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Effects of different topical agents on enamel demineralization around orthodontic brackets: an *in vivo* and *in vitro* study

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ABSTRACT

Background: The aim of this study was to evaluate the *in vivo* and *in vitro* effects of a casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) and fluoride containing topical agents in reducing enamel demineralization around orthodontic brackets, and to compare this with a control group.
Methods: Twenty-one patients and 60 extracted premolars were divided into three groups: two experimental and one control. Tooth Mousse® (CPP-ACP gel; GC-Corp, Tokyo, Japan) and Fluoridin N5® (Fluoride gel; Voco-GmbH, Cuxhaven, Germany) were applied to tooth surfaces around orthodontic brackets in the experimental groups. Teeth were extracted after 60 days to evaluate the *in vivo* effects of the testing materials. For the *in vitro* experiment, samples were cycled for 14 days through a daily procedure of demineralization. All teeth were sectioned and evaluated by superficial microhardness analysis. An indentation was made from two positions (occlusal-cervical) and one depth (10 μm).
Results: Comparisons of occlusal and cervical microhardness scores for all specimens showed no statistically significant side differences. A multiple comparison test showed that the use of CPP-ACP and fluoride containing topical gels were more significantly efficient than the control group ($p < 0.001$). No significant differences were detected between CPP-ACP and the fluoride groups against demineralization.
Conclusions: *In vivo* and *in vitro* evaluations indicated that CPP-ACP and fluoride containing agents successfully inhibited caries around orthodontic brackets.

Keywords: Demineralization, fluoride, casein phosphopeptide-amorphous calcium phosphate.

Abbreviations and acronyms: ANOVA = analysis of variance; CPP-ACP = casein phosphopeptide-amorphous calcium phosphate.

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INTRODUCTION

Despite advances in orthodontic materials and treatment mechanics, the placement of fixed appliances is still linked with a high risk of developing white spot lesions.[1,2] During orthodontic treatment, plaque accumulates around the brackets because of inadequate oral hygiene, which is common in pubertal people.[2] Patients with fixed orthodontic appliances have an elevated risk of tooth caries, and enamel lesions can occur within a month, irrespective of mechanical plaque control and whether or not fluoridated dentifrice is used.[3,4] Caries lesions around orthodontic brackets could be reduced,[3,5] or even completely inhibited, when a fluoride dentifrice is used with a mouthwash.[4]

Fluoride ions can be incorporated into the hydroxylapatite structure of tooth enamel by the replacement of hydroxyl groups or by redeposition of dissolved hydroxyl-apatite as less soluble fluoridated forms, such as fluorapatite or fluorhydroxyl-apatite.[6] During orthodontic treatment, fluoride could be administered to the teeth in various ways, including topical (fluoridated toothpaste, mouthrinse, gel and varnish) and adhesive (fluoride releasing cements and elastomeric modules and chains) methods.[7] Topical fluoride agents have been shown to decrease enamel demineralization *in vitro* and in clinical studies.[8,9] The efficiency of regular application of fluoride varnish appears to reduce lesion formation on bracketed maxillary incisor teeth.[10] Fluoride gels also provide additional preventive benefits when brackets have been bonded with composite resin cement.[11]

Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) is reported to have topical anticariogenic effects because of its ability to stabilize calcium and

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phosphate in an amorphous state, preventing the growth of calcium phosphate to the critical size required for precipitation.[12–14] In recent years, CPP-ACP nano-complexes have also demonstrated anticariogenic properties in both animal laboratory and human *in situ* experiments.[15,16] Calcium and phosphate ions are released from ACP materials, especially in response to changes in the oral environment caused by bacterial plaque or acidic foods, which can be deposited into the tooth structures as an apatite mineral and is similar to the hydroxyl-apatite found naturally in teeth.[17]

Sudjalim *et al.*[18] carried out a laboratory study to investigate the effect of sodium fluoride and 10% CPP-ACP on enamel demineralization adjacent to orthodontic brackets, and found that the use of both agents should be recommended for all orthodontic patients to provide preventive actions and potentially remineralize early enamel demineralization.

In vitro studies have demonstrated the remineralization potential,[17,19] shear bond strength of brackets[20,21] or lingual retainer composites[22] of ACP-containing materials. However, no *in vivo* comparative studies have investigated the efficiency of fluoridated or CPP-ACP containing topical gels on enamel demineralization around orthodontic brackets.

