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Research Article

Effect of nano theobromine incorporated into a primer on enamel density (An Invitro study)

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Abstract: The current study aimed to evaluate the enamel density after applying an orthodontic primer containing nano theobromine powder. **Materials and methods**: The sample consisted of 24 lower premolar teeth distributed into three groups (eight teeth in each group). After demineralization, the specifically prepared enamel area was covered either with a 3MTM Transbond orthodontic primer for control or with the same primer incorporated with 10% or 15% nano theobromine powder for the experimental groups. The crowns were sectioned horizontally and longitudinally creating 48 right and left segments. Teeth were subjected to radiological densitometry analysis in horizontal and vertical directions to evaluate the enamel density and subsequent remineralization. **Results**: A significant change in enamel density values was recorded in teeth covered with a primer containing 15% nano theobromine ($p>0.05$), with values higher than that of the baseline (105.00±22.59, 96±23.8 grayscale). **Conclusion**: Both incorporating groups particularly 15% nano theobromine showed an increase in enamel density and subsequent remineralization. This modified orthodontic adhesive may be useful for preventing early enamel demineralization and supporting clinicians with minimally invasive procedures.

Keywords: Nano theobromine, Orthodontic primer, Remineralization

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INTRODUCTION

The bonding of orthodontic brackets to tooth surface enamel has been an important issue since the introduction of direct bonding in orthodontics. Due to limited accessibility to clean tooth surfaces during treatment, bacterial adhesion, colonization, and proliferation around orthodontic brackets specifically *Streptococcus mutans* increase. The products of these bacteria lead to the demineralization of enamel and increase the risk of dental caries [(1,2)]. The modern preventive objective is to reduce or stop the progress of dental caries around orthodontic brackets using non-invasive methods such as antibacterial and/ or remineralization agents [(3,4)].

One of these promising aspects is the orthodontic bonding with materials that contain potential remineralization agents that prevent enamel demineralization around orthodontic brackets [(5,6)]. Previous studies have focused on incorporating fluoride which plays a significant role in the reduction of dental caries[(7–9)]. The disadvantage of such an approach is that the majority of fluoride is released during the setting process, with a minor quantity of long-term release [(10,11)]. Moreover, some studies suggested that fluoride may reduce the shear bond strength of brackets[(12,13)]. Other agents, such as silver, titanium oxide, and quaternary ammonium, have also been incorporated into bonding systems [(14–17)]. However, some of these agents impaired the mechanical properties of the materials [(15)].

A new class of compounds derived from natural resources has been extremely successful in the development of new medications. One of these products is theobromine derived from theobroma cacao. In the calcium and phosphate-rich medium, theobromine forms larger hydroxyapatite crystallites. Subsequently, the enamel is reinforced and becomes less susceptible to acid attacks of bacterial products and dental caries.

There have been recent attempts to replace fluoride with theobromine as a remineralizing agent and as a potential substitute for fluoride, particularly in mouthwashes and toothpaste. They recorded that following the application of theobromine, the microhardness of the enamel surface increased, indicating that theobromine generally has protective properties for enamel [(18,19)].

Limited information is available about the nano natural products incorporated in orthodontic adhesive, particularly in the primer rather than the composite itself especially those that have remineralizing effects [(20)]. According to our best knowledge, no study evaluated the effect of adding nano theobromine product into the orthodontic primer. Thus, this manuscript aimed to assess the effect of nano theobromine incorporated in the orthodontic primer on the enamel density (ED) and consequently on enamel remineralization. The null hypothesis is that there is no significant effect on ED after the application of orthodontic primer incorporating nano theobromine.

MATERIALS AND METHODS

Study sample

The ethical clearance was obtained from the Research Ethics Committee of the College of Dentistry, University of Mosul in 2023 (UoM.Dent.23/31).

