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Original Article

Infiltration level of CPP-ACFP (MI Varnish™), self-assembling peptide P_{11}-4 (Curodont™ Repair), and newly synthesized fluorinated self-assembling peptides in artificial enamel initial carious lesions on smooth surfaces assessed by laser confocal microscopy – an in vitro study

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Abstract

Aim: The aim of this study was to compare the lesion depth and level of penetration of CPP-ACFP, SAP P_{11}-4 and 2 newly synthesized self-assembling peptides by measuring the linear depth of fluorescence.

Materials and methods: A total of 30 enamel samples were prepared from orthodontically extracted permanent premolars. Three zones were provided: a sound enamel surface (SC), a demineralization zone (DC), and a remineralization zone (TA). The samples were randomly divided into five groups (n=6): group 1 – Gr V (MI Varnish™), group 2 – Gr CR (Curodont™ Repair), group 3 – IU1, group 4 – IU2, and group 5 – Gr NT (control group, no treatment). Lesion depth was assessed by measuring the linear penetration depth of the fluorescent dye by laser confocal microscopy.

Results: A statistically significant reduction in the depth of the lesion was found in the experimental groups compared to the control group, which was not treated ($p<0.001$). The depth of the lesion reveals the degree of mineralization and the cariostatic effect in the experimental groups. When measuring the confocal images, the results showed that there was a similar degree of remineralization for CPP-ACFP and for SAP P_{11}-4, IU1, and IU2 for the two time points DC and TA, but without statistical significance between them ($p=1.00$). The samples treated with IU2 showed the highest degree of remineralization, followed by CPP-ACFP, SAP P_{11}-4 and IU1.

Conclusion: The self-assembling peptides have a significant level of infiltration and potential for remineralization of artificial carious lesions in permanent teeth. The combination of SAP with fluoride has an additive effect.

Keywords

self-assembling peptide, Confocal laser scanning microscopy, incipient carious lesions

Introduction

Tooth decay is still the most common disease and is a leading public health problem. The goal of modern dentistry is non-invasive treatment of initial carious lesions through various strategies in an attempt to prevent disease progression and improve esthetics, strength, and function. Recent approaches focused on the application of remineralizing agents to incipient carious lesions aiming to control demineralization and promote remineralization. Remineralizing agents create a supersaturated environment around the lesion, thereby preventing mineral loss and directing calcium and phosphate ions to the loose zones. The development of modern remineralization agents has advanced significantly in the recent years, with many of them already in clinical use, while others at various stages of development. Calcium-phosphate remineralization systems have been proven to have beneficial effect, and the scientific evidence is abundant. They use a complex of casein phosphopeptide for stabilization and delivery of bioavailable calcium, phosphate, and fluoride ions. A product used in the clinical setting is MI Varnish™ – a fluoride varnish associated with casein phosphopeptide-amorphous calcium phosphate (CPP-ACFP). Recently, efforts have been directed towards the development of regenerative therapies as an alternative approach in the management of non-cavitated lesions, in which biomineralization of the affected dental tissues occurs. Self-assembling peptide (SAP P_{11}-4) is one alternative approach, recently introduced for the treatment of incipient carious lesions. It regenerates demineralized dental tissue through the new formation of hydroxyapatite, thus promoting the biomineralization of enamel lesions.[1]

Confocal laser scanning microscopy (CLSM) is an effective method for determining the carious lesion formation process and evaluating the changes generated by remineralizing agents in demineralized enamel.[2-4] CLSM measures various parameters of the lesions, such as their area, depth, and total fluorescence. Therefore, it provides information on important lesion characteristics (e.g. depth, mineral density and healing pattern).[5]

Aim

To determine lesion depth, extent, and percentage of penetration of CPP-ACFP, P_{11}-4 and the newly synthesized fluorinated self-assembling peptides (IU1 and IU2) by measuring the linear depth of fluorescence.

