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Effects of Nanohydroxyapatite and Fluoride varnish on the Microhardness of the Artificially Induced Enamel Lesion: *An In-Vitro Study*

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Abstract:

Study objective: To evaluate the ability of nanohydroxyapatite and fluoride varnish to remineralize an artificially induced enamel lesion. **Methods:** Fifty-four extracted premolar teeth were randomly allocated into three main groups (n=18 each); nanohydroxyapatite (n-HAP), fluoride varnish (FL) and untreated control group (C). These groups were further classified into three subgroups (n=6) based on the storage time in artificial saliva (1 week, 2 weeks, 4 weeks). All teeth subjected to pH cycling procedure for induction of white spot lesion. The n-HAP group was treated by immersing the samples in 10% n-HAP solution for three minutes before storing them in artificial saliva. This procedure was repeated daily. The second group treated with fluoride varnish and the third group left untreated. The microhardness was measured at baseline, after pH cycling procedure and after 1-week, 2-weeks, 4-weeks from first time of application of treatment agents and the statistical analysis was performed for the post treatment periods (1, 2, and 4-weeks) to assess the progression of remineralization, with baseline and after pH cycle data serving as reference points to establish the initial condition of the samples. **Results:** Statistical analysis of mean microhardness values demonstrated a significant difference in microhardness values according to different storage times only for the nanohydroxyapatite group while there were no significant differences in microhardness mean values for fluoride varnish and untreated group.

Conclusion: 10% n-HAP suspension more effective in remineralizing of WSLs as compared to fluoride varnish.

Key words: Nanohydroxyapatite, Microhardness, White spot lesion, Minimum invasive dentistry, Fluoride varnish.

Introduction

White spot lesions (WSLs) are a clinical term that refers to the early demineralization of enamel within both surface and subsurface layers that results from plaque accumulation in areas of stagnation in peoples exhibiting inadequate oral hygiene. Whenever the demineralization activity is not stopped, and then will eventually deepens and form a cavity (Marinelli et al., 2021). White spot lesion, also known as initial caries, early enamel caries, or smooth surface caries exhibit an opaque white appearance due to the mineral's loss in the subsurface enamel and altered light reflection as compared to intact enamel (Haberal, 2024). According to Cosma et al., 2019 WSLs are a common consequence observed in individuals wearing fixed orthodontic devices, primarily due to the positioning of the brackets, which promotes plaque buildup, resulting in the formation of the WSLs (Cosma et al., 2019). Recent treatments of WSLs emphasise the utilisation of remineralising agents to control demineralisation and promote remineralisation. The remineralising products form a supersaturated condition surrounding the carious lesion, which prevents mineral loss and concentrates calcium and phosphate ions in the unfilled regions (Chokshi et al., 2016). In a study conducted by Reynolds, 2008 it was found that fluoride ions play a significant role in preventing enamel demineralization. This is attributed to the fluorapatite formation in enamel, which is facilitated by the existence of phosphate and calcium ions produced as a result of organic acids generated from dental plaque activity. Nanohydroxyapatite (n-HAP) was employed in remineralisation process due to its biocompatibility and bioactive properties, which result in the precipitation of particles resembling the enamel crystal in structure (Krishnan et al., 2016). Nanohydroxyapatite (n-HAP) suspension had good potential to enhance crystal morphology, remineralise, and halt the demineralization process (Lei et al., 2019). The hardness measurement constitutes a viable approach for assessing the enhancement of WSLs. While this measurement method does not directly

demonstrate the alterations in the minerals content of the WSLs throughout treatment, it may indicate beneficial improvements in the treatment of WSLs (Zakizade et al., 2020). Regarding the effect of nanohydroxyapatite suspension on WSLs at different storage time intervals in artificial saliva, no Iraqi study had been found. So, the study objective was the evaluation of the WSLs microhardness after treatment with nanohydroxyapatite suspension at various storage times in artificial saliva in comparison to the fluoride varnish. The null hypothesis suggests no significant differences of microhardness after treatment with nanohydroxyapatite suspension at different storage time intervals.