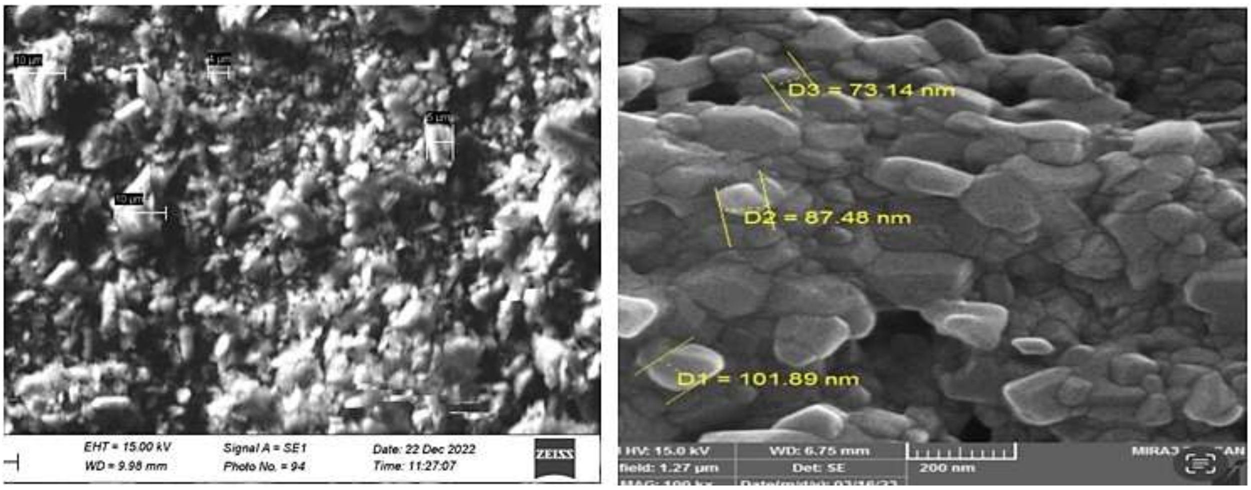
Samples consisted of 24 extracted human lower premolars, which, had normal sizes and shapes. These were taken out of patients between the ages of 16 and 28 to be used in orthodontic treatments. The teeth were collected from private clinics and Dental Health Centers in Mosul city.

The selection criteria for teeth included the absence of hypo-plastic areas, caries, attrition, cracks, restorations, and any history of previous treatment with orthodontics or endodontia (21). The labial surface of the teeth was evaluated using a ×5 magnifier to check the chosen teeth. Soft tissue remnants were cleaned up and eliminated. After extraction, the samples were kept at room temperature (about 22 C°) in distilled water. For the remaining research experiments, the water was replaced every week to prevent microbial growth (22). The teeth were randomly divided into:

- Control group; consists eight teeth subjected to 3M™ Transbond XT™ without any modifications
- Nano-theobromine 1; consists of eight teeth using primer mixed with nanotheobromine at a concentration of 10%.
- Nano-theobromine 2; consists of eight teeth using primer mixed with nanotheobromine at a concentration of 15%.

Preparation of the Modified Adhesives

The nano theobromine powder was prepared from theobromine (Sigma-Aldrich, Italy) by mechanical milling. Ball milling is a mechanical milling method to fabricate nanomaterials, one of the most widely used top-down techniques to produce nanoparticles is mechanical milling (23). The measurement of particle size was conducted under ESM after milling and it was approximately 75.49 nm (Fig.1).



(A) **(B)**

Figure (1): SEM image represented Nano-theobromine particles (A) before milling, (B) after milling.

The primer with 10% nano theobromine was obtained by incorporating 10 % per weight of theobromine (Sigma-Aldrich, Italy) weighed on a 0.003 g precision scale of (AUM-150-l), into the 0.03 g of the primer. We obtained the primer with 15% nano theobromine by incorporating 15 % per weight of theobromine (Sigma-Aldrich, Italy) weighed on a 0.006 g precision scale of (AUM-150-l), into the 0.034 g of the primer. To achieve a uniform color distribution and complete wetting of the nanoparticles within the resinous material, manual mixing with a plastic spatula was done for about 60 seconds in a semi-dark environment. After that, the nano theobromine-modified primer was then transferred to a sterile disposable syringe and covered with dark-colored tape [24]. The homogeneity was checked under SEM.

Demineralization protocol

Each crown was coated with an acid-resistant varnish (nail varnish) in a group of demineralized samples, leaving the middle third of the buccal surface open to view an enamel window measuring about 5 × 5 mm. As a result, the majority of the tooth crown was sealed with an acid-resistant varnish, leaving only the exposed enamel available to acid attack. [25]. Each tooth was individually immersed in 40 ml on a demineralization solution containing 2mlM $CaCl_2$, 2.2mlM KH_2PO_4, 0.05M acetic acid, with pH 4.4 adjusted by 1M KOH at 37c for 96 hours to develop initial artificial carious lesions [26].

