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Original research

EFFECTS OF NANO-HYDROXYAPATITE DENTIFRICES WITH AND WITHOUT FLUORIDE ON PRIMARY TEETH ENAMEL: A MICRO-CT AND A SEM STUDY

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ABSTRACT

Objectives: The ideal remineralization system should provide sufficient ion reserves to provide mineral gain on deeper surfaces, as well as being biocompatible and easy to apply with no adverse effect. The aim of this study was to evaluate the remineralization efficiency of dentifrice form of nano-hydroxyapatite alone and its combination with fluoride on initial enamel lesions in primary teeth by micro-CT and SEM.

Materials and Methods: The enamel surfaces of 30 extracted primary incisors were treated with nano-hydroxyapatite based (nHAP), nano-hydroxyapatite-fluoride-containing (nHAP+F) and fluoride-containing dentifrice (F), respectively after the pH cycle. Mineral gain and surface properties were evaluated by micro-CT and SEM.

Results: nHAP group provided more mineral gain than fluoride containing paste groups but there was no statistically significant difference ($p>0.05$) according to the micro-CT evaluations. In SEM images, the micropores were completely covered with an apatite layer and mineral precipitations were observed to be wider and more pronounced in the nHAP group. Calcium fluoride crystalline structures exhibited non-homogeneous globular precipitations in the F Group.

Conclusions: It was concluded that nHAP dentifrice is as effective as fluoride dentifrice on initial enamel lesions in primary teeth with less caries-resistant enamel surface and therefore it can be safely used.

Keywords: Dentifrices, hydroxyapatite, microcomputed tomography, preventive dentistry, tooth remineralization.

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INTRODUCTION

Early childhood caries (ECC) is a special form of severe dental caries that affects young children and even infants.[1] Progression of caries may result in premature loss of primary anterior teeth leading to impaired quality of life.[2-53] In order to manage the progression of ECC, the effectiveness of preventive treatments varies depending on the early diagnosis of the lesion, as well as their ability to be applied in non-invasive methods and daily routine. Today, dentifrices can serve this purpose thanks to its easy accessibility and widespread use.[3-7]

Fluoride (F), which forms a more resistant fluorapatite structure than hydroxyapatite against acid attacks, is the most widely used agent for remineralization.[3-7] It has been understood that the mechanism of remineralization with topical fluorides is limited to the increase in surface hardness and resistance to demineralization only as a result of chemical and physical modification of the existing apatite surface, rather than recovering the lost minerals to form a new apatitic layer.[6-8,10]

Based on the knowledge that superficial remineralization has a limited impact on improving aesthetic and structural properties, it is accepted that the ideal remineralization system should provide mineral recovery on deeper surfaces.[7] Hydroxyapatite (HAP) has started to be used in the reconstruction of tooth enamel as a biocompatible and bioactive material.[11-15] Following the advances in nanotechnology, the initial enamel lesions could able to be repaired with nano-sized hydroxyapatites (nHAP), based on the knowledge that the surface porosities were better retained by increasing the surface areas of the hydroxyapatites which were incorporated into dentifrices.[11,16,17] In addition to several studies demonstrating that its use alone provides remineralization[8,10,12,17-19], there are also studies comparing the use of materials alone with their combined use to determine if nHAP has a synergistic effect with fluoride.[20-23]

Scanning electron microscopy (SEM), which allows a detailed examination of the topographic structure of the enamel surface is used as the gold standard in many in-vitro studies.[9,17,24,25] Besides SEM, micro-CT examination is also an innovative and non-invasive procedure. Micro-CT has the ability to determine the mineral loss of the initial enamel lesions and make quantitative measurements. However, there was no study evaluating the effect of remineralization of nHAP and fluoride-containing dentifrices on enamel lesions in primary teeth using micro-CT.

In this *in-vitro* study, it was aimed to evaluate the remineralization effect of the nano-hydroxyapatite dentifrice alone and with a combination of fluoride on the initial enamel lesions by micro-CT and SEM.

MATERIALS AND METHODS

30 extracted maxillary primary incisors were used in this *in-vitro* study. The written consent forms were signed by the parents. Ethics committee approval was obtained from the Clinical Research Ethics Committee (36290600/104). The study followed the principles of the Declaration of Helsinki.