Material And Methods

Sample

The teeth sample used in the study included fifty-four sound, caries-free premolar teeth extracted due to orthodontic treatment. Teeth were extracted a traumatically, rinsed with de-ionised water, and subsequently all teeth wiped with acetone to remove debris. After that, the samples were cleaned by rubber cups with non-fluoridated pumice and a conventional low speed handpiece. Under the magnifying lens, the teeth were then examined to ensure that they were free from fractures or cracks. To prevent bacterial and fungal growth and minimise enamel brittleness, teeth samples were kept at room temperature in twenty millilitres of deionised water containing 0.1% thymol until use (Barbakow et al., 1987).

Preparation Of The Sample

Using an orthodontic ruler, six millimetres in diameter circular window was formed as well as standardised on the buccal side of the samples. To identify the central region of the buccal side of the samples, two imaginary lines were drawn: first line extending from the tip of the buccal cusp to the cervical line, as well as the other line extending from the mesial to the distal surface at the most prominent curvature of these teeth. Following that, an adhesive tape circle with a 6 mm-diameter was cut and affixed to the buccal surface of the tooth. After the application of acid-resistant nail polish, the adhesive tape was removed, creating a window on the buccal surface of teeth. The abrasive paper had been affixed in a manual apparatus, and the window of each sample was subjected to polishing in same direction in a total of 10 times and this process made it possible to measure the microhardness of tooth on flat surface (Al-Sayyab, 2000), Figure (1).

White Spot Lesion Induction

By using the pH-cycling procedure, white spot lesions were created in the enamel of

the samples. The demineralising solution was prepared by mixing 1.0 M/L of calcium chloride, 2.0 mM/L of phosphate chloride, and 0.075 M/L of acetic acid with the adjustment of the pH to 4.3 at 37 °C. The remineralising solution was made by mixing 1.5 mM/L calcium nitrate, I50 mM/L potassium chloride, and 0.9 mM/L potassium phosphate with a pH adjustment to 7 at 37 °C (Featherstone et al., 1986). The samples were immersed in 20 ml of the demineralisation solution at 37°C for six hours and kept in an incubator. After that, the samples were washed for one minute with running, de-ionised water. The samples were then immersed in a remineralising solution at 37°C for seventeen hours and placed in an incubator. The entire procedure was repeated for ten days on daily. The teeth samples were then with rinsed with de-ionised water, and an examination was conducted for the samples under stereomicroscope to ascertain any microscopic alterations associated indicative of caries development that could be seen with the naked eyes as white spots lesion after drying.

Treatment Groups After White Spot Lesion Formation

Fifty-four extracted premolar teeth were randomly allocated into three main groups (n=18) that are nanohydroxyapatite (n-HAP), fluoride varnish (FL), and untreated groups (C) and these groups were further classified into three subgroups (n=6) based on the storage time in artificial saliva (1-week, 2-weeks, 4-weeks). The n-HAP group was treated by immersing the samples in a 10% n-HAP solution for 3 minutes, after that, the samples stored in artificial saliva. This treatment regimen was conducted on a daily basis. The second group was subjected to the fluoride varnish treatment by painting it in a thin layer with a tiny brush on the window created on the samples buccal surface, and the third group left untreated.

Microhardness Testing

The microhardness of the samples was measured by Vickers device (Model: TH715, SN: 0003, Beijing Time High Technology Ltd, China) in the Department of Material Engineering at Technology University with a load of 500 gram for 30 seconds, Figure (2). The microhardness was measured at baseline, after pH cycle procedure and after the treatment of the teeth sample with study materials at different storage times (1 week, 2 weeks, 4 weeks). Three Vickers indentations were made per specimen, and the hardness value was calculated by averaging the three readings (Hashim and Mansoor, 2024). The sample under the indenter of Vickers shown in Figure (3).