Materials and methods

To achieve the aim of this study, we collected 30 intact permanent premolars extracted for orthodontic indications from patients aged 14 to 20. A total of 30 enamel specimens were prepared from them with included vestibular surface. A schematic representation of the preparation technique of the specimens is presented in **Fig. 1**. A 5×5 mm window was provided for the experiment. The proximal 2 mm of the surface were covered with acid-resistant nail polish (Top coat, Victoria beauty) for insulation and control in the next stages of the trial. One third of the experimental window was covered with acid-resistant nail polish to provide a sound control (time point – SC). This stage was followed by laboratory formation of artificial carious lesions in the enamel with a demineralization solution with the composition: 1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4, 50 mM acetate buffer, pH 4.3 at 37°C for 5 days. The second third of the provided window after creating the artificial lesion was covered with acid-resistant nail polish to provide a demineralized control (time point – DC). The rest of the artificial lesion was used for treatment with the selected agents (time point – TA).

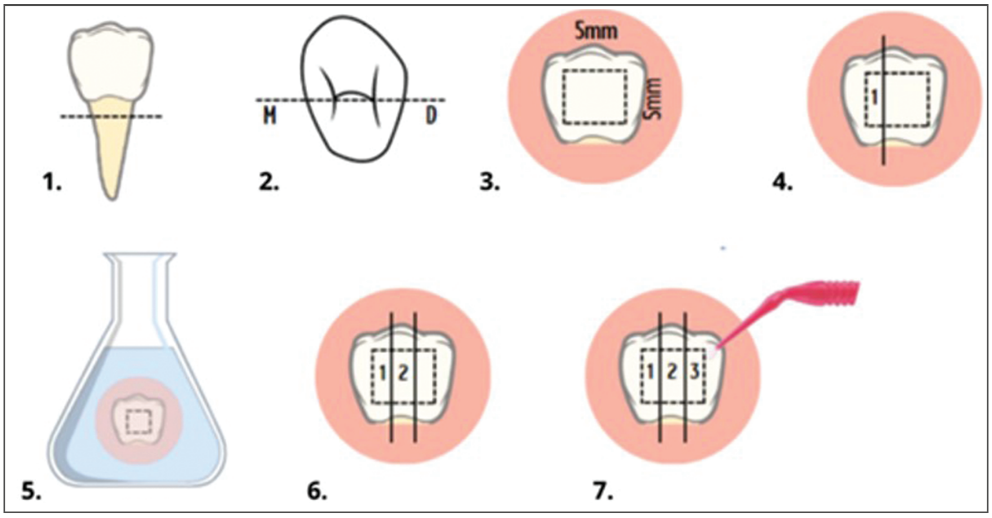

Figure 1. Schematic representation of the specimen preparation technique. **1**: Cutting 1 mm below the EDJ; **2**: MD cutting of the coronal part; **3**: Inclusion in a template made of fast-curing plastic; **4**: 1-sound control; **5**: Creating an artificial carious lesion; **6**: 2-demineralized control; **7**: 3-treatment area.

The enamel samples were randomly distributed in five groups (n=6). Randomization was achieved using a computer-generated permuted block sequence.

- Group 1. Gr V – Treatment with MI Varnish™ (GC Corporation, Tokyo, Japan) – samples were rinsed with distilled water, then air-dried for 20 seconds. A thin, even layer of varnish was applied once with an applicator. The varnish was allowed to interact with the enamel surface for 5 minutes.
- Group 2. Gr CR – Treatment with Curodont™ Repair (Credentis AG, Windisch, Switzerland). The clinical protocol indicated by the manufacturer was applied except for the etching procedure, which was modified according to the experimental study protocol described by Wierichs et al.[6] The enamel surface was treated with 2% NaClO for 20 seconds, followed by rinsing with water and drying. Etching gel 37% orthophosphoric acid was applied for 5 seconds, then rinsed with air water spray for 30 seconds and dried with air for 20 seconds. A single dose of Curodont™ Repair dissolved in 0.05 mL of distilled water was applied with a microapplicator and left to interact with the enamel surface for 5 minutes.
- Group 3. Gr IU 1 – Treatment with a modified analogue of P_{11}-4 containing one covalently bound F atom. The specified Curodont™ Repair application protocol was applied.
- Group 4. Gr IU 2 – Treatment with a modified analogue of P_{11}-4 containing 2 covalently bound F atoms. The specified Curodont™ Repair application protocol was applied.
- Group 5. Gr NT – Control group, no treatment.