Groups were planned according to remineralization materials as F group, nHAP+F group and nHAP group. Remineralization materials were tested on the three divided windows of the same sample as -intact enamel without any treatment (control group), initial enamel lesion not exposed to the pH cycle (demineralization group) and initial enamel lesions exposed to pH cycle and dentifrice application (remineralization group). Teeth were randomly divided into three groups (n=10).

The surfaces of the teeth were examined by a stereomicroscope (Leica MZ12, Meyer Instruments, Houston, TX, USA) to ensure that there was no visible structural defect. Teeth were brushed with a soft brush under water and stored in 0.1% thymol solution.

Prior to the experiments, the teeth were embedded in acrylic molds to cover all surfaces except buccal enamel surfaces. All parts were coated with 2 layers of acid-resistant nail polish except the 3x3 mm area on the buccal surfaces. The area of 3x1 mm thickness in the middle of the buccal surface was covered with 2 layers of nail

polish to be used as a control group. The other two areas of 3x1 were exposed to acid gel in order to create an initial enamel lesion (Figure 1).

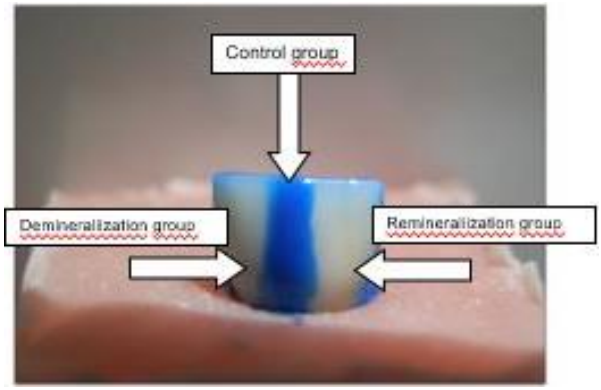

Figure 1: Intact enamel without any treatment (control group), initial enamel lesion not exposed to the pH cycle (demineralization group) and initial enamel lesion, exposed to pH cycle and dentifrice application (remineralization group) surfaces in the tooth sample

Initial enamel lesion creation

The acid gel technique used in the formation of the initial lesions contains -8% carboxymethyl cellulose and -0.1 mol/l lactic acid and its pH was adjusted to 4.5 with potassium hydroxide (KOH). Specimens were placed in a container containing acid gel and incubated for 96 hours in an oven (Kottermann, 3044, Germany) with a temperature of 37°C.

pH cycling

One of the two remaining windows with the initial enamel lesion was closed with nail polish after acid gel application and used for surface evaluation prior to the pH cycle. The last window which was left open with the nail polish uncoated surface was used for surface evaluation after the application of pH cycle and remineralization materials together.

During the demineralization-remineralization cycle applied to the samples in order to simulate the pH changes occurring in the oral environment throughout the day; 3-hour demineralization solution (2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4, 0.05 mM acetic acid, pH 4.4 with KOH), 2-hour remineralization solution (1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4 (0.15 mM KCl, pH 7.0), 3-hour demineralization solution, and 16-hour remineralization solution was applied, and 24-hour pH cycle was completed.[18]

Remineralization materials used in the study were Colgate Optic White (Colgate&Palmolive Company, 1000 ppm NaF) for F group, Ultradex Recalcifiying and Whitening (Periproducts, 1% nHAP + 1000 ppm NaF) for nHAP+F group and Apacare Apakids (Apagard, 1% nHAP) for nHAP group. Remineralization materials were weighed on a sensitive balance and then they were gently applied on the teeth twice daily (at 9:30 am and 7:30 pm) and for 2 minutes during the pH cycle (7 days) using a standard-sized applicator.

Each test material was removed under running water after application, then the samples were placed back into the containers. In order to maintain the effectiveness of the demineralization and remineralization solutions, the solutions were freshly prepared and renewed daily.

Micro-CT Analysis

Hydroxyapatite blocks (Skyscan, Kontich, Belgium) obtained from calcium hydroxyapatite (HAP) crystal were used to calibrate the micro-CT and to prevent x-ray hardening artifact.