Nanohydroxyapatite solution Ca10 (PO4)6 $(OH)_2$ Preparation:

This study used a 10% concentration of n-HAP suspension solution. After dissolving 100 grammes of nanohydroxyapatite powder in 2 mol of HCl, a litre of de-ionized water was added to prepare a 10% concentration of n-HAP suspension solution. NaOH was added to correct the pH of 7. The end product was a suspension solution that contained salts that had been removed from the solution with a separating funnel to gain a 10% n-HAP suspension solution (HUANG et al., 2009).

Statistical Analysis:

The data was analysed in several steps. A descriptive statistic that includes the means and standard deviation. A one-way ANOVA test was performed to compare the various tested groups after the treatment. Pairwise post hoc test was used when One-way ANOVA test was significant.

Results

Microhardness means values and standard deviation at the baseline and after demineralization for n-HAP; FL and C groups at different storage time are demonstrated in Table 1. A one-way ANOVA test after treatment demonstrated a significant difference in microhardness values according to different storage times only for the n-HAP group while there were no significant differences in microhardness mean values for FL and C groups as shown in Table 2. Pairwise post hoc test for n-HAP group show significant differences between the microhardness values of 2-weeks and 4-weeks storage time intervals as shown in Table 3.

Discussion

Initial enamel lesions, resulting from mineral demineralization on the enamel surface, represent the initial stage of tooth decay, which is restricted to enamel and does not extend to the deeper tissues. Early enamel lesions can be remineralized and reversed (Gürbüz, 2021). Indeed, the remineralization process of the early carious lesion with preventive agents will reduce or prevent cavity formation while preserving the structural integrity of the tooth. The microhardness serves as a criterion to determine the ability of different remineralizing agents to stop the demineralization and enhance lesions remineralisation (Kooshki et al., 2019). The microhardness in this study was conducted by the use of Vickers device, which yielded a positive outcome consistent with the findings of previous studies (Kashmoola and Qasim, 2021; Al Qaysi et al., 2023). The microhardness of teeth sample treated with n-HAP solution was increased. The results could be attributes to the increased of phosphate and calcium ions deposition from n-HAP into

porous enamel, thereby occluding the porosities in the enamel (Kidd and Fejerskov, 2004). Additionally, n-HAP demonstrates a significant morphological resemblance to the natural apatite crystals present in the dental structure, since hydroxyapatite crystals within the dental tissue are typically manifested as needle-like crystals form. Consequently, this property will facilitate the development of synthetic apatite on the surface of enamel (Wakwak et al., 2021). This finding agreed with those of Amaechi et al., 2021 who concluded that n-HAP may serve as a reservoir for phosphate and calcium ions, maintaining a sustained topical super saturation condition of these ions in relation to the tooth minerals over time. Furthermore, this result confirms those of Carvalho et al., 2014 who illustrated the efficacy of n-HAP in the rehardening of early carious lesion following an artificial cariogenic challenge . At 1-week and 4-weeks after the application of n-HAP there was increased in microhardness mean values and these results consistent with the findings of the research conducted by AL Again et al., 2018 who found that there was increased in microhardness for the 1-week and 4-weeks stored subgroups. Nanohydroxyapatite initially promotes minerals deposition, which increase microhardness. However, during the remineralization process, the newly formed mineral layers may still be immature or less crystalline. This could result in temporary decrease in the microhardness as the deposited minerals undergo restructuring or recrystallization and this why there was decrease in the microhardness in 2-weeks after the initial increased in the microhardness in the 1-week. After the use of sodium fluoride varnish, the microhardness means values increased. This may be related to the way in which the fluoride ions react with the enamel surface to form compounds that contain fluoride, primarily CaF_2 which act as fluoride ion reservoirs and prevent further dissolution of enamel during acidic attack. These results agreed with those of Chaudhary et al., 2013 and Esfahani et al., 2015. At the 1week and 2-weeks from the application of fluoride varnish there was increased in microhardness mean value and these results consistent with the findings of Oliveira et al., 2019. At 4-week there was decrease in the microhardness mean value and these results disagree with the findings of Kamal et al., 2018 who found that at 4-week there was increase in the microhardness mean value.