The nail polish was not removed after the treatment. Each sample was placed individually in a sterile glass container in 30 ml of artificial saliva for a period of 30 days at room temperature. The artificial saliva was replaced daily. The composition of the artificial saliva was the same as described by Schmidlin et al.[7]: 2.4 g KCl, 1.7 g NaCl, 0.1 g $MgCl_2 \times 6H_2O$, 0.2 g $CaCl_2 \times 2H_2O$, 0.2 g KSCN, 0.7 g KH_2PO_4, and 0.1 g H_3BO_3 in 1000 mL distilled water, with the pH adjusted to pH 7.0 by adding 4NNaOH.

Following the 30-day remineralization period, the nail polish was carefully and completely removed with acetone from the samples in all groups. The samples were washed for 10 minutes in an ultrasonic bath with distilled water and immersed for one hour in a freshly prepared solution of 0.1 mM rhodamine B. All samples were washed with distilled water to remove excess rhodamine B solution.

The samples were observed using a Leica TCS SPE confocal laser scanning microscope (CLSM). A 532 nm helium-neon laser was used with a rhodamine filter to excite the dye rhodamine B. The captured images were analyzed using the built-in Image J software. Fifty 10 µm confocal sections with a depth of 490 µm (from 00 to 49) were taken for each of the studied areas in the z plane (z stack). For each sample, an image of the three areas was captured: sound control (SC), demineralized control (DC), and treatment area (TA). Lesion depth was assessed by measuring the linear depth of dye penetration. Lesion depth was defined as the distance from the lesion surface to the deepest point of fluorescence.

This study has been approved by the Committee for Scientific Research Ethics, Medical University of Plovdiv, Bulgaria (Reference N:P-1463, Protocol of approval N:3/20.05.2021).

Statistical analysis

Descriptive statistics were used to summarize the study variables. Continuous variables were reported as means ± standard deviations (SD). To assess the normality of the quantitative data, the Shapiro-Wilk test was performed. Data followed normal distribution and were analyzed using parametric tests – paired t-test to compare two group means where the samples were the same in each group and measured two times to see the changes to an intervention; one-way ANOVA for significant differences between the means of two or more independent groups. A level of $p<0.05$ was considered statistically significant for all analyses. Data were analyzed using the Statistical Package for the Social Sciences (SPSS, v. 26).

Results

The lesion depth, degree, and percentage of penetration of CPP-ACFP, P_{11}-4, IU1, IU2 were determined by measuring the linear depth of fluorescence in micrometers (µm) using the built-in Image J software.

When comparing the average values, the highest values were found after the creation of an artificial carious lesion for all five groups from 285±46 µm to 262±58 µm. In the first zone of the experimental window, sound control (SC), no penetration of fluorescent substance was observed. After treatment and soak in artificial saliva, reduced fluorescence was observed in the experimental groups treated with the respective medication. For the control group, such a change was absent, due to the penetration of fluorescent substance at the same depth, analogous to a demineralized surface **(Table 1)**.

We observed statistically significantly lower levels of TA (93.33±26 µm) compared to DC (283±49 µm) in group V (paired t-test=6.80, p=0.001). A statistically significant difference was also registered in group CR of DC and TA area (n=6), respectively (paired t-test=8.60, p<0.001). And for the CR group, paired t-test reported statistically significantly lower levels of TA (78±18 µm) compared to DC (285±46 µm) (paired t-test p<0.001). This was also found in the newly synthesized peptides for Gr IU1 TA (87±16 µm) compared to DC (277±44 µm) (paired t-test=12.44, p<0.001) and for Gr IU2 TA (102±29 µm) compared to DC (263±27 µm) (paired t-test=15.95, p<0.001). In the control group, no differences were found in the two study areas. The initial values of the demineralized surface coincided with the area designated for treatment, since in the control group the untreated surface remained and, accordingly, the artificial carious lesion remained at the same depth **(Fig. 2) (Table 1)**.