Primer Application and Tooth Sectioning

The 3M transbond adhesive primer mixed with (10% or 15%) nano theobromine was applied to the exposed window of the demineralized enamel according to the groups, then, cured for 20 seconds and immersed in artificial saliva as the storage medium and changed the saliva every 48 hours [27], and research adjusts the Ph (4.4) of saliva regularly. After 90 days, the researcher sectioned the crowns firstly from the roots with a low-speed double-sided diamond disk and continuous water spray irrigation. After that, each crown was cut in a buccolingual direction from lingual to halfway; then, the two parts were separated using osteotome and hammer to have clean cuts as shown in Figure 2.

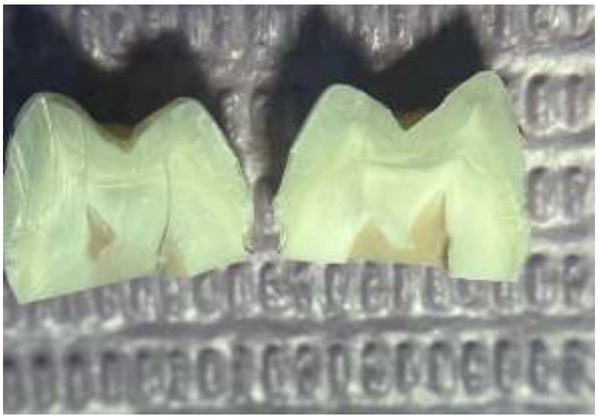


Figure (2): The crown after cutting

Densitometry Estimation and Analysis

After sectioning procedures, the densitometry analysis was conducted. Teeth subjected to radiological densitometry analysis in Al-Rasheed Radiograph Center in Mosul city using the x-ray system that consists of a Dental X-ray tube machine (Dyson, DYS-M, ZHEJIANG GETIDY, China), Dental X-Ray sensor (Carestream, RVG 5200, United States) that was used for this analysis. X-ray imaging was undertaken using a standardized technique. The standardization included the distance between the source and the object (20 cm), the Voltage (70 K.V.), the milliampere (20 mA), and the time of exposure (0.3 seconds). In this study, the X-Ray imaging sensor was fixed on a table and the specimen was placed above it. The X-ray tube was directed perpendicularly to the specimen and sensor. The area at which the crown resides was carefully positioned in the middle of the sensor.

In current work, The X-ray processing software (Carestream, C.S. Imaging Software version 7.0.3, United States). Specific features in the software including the densitometry analysis were utilized. Depending on the tone of each pixel in the radiographic image, this feature uses the gray tone in the grayscale as an indicator of bone density. Every pixel has 256 values representing its grayscale tone, which ranges from zero (pure black) to 255 (pure white). Therefore, the software uses each pixel's grayscale value to calculate bone density relative to other pixels.

In each specimen, a reference line was detected from the highest point in the occlusal surface to the gingival region of the deepest point. Then, to detect the midpoint of the reference line, the obtained distance was divided into two equal distances. Then two millimeters above and below the midpoint, a line was drawn between them to estimate the enamel density. To measure enamel density in a horizontal direction the same point was used to measure the distance from the outermost surface of the enamel to the enamel-dentinal junction by measuring it horizontally as shown in Figure 3.

