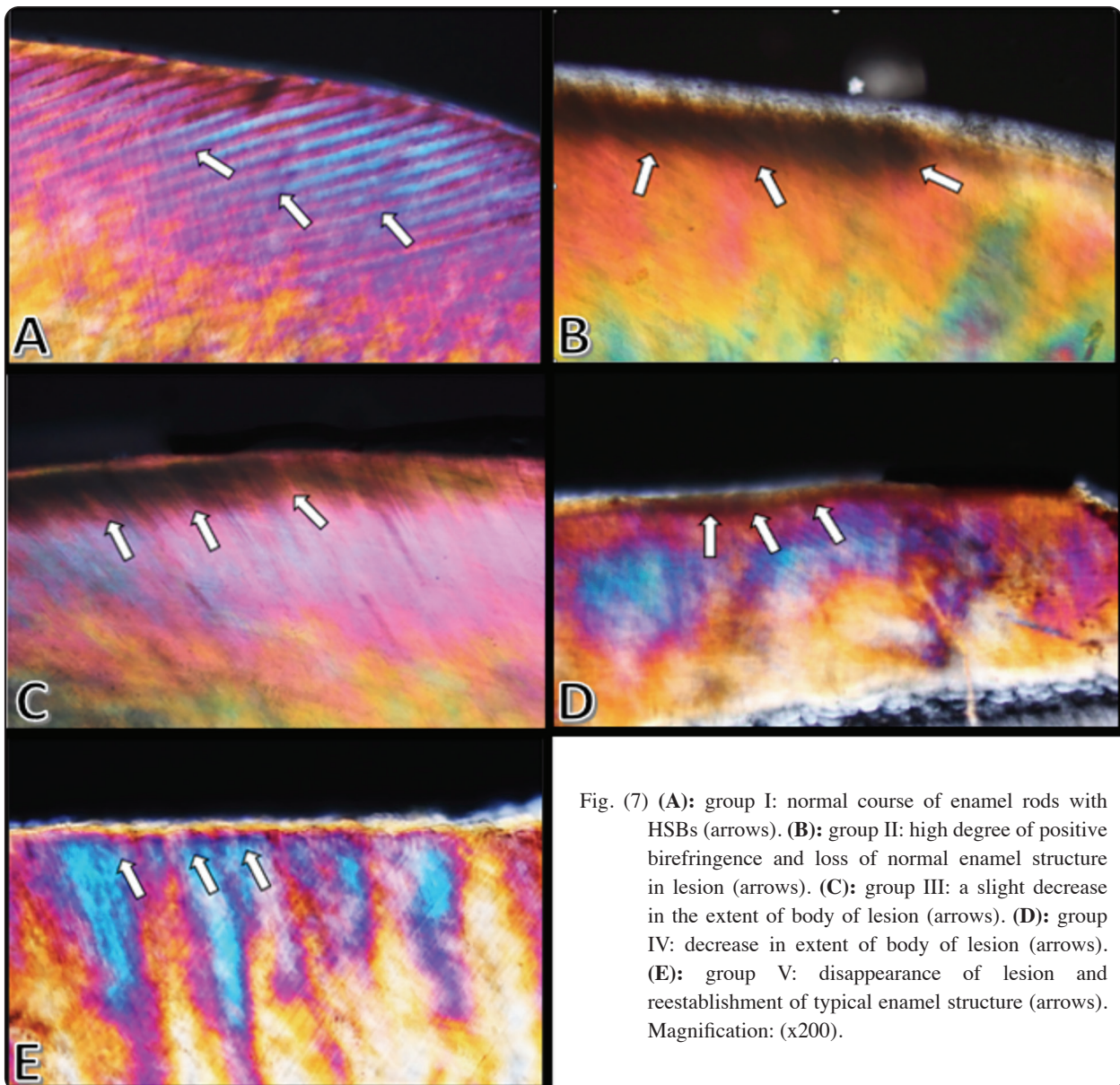


Fig. (7) (A): group I: normal course of enamel rods with HSBs (arrows). (B): group II: high degree of positive birefringence and loss of normal enamel structure in lesion (arrows). (C): group III: a slight decrease in the extent of body of lesion (arrows). (D): group IV: decrease in extent of body of lesion (arrows). (E): group V: disappearance of lesion and reestablishment of typical enamel structure (arrows). Magnification: (x200).

DISCUSSION

There have been significant efforts to minimize carious lesions advancement, while clinical studies are to be the gold standard; *in vitro* models have played a crucial role in cariology research by providing a controlled and reproducible environment to estimate the value of remineralizing agents in limiting carious lesions progression³⁰. In addition, early enamel lesions remineralization is important to protect other dental tissues³¹. The choice of enamel as the tooth structure in this study rather than dentin is justified by its significance as the primary barrier against caries progression as Enamel is the outermost layer of the tooth³².

In our study the teeth were preserved in distilled water at room temperature until beginning of investigation to prevent dehydration that could affect the measurements as well as for standardization^{29,33}. In the current study artificial caries formation was chosen as artificially created lesions that resemble caries can be compared to the first ultrastructural changes that can be seen during the caries process³⁴. It is also more uniform, controlled than natural dental caries and reproducible³⁵.