The within-group analysis **(Table 1)** demonstrated that there was no statistically significant difference for time point DC (demineralized area, depth of the created artificial carious lesion) and for the five groups (p=0.848). After treatment and soak in artificial saliva, the results obtained showed statistically significant differences for the four experimental groups treated with CPP-ACFP, SAP P_{11}-4, IU1 and IU2 for both time points DC and TA, but without statistical significance between them (p=1.00). Although no statistical significance was found in the experimental groups, the best result was shown by the samples treated with IU2, followed by CPP-ACFP, SAP P_{11}-4, and IU1. For the control group, the carious lesion remained at the same depth as the baseline value during demineralization. A statistically significant difference was found between the control and all experimental groups (p<0.001).

Discussion

Confocal laser scanning microscopy can be an effective method for determining the process of carious lesion formation, including demineralization and remineralization.[3] The present research used a confocal laser scanning microscope (CLSM) – Leica TCS SPE to evaluate the changes generated by remineralizing agents in demineralized enamel. Its advantages include easy and rapid preparation of enamel sections and the ability to examine subsurface changes in dental specimens.[4] CLSM measures various parameters of lesions, such as their area, depth, and total fluorescence. Therefore, it can provide information on important characteristics (e.g. depth, mineral density, and healing pattern) of the lesions. The obtained surface images of the studied surfaces are similar in nature to those of SEM, but with improved resolution.[2] The removal of out-of-focus blurring allows much more information to be obtained than fluorescence microscopy techniques, with images capable of 3-D reconstruction of the specimen.

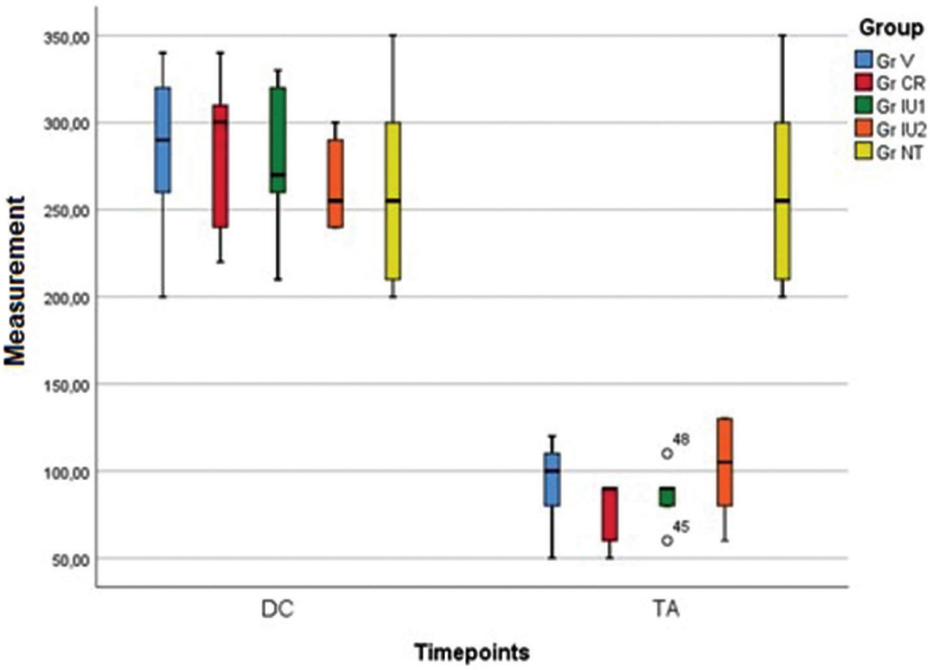

Figure 2. Box-plot diagram of DC and TA measurements by groups.