The aim of this study was to evaluate the *in vivo* and *in vitro* effects of CPP-ACP and fluoride containing topical gels in reducing enamel demineralization around orthodontic brackets and compare this with a control group. The research hypothesis of this study states that CPP-ACP and fluoride containing topical gels can significantly reduce the overall amount of demineralization around orthodontic brackets under laboratory conditions and in the mouth. This study was approved by the Research Ethical Committee at the Erciyes University Faculty of Dentistry, Kayseri, Turkey.

MATERIALS AND METHODS

In vivo experiment

Twenty-one orthodontic patients, aged 13–17 years (mean: 15.05 ± 1.70 years), scheduled to have four first premolar teeth extracted for orthodontic reasons were invited to participate in the study and consent forms were signed. This study was organized as a parallel group design with two groups receiving the experimental protocol and the other group acting as the control. Power analysis was established by G*Power Version 3.0.10 software (Franz Faul, Universität Kiel, Germany). Based on a 1:1 ratio among groups, the total sample size of 21 patients would give more than 80% power to detect significant differences with 0.40 effect size and at $\alpha = 0.05$ significance level. Patients were divided into three groups, each group consisting of seven patients. Block randomization was used in each group. For group standardization, before starting the procedure, all the patients' teeth were evaluated clinically and radiographically to determine the baseline caries risk. Nine participants (42.85%) were male and 12 (57.15%) were female.

The salivary flow rate and buffer capacity were recorded. Criteria for inclusion in the study were no active caries lesions, normal salivary flow rate (>1.0 mL/min) and buffer capacity (final pH: 6.8–7.5). All patients received full mouth cleaning to remove plaque in preparation for bonding. There were no visible signs of caries, fluorosis, or developmental defects. To evaluate the baseline demineralization values of all selected teeth, a portable battery powered laser fluorescence device (DIAGNOdent Pen®; KaVo, Germany) was used. The two groups' scores were less than 13. This indicates that there was no demineralization; both were equivalent for caries risk.

Twenty-eight brackets (Dyna-Lok series, 100-gauge mesh, 3M Unitek) were bonded for each group (14 maxillary and 14 mandibular first premolars). Brackets were applied with one of the following methods, both *in vivo* and *in vitro* experiments: after surface preparation with a 37% phosphoric acid gel (3M Dental Products; St Paul, Minnesota, USA), liquid primer of the Transbond XT (3M Unitek; Monrovia, California, USA) was applied to the etched surface and the stainless-steel orthodontic premolar brackets were bonded to teeth with Transbond XT (3M Unitek). Any excess resin was removed with an explorer before the resin was polymerized. A light emitting diode (Elipar Freelight-2, 3M ESPE; Seefeld, Germany) was then used to cure the specimens for 20 seconds.

Patients were divided into three groups: two experimental and one control. Tooth Mousse® (CPP-ACP containing topical gel; GC Corp, Tokyo, Japan) and Fluoridin N5® (sodium fluoride containing topical gel; Voco GmbH, Cuxhaven, Germany) were applied to the tooth surfaces of patients in the experimental groups. Agents formed were left undisturbed for five minutes on the enamel surfaces. No agent was applied to the tooth surfaces of the control group.

No adverse events or side effects in each intervention group were determined. After 60 days, the brackets were removed. The teeth were extracted and stored in a refrigerator in flasks containing gauze dampened with 2% formaldehyde (pH 7.0). Demineralization in enamel around the brackets was evaluated by a cross-sectional microhardness method as previously reported.[1,23–25] During the experimental period and three weeks prior, all subjects brushed their teeth with a non-fluoridated dentifrice. They received no instructions regarding oral hygiene, kept their usual habits, and were instructed not to use any antibacterial substance.

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In vitro experiment

Sixty caries-free human maxillary premolars extracted for orthodontic reasons were stored in 0.1% thymol and stored in a refrigerator (maximum one month). Teeth with hypoplastic areas, cracks, or gross irregularities of enamel structure were excluded from the study. The criteria for tooth selection dictated no pretreatment with a chemical agent such as alcohol, formalin, hydrogen peroxide, and so forth. Soft tissue remnants and calculus were removed from the teeth after cleaning with a fluoride-free pumice and rubber cup. The teeth were then disinfected in Streck Tissue Fixative (Streck Laboratories, Inc., Omaha, Nebraska, USA) for two weeks. This solution has been shown to have antimicrobial effects while not exhibiting deleterious effects that could compromise the histological features of artificial demineralization.[26]

All brackets were bonded to the teeth as explained above. Teeth were painted with an acid resistant varnish to a distance of 2 mm from the bracket margin as measured by a ruler, so that most of the crown was covered by acid resistant varnish and only the exposed enamel could be attacked by acid.