The parameters were set to 100 kvp, 100 mA and 13.7 micrometers/pixel and the rotation step was 0.5 to match the scan values of the HAP blocks. For other parameters, X-ray hardening correction was applied, optimal contrast settings were set to 0-0.05 and used for reconstruction of samples. Each scanned tooth was then reconstructed using NRecon software. In total, 726 2-dimensional axial projections were obtained for each image group. These two-dimensional axial projections were then prepared for analysis by the manufacturer's software, Computer Tomography Analyze (CTan) (version 1.14,4,1 Skycan, Kontich, Belgium).

CTan software was used for advanced volumetric analysis and depth measurements of samples reconstructed with NReconstruction (NRecon) software. The starting and ending zones were selected for each tooth on the program. Since there are 3 different regions on a tooth in the present study, the area to be analyzed in CTan program was divided into left, middle and right. Afterwards, different Region of Interest (ROI) in the selected area was determined for the measurement of mineral density and gray color

values by applying adaptive interpolarization for each tooth.

The selected ROIs were then calculated using the CTan program for Bone Mineral Density (BMD). Mineral concentrations were calculated automatically by the program using the calibration curve generated with the aid of HAP calibration blocks. The densities of ROIs were then compared. Images, photos and videos were prepared with CTvox (version 2.7.0.).

SEM Analysis

Following the micro-CT analysis, 3 randomly selected samples from each group were taken to the Metallurgical Engineering Department of the Middle East Technical University for SEM examination. After all surfaces were coated with gold, images were taken from three different regions on each sample in ×500, ×2500 and ×5000 magnifications.

Statistical Analysis

SP IBM SPSS Statistics Version 22 package program was used to evaluate the results of the study. "One Way Analysis of Variance (ANOVA) Test" was used to evaluate the mineral density difference between the groups. When examining the difference between the groups; 0.05 was used as the level of significance.

RESULTS

Micro-CT Findings

Intra-group and intergroup comparisons of the mineral density measurements of all three surfaces -intact enamel without any treatment (control group), initial enamel lesions not exposed to the pH cycle (demineralization group) and initial enamel lesions exposed to pH cycle and dentifrice application (remineralization group)- were made by micro-CT.

Within each F, nHAP+F and nHAP groups, the mineral density of the demineralization groups was significantly lower than the other two groups ($p<0.05$). There was no significant difference between the remineralization group and the control group. Although there was no statistical difference, remineralization groups had higher mineral density in fluoride dentifrice (F) and nano-hydroxyapatite dentifrice (nHAP) than the control group. In the nHAP+F group, the control group had a higher mineral density than the remineralization group.

When the mineral gain of three dentifrices on initial enamel lesions after the pH cycle was examined between the groups, the values were; F group was found to be 0.0965 g/cm^3, nHAP+F group was 0.0978 g/cm^3 and nHAP group was 0.1162 g/cm^3. No significant difference was found between the groups according to the mineral density difference between the remineralization and demineralization groups. In general terms, it can be said that the order of gain in mineral amount between the groups is nHAP> nHAP+F> F (Figure 2).

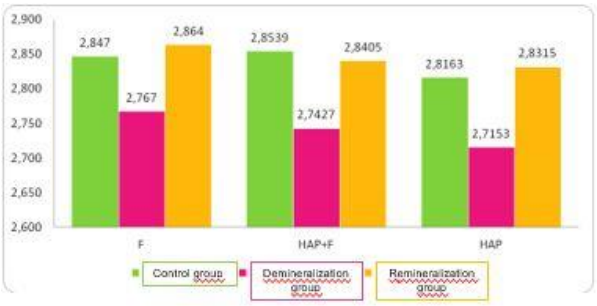

Figure 2. Comparison of mineral density difference between remineralization-demineralization-control groups in all treatment protocol

The increase in mineral content of the initial enamel lesions in the dentifrice group containing nano-hydroxyapatite was higher than the paste groups containing 1000 ppm F, although not statistically significant (Figure 2, Table 1).

Table 1: ANOVA test results for intergroup difference

	N	Med.	Std. Dev.	Min.	Max.	F value	P value
F	10	0.016500	0.0458191	-0.0610	0.0960		
HAP+F	10	-0.013400	0.0767003	-0.0850	0.1730	0.496	0.615
HAP	10	0.015200	0.0964743	-0.1340	0.2300		

SEM Findings

In the enamel samples demineralized to form an initial enamel lesion, it was found that the tooth enamel surface had a smooth and uniform structure before being exposed to any remineralizing agent and that the enamel prisms were viewed as key holes.