Table 1. Inferential statistics of the variables DC and TA within and between groups

	Gr V (CPP–ACFP)	Gr CR (P_{11}-4)	Gr IU1	Gr IU2	Gr NT (control)	p^1- value
DC, mean±SD	283±49	285±46	277±44	263±27	262±58	0.848
TA, mean±SD	93±26	78±18	87±16	102±29	262±58	<0.001
p^2- value	0.001	<0.001	<0.001	<0.001	1.000	

p^1: significance of the difference between groups (one-way ANOVA test); p^2: significance of the difference within groups (paired t-test); significance level p<0.05

In this study, the depth of the lesion prior to and following treatment was assessed within a specific experimental window for each specimen to detect progression or regression of the lesion. This avoided structural differences between teeth. The depth of the lesion corresponded to the depth of demineralization. The created artificial carious lesions were of the same depth prior to randomization and allocation to groups.

After measuring the confocal images, it was proven that there is a reduction in the depth of the lesion following the application of the four agents. The depth of the lesion revealed the degree of mineralization and the cariostatic effect in the experimental groups. The result showed that there is a similar degree of remineralization for CPP-ACFP, SAP P_{11}-4, IU1, and IU2 without a significant difference (p>0.005). The results obtained are similar to other studies.[3,8-10]

The CLSM images were evaluated separately by another operator who was unaware of the randomization of the groups. The healthy area of the experimental window (intact enamel surface) appears pitch black, i.e. there is no fluorescence **(Fig. 3)**. This proves that rhodamine B does