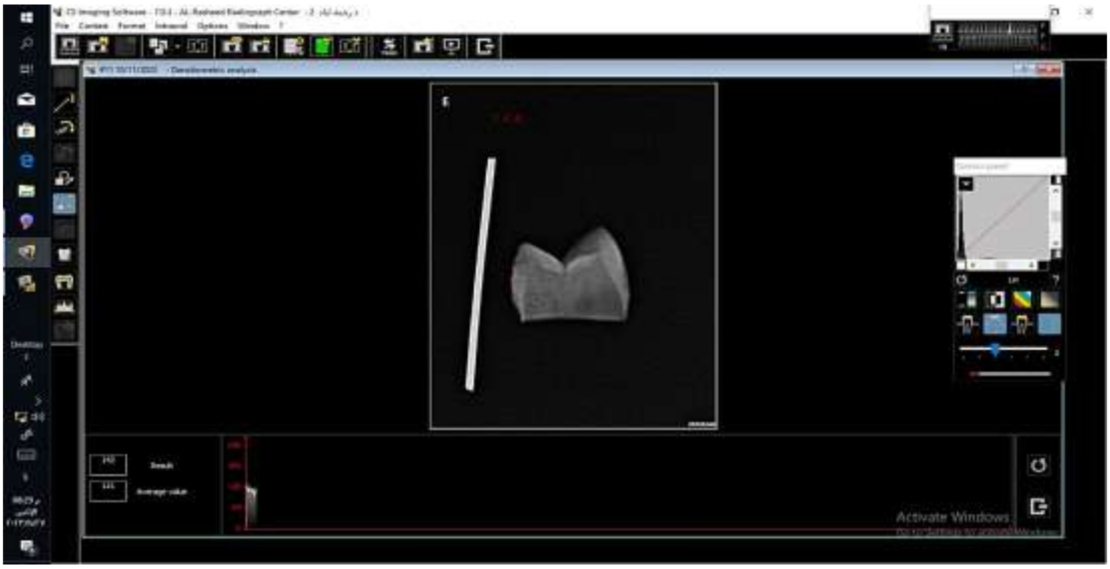


Figure (3): The screenshot shows the software and the approach to detecting the vertical measurements.

Five teeth specimens were reassessed after scoring them. Scores were assessed twice, at baseline, and after 15 days from the first reading. Moreover, an experienced reader scored the same teeth to investigate the inter-examiner calibration variation. They. Intraclass correlation was conducted to assess the intra and interexaminer calibration procedures. The results showed that the intraclass correlation was 0.85 (p<0.05) for the intraexaminer and 0.80 (p<0.05) for the interexaminer calibrations.

Statistical Analysis

The data of the current study were analyzed by utilizing a statistical computerized program (IBM SPSS statistics Version 26). The Shapiro-Wilk test was used to assess the data distribution for the study groups. Descriptive statistics that contain the minimum and maximum values, mean, and standard deviation. A two-way analysis of variance (ANOVA) was done to recognize any significant differences among various groups. Post hoc testing using Tukey's HSD was used mainly with post hoc comparisons in the analysis of variance (ANOVA). The *p*-value was set to be ($p < 0.05$) for the significance level.

RESULTS

Densitometry Assessment

Distribution along enamel density and remineralization Groups

The assessment of data normality of the distribution has shown that most data along ED and remineralization groups followed a normal distribution. These data included the mean, standard deviation, minimum, and maximum values of the ED for all the study groups as shown in Table 1. The description of the groups revealed that the orthodontic adhesive modified with 15% nano theobromine group had the highest ED mean value followed by the adhesive modified with 10% nano theobromine group. In comparison, the control group showed the lowest mean value.

Table (1): The descriptive statistics for the ED measurement groups

Variable	Mean	SD	Minimum	Maximum
Control in Vertical Direction	75.86	26.90	46.00	121.00
Modified with 10% Nanotheobromine Vertical Direction	93.46	26.33	56.00	140.00
Modified with 15% Nanotheobromine Vertical Direction	96.26	23.68	64.00	144.00
Control in Horizontal Direction	92.86	21.67	64.00	123.00
Modified with 10% Nanotheobromine Horizontal Direction	99.06	21.48	71.00	146.00
Modified with 15% Nanotheobromine Horizontal Direction	105.00	22.59	78.00	162.00

Measurement is in grayscale

Analysis of Variance (ANOVA) for ED among the Study Groups

A two-way ANOVA was performed to evaluate the effects of measurement direction and nano theobromine concentration on enamel density (Table. 2). The result showed that there was a significant difference between enamel density for different concentrations of nano theobromine ($p= 0.015$) with non-significant effect of measurement direction. Whereas, there was a statistically non-significant interaction between the effects of concentration and measurement direction ($p= 0.670$).

A more specific multiple comparison by Tukey's HSD test was conducted among all the study groups as demonstrated in Table 3. Post hoc testing using Tukey's HSD test indicated that enamel density was at a higher concentration of 15% than they were at a concentration of 10% ($p = 0.010$). There was no significant difference between the enamel density of the control and the concentration of 10% groups ($p = 0.223$). It is important to mention that Eta squared (η^2) ranges from 0 to 1, with values of 0.01-0.059, 0.06-0.0139, and 0.14 above representing small, medium, and large effect sizes, respectively (Tables. 2 and 3).