Samples in the current investigation after the suitable demineralization procedure were exposed to daily renewed artificial saliva at a pH of 7 for the duration of the month-long trial. This method was carried out by many studies^{17,36,37}. The freshly prepared artificial saliva was replaced daily in order to ensure ionic balance and maintenance of pH³⁸ and to prevent bacterial contamination¹⁷ and also to avoid structural changes during intervention phase²⁹. Evaluation of the specimen was done at four weeks as according to²⁷ assessment of remineralization pattern was done at one week and four weeks intervals and greater improvement of remineralization was found at four weeks suggesting that long time helped to gain more benefits from the remineralizing regimens used.

In the current study, SEM evaluation of the demineralized group showed destruction of the

prism pattern, irregular enamel surface with scattered porosity. Other acids as citric or lactic acids were previously to predispose enamel surface dissolution and erosion foci^{39,40}. This was previously explained by⁴⁰ who documented that acid application dissolves calcium and phosphate creating gaps which leads to the formation of enamel porosity.

Results obtained from EDXA in our study aligned with SEM findings, providing further support for the demineralization process observed. The demineralization group exhibited a decreased Ca/P ratio, indicating a chemical breakdown of enamel hydroxyapatite. The drop in pH below a certain threshold level can lead to an acidic environment that initiates the demineralization process. Acidic conditions can dissolve the hydroxyapatite mineral component of enamel, resulting in loss of calcium and phosphate ions. This chemical dissolution leads to enamel structure demineralization³⁵. SEM-EDXA results were also in agreement with⁴¹.

SEM results were also verified by the polarized light microscope results that revealed a relatively high degree of positive birefringence with loss of normal enamel structure in lesion which indicates significant changes in the optical properties and structural integrity of the enamel. This was in accordance with the study done by⁴² who used the same demineralization protocol however the authors did not measure the lesion depth.

The SEM evaluation of the artificial saliva group in our study showed relatively some favorable surface changes in comparison to the demineralization group. This indicates a remineralization pattern however to a much lesser amount than fluoride group and Curodont group. EDXA results supported the SEM result with minimal increase in Ca/P ratio in comparison to the demineralized group but this increase was statistically non-significant. This proposes that an amount of remineralization has occurred in each group; thus, the role of artificial saliva cannot be unnoticed. This can be explained that artificial saliva was not able to increase

calcium and phosphate delivery in comparison to the remineralizing regimens applied⁴³. This was in correlation with other studies^{44, 45, 46}. The polarized light microscope results verified the SEM-EDXA results with slight decrease in extent of the body of lesion verified by using image J Software where the average of the lesion depth was 49.05 μm .

The SEM of fluoride group showed distinct surface coating deposits in the form of globules with varying sizes. This was in accordance with the study done by⁴⁷ who reported that these globules are consisting of amorphous CaF_2 precipitates, which are fluoride reservoir and these globules coalesce and form a surface layer. In our study, fluoride showed an increase in mean Ca/P ratio compared to demineralized group and the artificial saliva group but this increase was not statistically significant. The increase in Ca/P ratio is due to fluoride's capacity to form fluorapatite crystals at which the fluoride attracts Ca and P ions. Nevertheless, fluoride remineralizes the surfaces resulting in lesion arrest without full depth remineralization⁴⁸.

The findings of polarized light microscopy in our study supported the limitations of fluoride in achieving full depth remineralization of carious lesions. We observed an obvious decrease in extent of lesion body and shift towards negative birefringence in the fluoride-treated group compared to the control group. Negative birefringence indicates a loss of normal enamel structure in lesion, suggesting that the lesion body was not fully remineralized. This supports the notion that fluoride's remineralization effect is primarily focused on the surface of the lesion rather than reaching deep into the lesion body. Using image J Software average lesion depth was 24.43 μm .

Curodont group revealed superior re-establishment of surface integrity. This result was in concomitant with⁴⁹ who concluded that SEM of samples of self-assembling peptide P11-4 showed wide regions of remineralized enamel in 93% of samples. EDXA

results revealed that the highest values of Ca and PO_4 weight percentage were recorded in Curodont group. Post hoc pairwise comparisons of Ca/P ratio showed Curodont to have significantly higher value than artificial saliva and demineralized groups. Our results were also verified by polarized light microscopy where there was complete disappearance of the lesion and reestablishment of typical enamel structure which indicates the diffusion of the material to the full depth of the lesion. The diffusion of the self-assembling peptide SAP P11-4 into demineralized enamel can be attributed to its monomeric form and low viscosity. These properties enable the peptide to penetrate the porous structure of demineralized enamel, allowing it to diffuse and distribute throughout the affected area to build a network leading to new hydroxyapatite nucleation sites^{50, 51, 52}.

In our study, the Curodont group showed superior results than fluoride group which is in agreement with⁵³ who demonstrated that Curodont exhibited high mechanical properties even at 125 μm depth. They emphasized that mechanical properties of Curodont were higher than those of the fluoride-containing material at 200 μm depth.

CONCLUSIONS

Both Sodium fluoride varnish and P11-4 were able to remineralize early enamel carious lesions. The treatment with P11-4 leads to remineralization of early enamel carious lesions more efficiently than sodium fluoride varnish. More in vitro and in vivo research are recommended in the future to explore P11-4's impact on enamel remineralization throughout a variety of time periods. As well as to simulate intraoral conditions, future research should use cariogenic microorganisms.

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