On the demineralized surfaces of all samples, it was observed that the surface layer of the enamel was disrupted and micropores were exposed in accordance with the prism structure. Deterioration of the integrity of enamel prisms and more dissolution in the central part of the prisms than the periphery part resulted in honeycomb-like images in these regions. However, it has been observed that the prism periphery has also disappeared and large pits have been formed in some regions.

It was observed that enamel surfaces were covered with newly formed CaF_2 crystals after the treatment of initial enamel lesions with F dentifrice (Figure 3a). It was observed that remineralization on initial enamel lesions did not show a homogeneous flat surface. It has been determined that small globules formed in some regions combined to form larger globules. However, the surface of some micropores was not covered and there were gaps in some places.

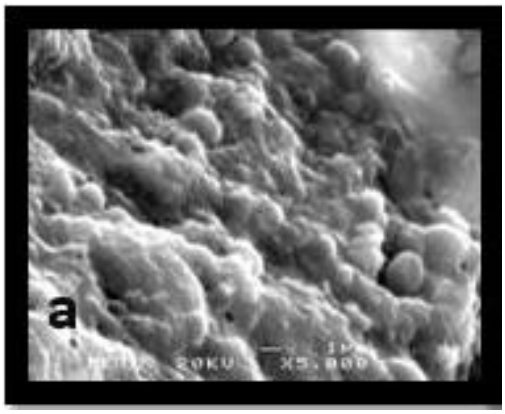

Figure 3a. Remineralization surface of the F group ×5000 magnification.

When the enamel surfaces treated with nHAP+F containing dentifrice were evaluated by SEM, it was determined that the whole surface had a homogeneous appearance and crystal accumulations of different diameters formed on the new surface layer (Figure 3b).

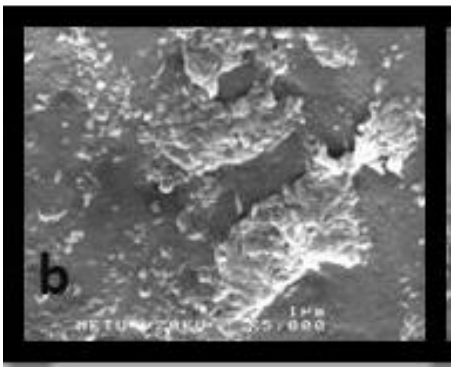

Figure 3b. Remineralization surface of the nHAP group ×5000 magnification.

In the samples treated with nHAP dentifrice, it was observed that the porous structure on the enamel surface formed after demineralization completely disappeared and the whole surface was covered with a new homogenous remineralization layer (Figure 3c).

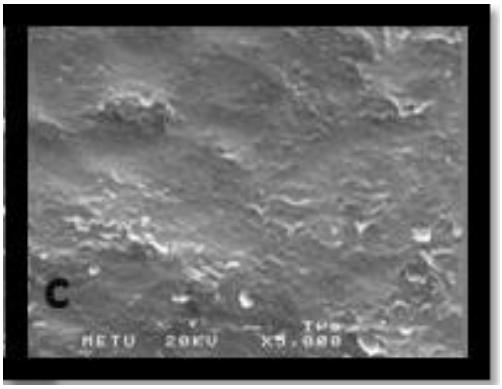

Figure 3c. Remineralization surface of the nHAP+F group ×5000 magnification.

The fact that the obtained surface cover provided the complete disappearance of the porous structure of the initial enamel lesions demonstrated that the thickness of this newly formed layer was higher than that observed after the application of F and nHAP+F dentifrice. It is also noteworthy that large scale mineral deposits were present in some regions.

DISCUSSION

The aesthetic restorations of the primary incisors may present some difficulties for both the practitioner and the pediatric patient. Therefore, in early childhood caries cases, early diagnosis, prevention, and even reversal of the initial enamel lesions have become increasingly important.[1-7] At this point, the concept of remineralization has come into the picture.[1,3-7]

The effectiveness of the method and the product used in protective programs should be

strong as well as easy to apply and low in cost, not requiring a professional application, reaching everyone with the individual application.[3,26] When the studies evaluating the effectiveness of remineralization of nano-hydroxyapatite in primary teeth are examined; it was observed that only three of them used dentifrice.[13-15] Only two of these studies had a fluoride comparison group[14,15] and generally surface hardness assessments were made.[12-15] In this context, in the present study, it was aimed to investigate the efficacy of different dentifrices due to their remineralization capacity through the evaluation of mineral changes on the enamel surfaces of primary teeth by micro-CT.