Sixty teeth were distributed into three groups: two experimental and one control. Tooth Mousse® and Fluoridin N5® were applied to the tooth surfaces of the experimental groups, while no agent was applied to the specimens in the control group. Agents were left undisturbed for five minutes on the tooth surfaces.

According to Sudjalim *et al.*,[18] four-hourly application of topical medicaments corresponds to approximately twice-weekly applications. Teeth from the control group were immersed in demineralization/remineralization solution without exposure to any topical solution. Teeth from the CPP-ACP and sodium fluoride topical agent groups were treated with a 0.5 mL smear of predetermined topical solution before immersion in cycling solutions to simulate the application of these topical solutions in a clinical setting. At four-hourly intervals, all enamel specimens were rinsed with deionized water and topical solutions reapplied to the specimens in the experimental groups and immersed into the solution.

Demineralization procedure

The demineralization procedure was adapted from the methods used by Hu and Featherstone.[7] The daily procedure of pH cycling included a demineralization period of six hours (9 am to 3 pm) and a remineralization period of 17 hours (4 pm to 9 am). Each crown was immersed individually in 60 mL of demineralization solution containing 2.0 mmol/L calcium, 2.0 mmol/L phosphates, and 75 mmol/L acetate at pH 4.3 for six hours at 37 °C. Specimens were then removed from the demineralization solution, rinsed with deionized water, and immersed individually in 40 mL of remineralization solution at 37 °C overnight (17 hours) to simulate the remineralizing stage of the caries process. The remineralization solution consisted of 1.5 mmol/L calcium, 0.9 mmol/L phosphates, 150 mmol/L potassium chloride, and 20 mmol/L cacodylate buffers at pH 7.0. This cycling procedure was repeated daily for 14 days.

The presence of demineralization in the control group was confirmed by laser fluorescent device (DIAGNOdent pen; KaVo, Biberach, Germany). When dried at day 14, demineralization was confirmed by the appearance of frosty white enamel. All teeth were removed from the solutions for bracket removal and sectioning to compare the microhardness values.

Cross-sectional microhardness analysis

One operator, who was blind from the group allocations, carried out the microhardness analysis (SO). The roots were removed 2 mm apical to the cemento-enamel junction, and the crowns were hemi-sectioned vertically into mesial and distal halves with a 15 HC (large) wafering blade on an Isomet low-speed saw (Buehler, Lake Bluff, Illinois, USA) directly through the slot of the bracket, leaving a gingival and an incisal portion. The teeth were embedded in self-curing EpoKwick epoxy-resin (Buehler), leaving the cut face exposed. The half crown sections were polished with three grades of abrasive paper discs (320, 600, and 1200 grit). Final polishing was undertaken with a 1 μm diamond-spray and a polishing-cloth disc (Buehler). A Shimadzu (Kyoto, Japan) HMV-700 microhardness tester under a 2 N load was used for the microhardness analysis.

An indentation was made in each half crown, from two positions and one depth. On the buccal surface, from the occlusal and cervical region, indentation was made at the edges (0 μm) of bracket. At these positions, indentations were made at a depth of 10 μm from the external surface of the enamel. The values of microhardness numbers found in the two half crowns were averaged.

Statistical analysis

Data analyses were performed using the Statistical Package for Social Sciences (SPSS, Version 13.0, SPSS Inc; Chicago, Illinois, USA) and Excel (Office 2007) (Microsoft Corporation; Redmond, WA, USA). The Shapiro–Wilk normality test and the Levene's variance homogeneity test were applied to the microhardness data. The data showed normal distribution, and there was homogeneity of variances between the groups.

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For *in vivo* evaluations, the teeth within individual patients were clustered, in analysis. The tooth surface is not an independent unit for statistical purposes because it is subject to similar conditions as the surrounding teeth. However, a multilevel design statistical test[27] showed the same results with a non-clustering comparison. Therefore, analysis of variance (ANOVA) was used to evaluate the effects of both topical agents (Tooth Mousse® and Fluoridine N5®) for *in vivo* and *in vitro* evaluations. For multiple comparisons, the Bonferroni *post hoc* test was used. The statistical significance level was set at $p < 0.05$.

To evaluate the intra- and inter-observer agreement, microhardness measurements were undertaken by two investigators using the same instrument at two separate times and Cohen's Kappa scores were determined.

RESULTS

The intra- and inter-examiner Kappa scores for assessment of microhardness were high with all values exceeding 0.80, which implies substantial agreement between the observers (Table 1).