Table (2): Two-way analysis of variance for ED between the study groups

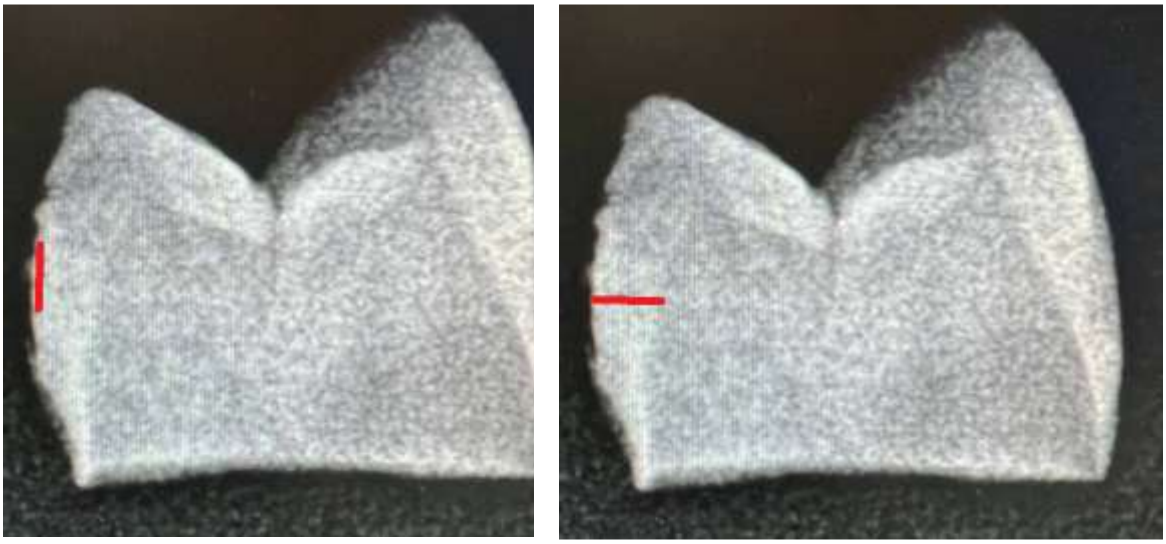
Variable	Sum of Squares	df	Mean Square	F	P-value	Partial Eta Squared
Direction	2419.62	1	2419.62	3.47	0.066	0.037
Concentration	6173.81	2	3086.90	4.42	0.015*	0.090
Direction × concentration	560.77	2	280.38	0.40	0.670	0.009

*Significant at p <0.05.

Table (3): The post Hoc test of the study groups

Variable		Mean Difference	St. Error	*P*- value
Control	Concentration of 10%	11.19	6.71	0.223
	Concentration of 15%	19.66	6.61	0.010*
Concentration of 10%	Concentration of 15%	8.46	6.50	0.398

*Significant at p <0.05.



(A) **(B)**

Figure (4): Illustration shows the screen measuring enamel density. (A) In a vertical direction, (B) In a horizontal direction after magnification

DISCUSSION

Tooth demineralization results in the loss of calcium and phosphorous from hydroxyapatite crystals. A reverse mechanism can be achieved if the pH is neutralized with adequate calcium and phosphate ions in the oral environment aiding in the rebuild of dissolved hydroxyapatite crystals [28]. Prevention of tooth demineralization focuses on increasing teeth resistance to acid attack by using various preventive aspects such as fluoride application.

New strategies aiming to reverse the progression of non-cavitated, demineralized lesions involve the topical use of remineralization agents. These agents provide an environment favorable for lesion repair and increase enamel resistance to acid challenge. The use of natural herbal products as an alternative to conventional treatment has increased in the last few years. Although several studies have been conducted to assess this (29,30) still, there is a need for conducting further research to establish the efficacy of these products particularly when incorporated with a dental composite.

The use of theobromine as a remineralization agent in this study was based on Nakamoto et al. who recorded that theobromine increases the size of apatite crystals, which is associated with enamel surface hardness. They further stated that theobromine is safer because of its low toxicity level when compared to fluoride (31). The current study was conducted to determine the ability of nano theobromine to promote the remineralization of artificially demineralized enamel by increasing enamel density. The results have shown that enamel density increased in demineralized enamel covered with an orthodontic primer containing 15% nano theobromine.