Recently, nHAP is considered one of the most biocompatible and bioactive materials; it has been studied as a biomimetic material that has demonstrated the potential effect of remineralizing of initial enamel caries under dynamic pH-cycling conditions.[12-14] The presence of hydroxyapatite material, which is the main structure of enamel, seems quite reasonable in terms of preventing the enamel demineralization and increasing its remineralization. HAP crystals in nanostructures have high biomimetic properties due to their composition, structure, morphology, body, and surface physical-chemical properties.[10,24,27] Dentifrices with nHAP are known to have a positive impact on the remineralization of initial enamel lesions, but there is no precise information on the repair mechanism of the lesions and more researches are needed.[17,27] Therefore, in this study, it was aimed to investigate the effectiveness of the remineralization of dentifrices with nano-hydroxyapatite in comparison to fluoride-containing.

Although the amount of fluoride in pediatric dentifrices varies between 250-500 ppm, it is stated that children with a high risk of caries under 6 years of age can use dentifrices containing 1000 ppm fluoride in the amount of swabs.[3,6] In addition, according to an in situ study and a systematic review evaluating the efficacy of different concentrations of fluoride dentifrices in remineralization of primary teeth enamel, it was stated that dentifrices containing at least 1000 ppm fluoride should be used in order to achieve effective remineralization.[29,30] For these reasons, in this study, a dentifrice containing 1000 ppm which is indicated to have optimum remineralization efficiency, was preferred.

In an *in-vitro* study by Itthagarun *et al*.,[18] in which they aimed to evaluate the effectiveness of remineralization on initial lesions of a dentifrice containing 10% nHAP with a dentifrice containing 950 ppm F, it was found that the same amount of reduction in lesion depth and the remineralization effects were similar. The fact that dentifrices with fluoride and nHAP did not show a significant difference between the mineral gain on initial enamel lesions in the present study also supports that view.

However, in the present study, although not statistically significant, the mineral density values of the remineralization group of nHAP dentifrice were higher than the fluoride dentifrice group. It was similar to an *in vivo* study which reported that the remineralization efficacy of nHAP was more successful than other dentifrices as a result of comparing the remineralization and reparability of different dentifrices on the enamel surface of premolar teeth.[31] In another study, the mineral gain of nHAP-containing dentifrices at the end of the 5-week cycle was found to be statistically significant than that of aminofluoride dentifrice on the initial enamel lesions on bovine specimens.[32]

There are also studies investigating whether there would be a synergistic effect by taking advantage of fluoride.[20,23,33,35] Whereas in a few studies the remineralization effect is reported to be increased[34,35], a number of investigators have found no synergistic interactions with fluoride.[20-23] In addition to those studies, it was reported that the biocompatibility of fluoride ions is adversely affected when used with nHAP.[36] In a study evaluating the remineralization effect of nHAP and nHAP+F combination in dentifrice form, initial enamel lesions were evaluated with microhardness and only HAP-containing dentifrice was found to be effective in increasing surface hardness.[20] In the present study, it was also detected that within the nHAP+F group,

mineral density values of the remineralization group could not reach to the baseline values. Consequently, based on micro-CT findings of the present study, it can be concluded that the addition of fluoride to the nHAP-containing dentifrices has no synergistic effect on remineralization. However, it can be stated that the gain in the mineral amount in the nHAP+F group was higher when compared to the F group, although not statistically significant.

In an *in vitro* study comparing the effect of dentifrices containing nHAP and sodiumfluoride on initial enamel lesions, it was observed that nHAP group had a unique form of remineralization.[12] While the remineralization surface of hydroxyapatite dentifrice was found to be smooth, it was found that the remineralization of fluoride dentifrice was irregular and resulted in non-homogenous layer, and some micropores did not close. It has been also reported that the modifications seen on the enamel surface were non-uniform irregular globular structures.[8,12,15] Similar to these studies, it was observed that the surface treated with fluoride dentifrice was irregular and rough in the present study.