Descriptive statistics and comparisons of microhardness between the occlusal and cervical regions of the three groups are shown in Table 2. Comparisons of occlusal and cervical microhardness for all specimens showed no statistically significant side differences ($p > 0.05$) for both the *in vitro* and *in vivo* evaluations. Therefore, occlusal and cervical microhardness scores for each specimen were pooled and the microhardness scores for each group for each evaluation type were obtained by calculating the mean of occlusal and cervical microhardness scores.

Descriptive statistics and the results of statistical tests for microhardness among the three groups are shown in Table 3. ANOVA showed statistically significant differences among the investigated groups, for both *in vivo* and *in vitro* evaluations ($p < 0.001$). According to multiple comparisons, statistically significant differences were determined between the Tooth Mousse® and the control ($p < 0.001$), and the Fluoridin N5® and the control ($p < 0.001$) groups. However, no statistically significant differences were detected between the Tooth Mousse® and Fluoridin N5® ($p < 0.05$) groups. The control group showed the lowest hardness scores (mean: *in vivo* = 205.661 ± 6.572; *in vitro* = 183.525 ± 9.190), while Tooth Mousse® (mean: *in vivo* = 286.929 ± 5.610; *in vitro* = 274.100 ± 7.704) and Fluoridine N5® (mean: *in vivo* = 288.018 ± 6.809; *in vitro* = 279.800 ± 10.582) showed significantly higher and comparable results.

Thus, the null hypothesis that CPP-ACP and fluoride containing topical gels can significantly reduce the overall amount of demineralization around orthodontic brackets, both in laboratory conditions and in the mouth, could not be rejected.

Table 1. Intra- and inter-examiner agreement in measurement of microhardness scores quantified by Cohen's Kappa

Observation type	Kappa score (K)
Intra-examiner agreement (Examiner 1)	0.82
Intra-examiner agreement (Examiner 2)	0.86
Inter-observer agreement	0.81

K <0.40 poor agreement; K = 0.41–0.60 moderate agreement; K = 0.61–0.80 substantial agreement; K >0.80 almost perfect agreement.

DISCUSSION

The results of this study showed that the application of Tooth Mousse® and Fluoridine N5® topical agents to tooth surfaces around orthodontic brackets were able to prevent demineralization of enamel, thereby suggesting its usefulness in the prevention of white spot lesions. To the best of our knowledge, this study is the first comparison of these topical agents against enamel demineralization around orthodontic brackets in an *in vivo* condition by microhardness testing.

The present *in vivo* and *in vitro* study evaluated the effect of two different preventive topical agents on demineralization on enamel around orthodontic brackets. Mineral loss was assessed by *in vitro* cross-sectional microhardness, a recognized analytical method. Cross-sectional microhardness was used to evaluate demineralization/caries because of the strong correlation ($r = 0.91$) between enamel microhardness scores and the percentage of mineral loss in caries lesions.[28]

Previously, the cariostatic effect of fluoride releasing materials were investigated by using a split-mouth design.[29,30] However, in the present study, the subjects in the experimental groups were randomly divided into two equal groups each received only 1, because the baseline clinical, radiological, salivary and laser fluorescence examinations showed that the patients were equivalent in regards to caries risk or demineralization activity. As suggested by Pascotto *et al.*,[23] the current experimental design was chosen instead of the split-mouth technique to avoid the carry-across effect due to fluoride or calcium and phosphate release by the agents on enamel around the brackets.

Pascotto *et al.*[23] observed reduced enamel hardness in the cervical region of the bracket compared with that in the occlusal area. *In vivo*, the explanation for this observation is greater dental plaque accumulation and difficulty in cleaning the area. *In vitro*, the explanation would be the less mineralization and the higher carbonate on the cervical surface than in the occlusal region.[23] Interestingly in the present study, in contrast to previous findings,[23,24,28,29] similar mineral loss was

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Table 2. Descriptive statistics and comparisons of the occlusal and cervical microhardness values of three groups for *in vivo* and *in vitro* evaluations

Evaluation type	Groups	n	Microhardness								Statistical evaluation (Wilcoxon test)
			Occlusal				Cervical				
			Mean	SD	Min	Max	Mean	SD	Min	Max	
In vivo	Control	28	207.143	7.199	198.000	221.000	204.179	9.813	182.000	221.000	NS p = 0.170
	Tooth Mousse	28	287.357	8.525	260.000	300.000	286.500	11.446	264.000	306.000	NS p = 0.789
	Fluoridine N5	28	288.786	10.785	268.000	303.000	287.250	10.193	269.000	302.000	NS p = 0.615
In vitro	Control	20	185.800	9.945	160.000	209.000	181.250	13.230	160.000	201.000	NS p = 0.176
	Tooth Mousse	20	274.500	11.879	257.000	306.000	273.700	10.147	251.000	295.000	NS p = 0.824
	Fluoridine N5	20	280.550	11.700	261.000	303.000	279.050	11.998	261.000	305.000	NS p = 0.537

n indicates sample size; SD: standard deviation; Min: minimum; Max: maximum; NS: not significant.