This is the first study that evaluated the effects of nano theobromine incorporated in the orthodontic adhesive Although direct comparison with the available studies was difficult, generally, the result is in agreement with the previous studies (32–34) that indirectly reported an increase in enamel density after the application of theobromine gel due to an increase in enamel microhardness. Also, the current results agree with Premnath et al. (35) and Shawky et al. (18) who assessed and compared the remineralization effects of theobromine and fluoride and recorded positive but lower remineralization effects of theobromine in comparison with fluoride. Also, it is interesting to mention that Shawky et al. (18) found that theobromine was able to increase the calcium and phosphate levels more than fluoride but the difference was not significant.

The results contradict those reported by Thorn et al. (36) who evaluated the remineralizing ability of different concentrations of theobromine at acidic and neutral conditions; they revealed that various concentrations of theobromine existing in a plaque fluid-like medium did not enhance remineralization.

The disagreement may be attributed to differences in the demineralizing agents which use continuous exposure instead of the pH cycling model (36). Earlier to that, Lippert et al. (37) compared the remineralization potential of theobromine on demineralized carious lesions. They concluded that theobromine did not provide remineralization under the selected conditions. These results are in disagreement with the current study showing that enamel density increased in theobromine groups.

According to the available literature, theobromine can improve the remineralization potential of a medium rich in calcium and phosphate. Theobromine concentration expressed in moles is 27 times lower than the fluoride concentration with equivalent remineralizing potential. Theobromine applied to enamel produces a smooth surface through remineralization (18). It is believed that the theobromine molecule attracts calcium and phosphate ions so that the deposition of calcium and phosphate occurs to form a new hydroxyapatite crystal called theobromine apatite (35). The improvement of the enamel surface and its remineralization during and even after orthodontic therapy is still a major concern in orthodontic practice. Although this study did not use a sophisticated approach, the improvement of enamel density was recorded mainly at a 15% concentration of nano theobromine. Thus, the null hypothesis was rejected, however, further studies are recommended to evaluate higher concentrations with further developed methods.

CONCLUSIONS

From this study, it can be concluded that the addition of nano theobromine powder particularly 15% may increase the enamel density and subsequently the remineralization ability of the orthodontic adhesive primer.

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Ethical statement: The ethical clearance was obtained from the Research Ethics Committee of the College of Dentistry, University of Mosul in 2023 (UoM.Dent.23/31).

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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تأثير الثيوبرومين النانوي المدمج في برايمر على كثافة المينا (دراسة مختبرية)

رحمة الزركي ,علي الخطيب, عمار الحمداني , روزيتا حسن

الملخص

الاهداف: هدفت هذه الدراسة إلى تقييم كثافة المينا بعد استخدام اللاصق التقويمي الذي يحتوي على مسحوق النانوثيوبرومين. **المواد والطرائق العمل** : تكونت العينة من ٢٤ سناً موزعة على ثلاث مجموعات (ثمانية أسنان في كل مجموعة). بعد إزالة تمعدن المينا، تمت تغطية المنطقه المعنيه المعدة خصيصًا إما باستخدام لاصق تقويمي نقي أو باستخدام نفس الماده مدمجة مع مسحوق النانوثيوبرومين بنسبة ١٠ % أو ١٥ % للمجموعات التجريبية. تم قطع التيجان أفقيًا وطوليًا لتكوين ٤٨ مقطعًا يمينًا ويسارًا. تم إخضاع الأسنان لتحليل قياس الكثافة الإشعاعي في الاتجاهين الأفقي والرأسي لتقييم كثافة المينا والأنسجة المعاد تمعدنها لاحقًا. **النتائج**: تم تسجيل زيادة ملحوظه احصائيا في كثافة المينا في الأسنان المطلية بالمادة التي تحتوي على ١٥ % نانوثيوبرومين (٢٣,٨±٩٦ وكذلك ٢٢,٥٩±١٠٥ تدرج رمادي) .**الاستنتاجات**: كلا المجموعتين اللتين تم اختبارهما وخاصة ١٥ % نانوثيوبرومين أظهرت نتائج واعدة فيما يتعلق بكثافة المينا وإعادة التمعدن اللاحق. لذا فبالإمكان ان يكون هذا اللاصق المعدل لتقويم الأسنان مفيدًا في منع إزالة تمعدن المينا في وقت مبكر ودعم الأطباء من خلال إجراءات طفيفة التوغل.