The most prominent advantage of nano-hydroxyapatite in preventive dentistry when compared to other agents is that it hardens the existing superficial layer and creates a remineralization mechanism where superficial porosities are covered with a uniform and thick new synthetic hydroxyapatite layer.[8-10,14-16] Similar to these studies, in the present study's SEM images of the enamel surfaces treated with hydroxyapatite-fluoride combination dentifrice, the whole surface had a homogeneous appearance and crystal accumulations of different diameters and the globular deposits did not cover the whole surface. The finding that the remineralization surface in the samples treated with nHAP dentifrice has a more intense mineral deposit than the intact enamel confirms the opinion that a more durable structure was formed than before.

CONCLUSIONS

Within the limitations of this study, based on the findings obtained from micro-CT and SEM images, it can be concluded that the remineralization mechanism of nHAP works by forming a layer with high mineral density by apatite deposition on the demineralized surface and repairing the crystal structure of the enamel by using a mineral deposition. In addition, it is thought that this layer may act as a mineral reserve against acid attacks and inhibit demineralization. The hypothesis that "nHAP has a similar fluor-like remineralization effect" is disproved as it creates more effective mineral exchange than fluoride dentifrice. It is thought that it can be recommended for effective prevention especially in children between the ages of 6 months-2 years to be used safely since there is no harm in swallowing hydroxyapatite. However, the findings need to be supported by further *in vivo* studies in order to clinically prove the efficacy of nHAP on remineralization.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest related to this study.

Florid İçeren ve İçermeyen Nano-hidroksiapatitli Diş Macununun Süt Dişi Minesi Üzerine Etkileri: Mikro-BT ve SEM çalışması

ÖZ

***Amaç**: İdeal remineralizasyon sistemi, ağız ortamına yeterli iyon rezervini sağlayarak daha derin yüzeylerde mineral kazanımını sağlamalı ve kolay uygulanabilir olmalıdır. Çalışmamızda; nanohidroksiapatit materyalinin macun formunun tek başına kullanımı ve floridle kombinasyonunun, süt dişi başlangıç mine lezyonlarında remineralizasyon etkinliğinin mikro-BT ve SEM ile in-vitro koşullarda değerlendirilmesi amaçlanmıştır. **Gereç ve yöntemler:** Çekilmiş 30 adet üst süt kesici dişin mine yüzeyine pH döngüsünü taklit eden bir düzenek içerisinde sırasıyla; nanohidroksiapatit esaslı (nHAP), nanohidroksiapatit-florid içerikli (nHAP+F) ve florid içerikli diş macunu (Kontrol Grubu) (F) uygulamasından sonra yüzeylerindeki mineral değişimlerinin tanısı ve miktarı Mikro-BT ile, yüzey morfolojisindeki değişimler ise SEM ile değerlendirilmiştir. **Bulgular:** nHAP grubunun florid içeren macun gruplarına göre yapılan*

ölçümlerde sayısal değer bakımından daha fazla mineral kazancı sağladığı ancak bu farkın istatistiksel olarak anlamlı olmadığı belirlenmiştir (p>0.05). nHAP içeren diş macunuyla tedavi edilen örneklerin SEM bulguları değerlendirildiğinde demineralizasyon sonrası yüzeyde belirgin olarak görülen mikroporları tamamen kaplayan bir apatit tabakası oluştuğu gözlenmiştir. F'li diş macunu grubundaki örnekler de ise; mine yüzeyinde düzensiz, homojen olmayan ve kalsiyum florür kristali olduğu tahmin edilen yapıların globüler olarak çökelmeler gösterdiği, bununla birlikte bazı mikroporların yüzeyinin örtülmediği ve yer yer boşluklar olduğu gözlenmiştir. ***Sonuçlar:*** *Nano-hidroksiapatit içeren diş macunlarının, çürüğe daha az dirençli mine yüzeyine sahip olan süt dişlerinde florid içeren macunlar kadar etkili bir remineralizasyon etkisi oluşturduğu ve çocuklarda floridli macunlara alternatif olarak güvenle kullanılabileceği düşünülmektedir.* ***Anahtar Kelimeler:*** *Diş macunları, hidroksiapatit, koruyucu diş hekimliği, mikrobilgisayarlı tomografi, diş remineralizasyonu*.

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