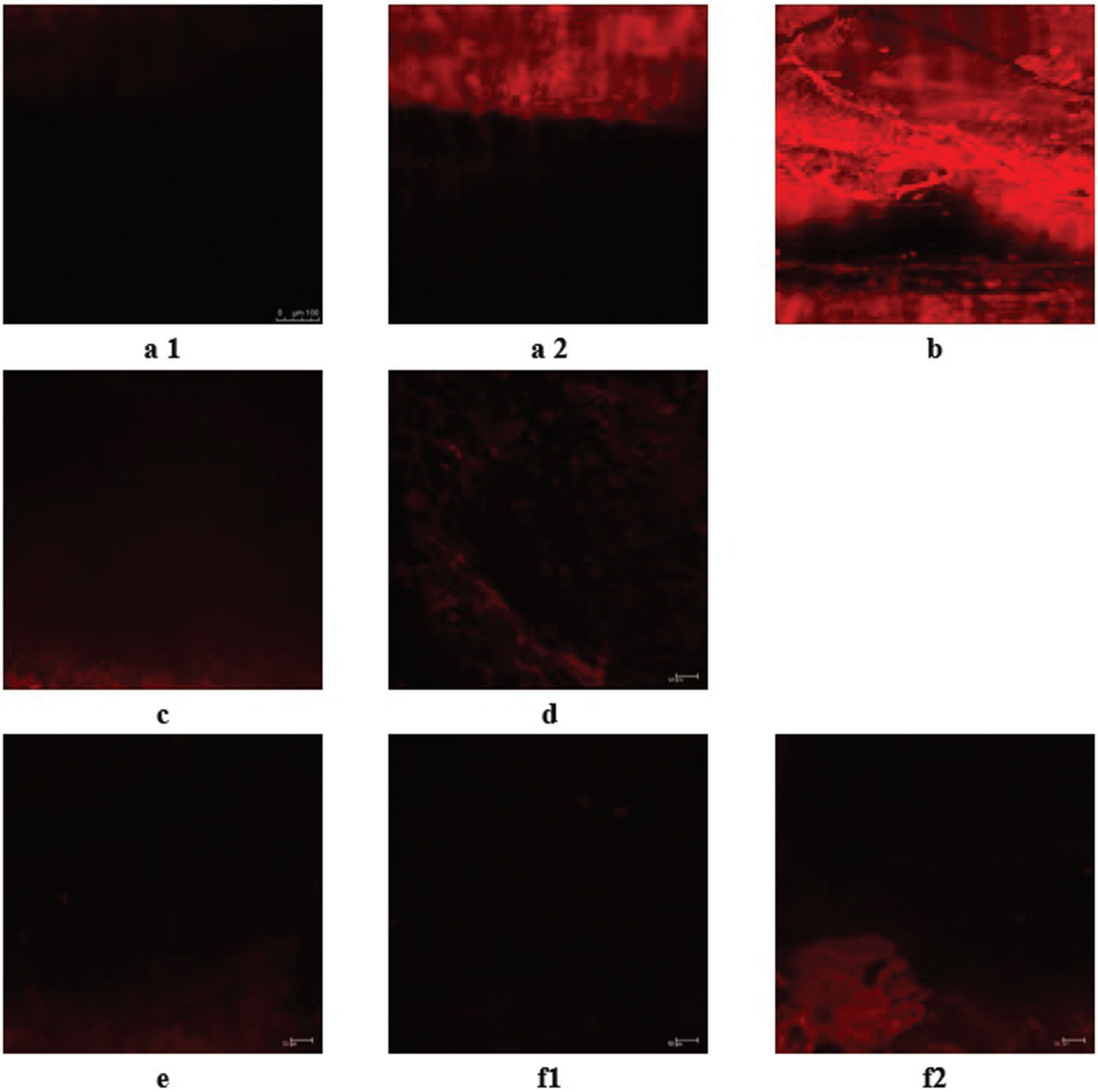

Figure 3. Confocal images of samples for each group. (**a1**) sound area (intact surface); (**a2**) depicts the boundary between untreated, healthy surface and demineralized surface; (**b**) demineralized surface (control group); (**c**) treated area for group CPP-ACFP; (**d**) treated area for group P_{11}-4; (**e**) treated area for group IU 1; (**f1**) - treated area for group IU 2; (**f2**) depicts the boundary between demineralized surface and treated area for group IU 2.

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not penetrate or poorly penetrates healthy tooth structure. According to literature data rhodamine B does not penetrate sound tooth structure or epoxy resin but is incorporated into demineralized tooth structure. The highest penetration of rhodamine B was observed after creating an artificial carious lesion and in the control (untreated) group, which means that rhodamine B penetrated and was retained throughout the depth of the lesion. Remineralized areas showed reduced fluorescence. Penetration of the dye decreased significantly as a result of an increase in the amount of minerals and a decrease in the degree of porosity in the demineralized surface. The results obtained are consistent with other studies.[10-12]

In a study by Goel et al.[13], a reduction of the lesion depth was observed after application of four remineralizing agents (fluoride varnish, CPP-ACFP, bioactive glass, and nanohydroxyapatite paste) to primary and permanent teeth. When comparing the amount of remineralization that occurred in each group between permanent and primary teeth, it was found that there was significantly better remineralization in primary teeth.

Savas et al.[3] investigated the effects of acidified phosphate fluoride (APF), self-assembling peptides, and silver diamine fluoride (SDF) on the regeneration of demineralized dental tissue on smooth surfaces by surface microhardness analysis, energy-dispersive X-ray spectroscopy, and confocal laser scanning microscopy. In all treatment groups, lesion depth and total fluorescence were significantly reduced after both seven and 30 days.

Kind et al.[14] using various analytical methods demonstrated that the self-assembling peptide P_{11}-4 diffuses into the subsurface lesion, assembles into higher-forming aggregates throughout the lesion volume, and supports the nucleation of newly formed hydroxyapatite nanocrystals and therefore leads to increased mineral density in the subsurface carious lesion. The penetration depth of P_{11}-4 into artificial lesions was visualized by confocal microscopy by comparing fluorescently labelled ATTO647-P_{11}-4 with placebo and unlabeled P_{11}-4. Artificial lesions treated with fluorescently labeled P_{11}-4 showed significant fluorescence covering the enamel sample depth from 103 to 154 µm. The placebo group and unlabeled P_{11}-4 showed no fluorescent signal.

Conclusion

The self-assembling peptides have a significant level of infiltration and potential for remineralization of artificial carious lesions in permanent teeth. The combination of SAP with fluoride has an additive effect. A statistically significant reduction in lesion depth was found in the experimental groups (Gr V, Gr CR, Gr IU1, Gr IU2), compared to the control one (p<0.001).

The confocal laser scanning microscope can be successfully used as a method for assessing and quantifying the processes of demineralization and remineralization.

Acknowledgments

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