Table 3. Descriptive statistics and results of statistical tests for microhardness among three groups for *in vivo* and *in vitro* evaluations

Evaluation type	Groups	n	Microhardness (μm)				Statistical evaluation (Kruskal Wallis rank test)	Multiple comparisons	
			Mean	SD	Min	Max		Tooth Mousse	Fluoridine N5
In vivo	Control	28	205.661	6.572	194.000	221.000	***p <0.001	***p <0.001	***p <0.001
	Tooth Mousse	28	286.929	5.610	274.000	295.500			NS p = 0.798
	Fluoridine N5	28	288.018	6.809	268.500	297.000			
In vitro	Control	20	183.525	9.190	170.000	205.000	***p <0.001	***p <0.001	***p <0.001
	Tooth Mousse	20	274.100	7.704	256.000	293.000			NS p = 0.134
	Fluoridine N5	20	279.800	10.582	261.000	303.000			

n indicates: sample size; SD: standard deviation; Min: minimum; Max: maximum; NS: not significant; ***p <0.001.

observed at the cervical and occlusal region at 0 μm positions. Statistically significant microhardness differences were determined between tested materials and the control at the same position. The control group showed less hardness values that indicated more mineral loss than the tested materials.

The remineralizing potential of Tooth Mousse® has been shown in animal studies,[13,14,31] in *in vitro* studies[12,14,17,32] and in *in vivo* studies.[15,16] The use of CPP-ACP containing toothpaste would be beneficial for patients with enamel demineralization because it might remineralize existing enamel lesions and also prevent the development of further white spot lesions. Kumar *et al.*[33] indicated that CPP-ACP containing Tooth Mousse® remineralized initial enamel lesions and showed a higher remineralizing potential when applied as a topical coating after the use of fluoridated toothpaste. In a different area, Giulio *et al.*[34] determined that topical applications of Tooth Mousse® could be effective in promoting enamel remineralization after interdental stripping.

The *in vitro* results of the present study have demonstrated that Tooth Mousse® enhances the prevention of tooth structure from demineralization or remineralization of artificially formed enamel lesions. This is consistent with a previous *in vitro* study[18] which evaluated the effects of sodium fluoride (NaF) and 10% CPP-ACP on enamel demineralization adjacent to orthodontic brackets. It found that the application of CPP-ACP, NaF, or CPP-ACP/NaF can significantly prevent enamel demineralization when orthodontic composite resin is used for bonding.

The buffering capacity of the Tooth Mousse® agent could explain the aforementioned preventive demineralization action. It has been reported that casein is able to buffer plaque acid either directly or indirectly through bacterial catabolism.[31] It appears that this agent releases basic amino acids which are able to accept proton ions. It has been proposed that the anticariogenic mechanism of CPP-ACP is due to localization of ACP at the tooth surface which then buffers the free calcium and phosphate ion activities, thereby helping to maintain a state of supersaturation with respect to the enamel. This depresses demineralization and promotes remineralization.[14]

In a recent systematic literature review evaluating the effectiveness of fluoride in preventing white spot lesion development during orthodontic treatment, it was shown that the use of sodium fluoride during orthodontic treatment could reduce the severity of enamel demineralization surrounding orthodontic appliances.[35] Fluoride delivery methods which are thought to reduce the demineralization of enamel surrounding orthodontic brackets include the daily use of toothpastes, mouthrinses and/or gels with a high fluoride concentration or fluoride toothpaste in combination

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with chlorhexidine mouthwash.[23] The current preventive effects of this material investigated in *in vitro* and *in vivo* conditions were in accordance with the previous results that showed fluoride containing materials have a higher remineralizing potential than other protective agents.

In the present study, two commercially available protective topical agents (Tooth Mousse® and Fluoridine N5®) consisting of different properties, prevented the variation of demineralized enamel lesions around bracket bases over 14 days in an *in vitro* demineralization process and in patients' mouths during a 60-day follow-up period.

CONCLUSIONS

With an *in vitro* and *in vivo* tooth bracket model, the following conclusions can be drawn: a fluoridated agent for orthodontic bonding significantly decreases lesion depth and prevents the development of enamel demineralization; the use of a CPP-ACP as a topical coating significantly reduces enamel mineral loss; white spot formation can be prevented by using CPP-ACP and fluoride containing topical gels; and no statistically significant differences were detected between the two groups against demineralization.

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