The capacity of artificial saliva to promote remineralization was noted, as specimens subjected to demineralization and

subsequently stored in artificial saliva exhibited elevated mean microhardness. However, this remineralizing effect, in comparison to the fluoride varnish and nanohydroxyapatite suspension was too small. These results agreed with those of Torres et al., 2012. Both fluoride varnish and nanohydroxyapatite enhanced the surface hardness of enamel, however, none of these agents achieves remineralization of the enamel to the extent of the sound enamel and nanohydroxyapatite exhibits greater capacity for increasing surface hardness compared to fluoride varnish, a finding that aligns with research conducted by Carvalho et al., 2014 and Aziznezhad et al., 2017.

Conclusion

According to analysis of the results, nanohydroxyapatite solution has higher remineralizing potential as compared to the fluoride varnish. Nanohydroxyapatite solution may serve as a viable alternative remineralizing agent and exhibits a considerable potential in the therapeutic management of incipient carious lesions.

Conflict of interest

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

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Figure (1): Creation of the window on the buccal surface of the tooth

Figure (2): Vickers microhardness tester

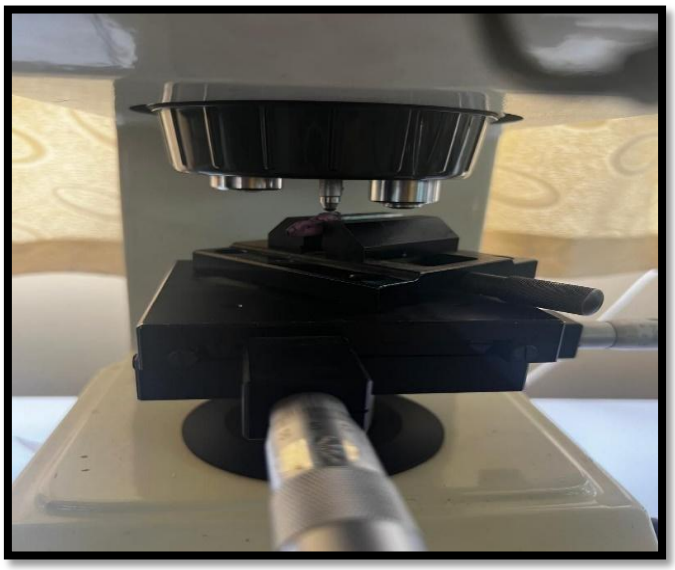

Figure (3): Sample under the indenter of Vickers device

Table 1: Microhardness (Mean and standard deviation) for the study materials at different storage time

Variables	Storage times	n-HAP (Mean and ±SD)	FL (Mean and ±SD)	C (Mean and ±SD)
Baseline	**1-week**	259 (19)	263 (39.9)	255 (63.7)
	2-weeks	267 (8.1)	257 (68.7)	278 (35.2)
	4-weeks	280 (29.6)	252 (29.9)	265 (18.7)
Demineralization	**1-week**	113 (39.7)	67 (21.6)	114 (45.8)
	2-weeks	68 (35.9)	141 (48.1)	110 (63.8)
	4-weeks	90 (27.5)	70 (40.5)	58 (13.9)

Footnotes:

Table 2: One-way ANOVA test for the study groups after treatment

	1-week	2-weeks	4-weeks	One-way ANOVA
Groups	**Mean (±SD)**	**Mean (±SD)**	**Mean (±SD)**	***P-value***
n-HAP	171 (23)	108 (45)	204 (68)	0.01
FL	140 (32)	186 (38)	129 (78)	0.1
C	129 (42)	128 (79)	76 (19)	0.055

Table 3: Pairwise post hoc test of different storage times for n-HAP group

	1-Week – 2-Weeks Mean difference (SE): *p-value*	1-Week – 4-Weeks Mean difference (SE): *p-value*	2-Week – 4-Weeks Mean difference (SE): *p-value*
n-HAP	64 (28) 0.09	- 32 (28) 0.5	-96 (28) 0.01