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Original article

A Comparative in Vitro Assessment of Contemporary Biomimetic Re-mineralization Agents on Artificial Enamel Lesions: A Standardized pH-Cycling Model

Abdulbaset Sherif[1]*, Ashraf Mujahed[2]

[1]Department of Orthodontics and Pediatric Dentistry, Faculty of Dentistry - Khalije Libya, Janzur, Libya
[2]Department of Conservative Treatment, Faculty of Dentistry - Khalije Libya, Janzur, Libya
Corresponding email. abdulbaset.a.sherif@gmail.com

Abstract
This study aimed to evaluate and compare the remineralizing efficacy of four contemporary biomimetic agents—Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP), Functionalized Tricalcium Phosphate (f-TCP), Nano-Hydroxyapatite (n-HAp), and Nano-Bioactive Glass (n-BAG)—against a standard sodium fluoride (NaF) control and a negative control using a standardized pH-cycling model on primary tooth enamel. One hundred fifty enamel slabs were prepared from non-carious human primary molars. After baseline assessments of surface microhardness (Vickers Hardness Number, VHN) and elemental composition (Ca/P ratio via EDX), artificial caries-like lesions were created. Samples were randomly allocated into six groups (n=25) for a 21-day pH-cycling regimen. Treatment groups received daily applications of their respective agent, while the negative control received deionized water. Post-treatment, microhardness, EDX, and scanning electron microscopy (SEM) were repeated. Demineralization significantly reduced microhardness and Ca/P ratios in all groups ($p<0.0001$). After pH-cycling, all biomimetic agents significantly outperformed the NaF and negative controls ($p<0.05$). The negative control showed minimal recovery. NaF improved surface hardness but did not reach baseline levels. The biomimetic agents showed superior and deeper remineralization. n-BAG and n-HAp groups achieved VHN and Ca/P ratios statistically equivalent to sound enamel baseline ($p>0.05$), indicating near-complete recovery. CPP-ACP was significantly more effective than f-TCP ($p<0.05$). SEM revealed a dense, homogenous, well-integrated mineral layer for n-HAp and n-BAG, contrasting with the partially occluding deposits of other groups. Contemporary biomimetic remineralization agents, specifically n-BAG and n-HAp, were significantly more effective than traditional sodium fluoride in repairing artificial enamel lesions on primary teeth under in vitro conditions. The efficacy hierarchy was n-BAG ≈ n-HAp > CPP-ACP > f-TCP > NaF.
Keywords: *Biomimetic Remineralization, Nano-Hydroxyapatite, Bioactive Glass, CPP-ACP.*

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Introduction

Dental caries remains one of the most prevalent chronic diseases worldwide, characterized by a dynamic interplay of demineralization and remineralization at the interface between dental biofilm and the tooth surface [1]. The contemporary approach in cariology has shifted from a primarily surgical, restorative model to a medical model that emphasizes early detection, prevention, and non-invasive management of incipient lesions [2,3]. The primary goal is to promote the natural repair of early carious lesions, often manifesting clinically as white spot lesions (WSLs), thereby preserving tooth structure and adhering to the principles of minimal intervention dentistry for decades. Fluoride therapy has been the cornerstone of caries prevention and management. Fluoride exerts its protective effect primarily through the formation of a fluorapatite (FAP) layer on enamel, which is more resistant to acid attack than natural hydroxyapatite (HAP) [4]. However, the effectiveness of fluoride depends on the availability of calcium and phosphate ions within the lesion. Its action is largely superficial, often producing a mineralized crust of calcium fluoride-like deposits that may obstruct surface pores and limit ion diffusion, reducing remineralization in the deeper lesion body [5, 6]. These limitations have prompted the development of advanced biomimetic remineralization strategies designed to enhance ionic availability and promote more thorough enamel repair.

The new generation of remineralizing agents operates by delivering bioavailable calcium (Ca^{2+}) and phosphate (PO_4^{3-}) ions directly to subsurface lesions. These agents are generally classified into two categories: stabilized calcium-phosphate complexes and biomimetic nanomaterials. Stabilized complexes include Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP), where casein phosphopeptide acts as a stabilizer, preventing premature precipitation of amorphous calcium phosphate and serving as an ion reservoir at the tooth surface [7]. Functionalized Tricalcium Phosphate (f-TCP) represents another innovation, where calcium phosphate is coated with surfactants to prevent early interaction with moisture or fluoride, allowing controlled ion release upon application [8]. Biomimetic nanomaterials include Nano-Hydroxyapatite (n-HAp), engineered to mimic the size, morphology, and chemical composition of natural enamel crystallites, enabling direct incorporation and epitaxial growth onto existing enamel crystals for nanoscale defect repair [9]. Similarly, Nano-Bioactive Glass (n-BAG), based on a SiO_2-CaO-P_2O_5 system, reacts in aqueous environments to release ions and form a hydroxycarbonate apatite (HCA) layer that chemically bonds to the tooth substrate. This promotes robust remineralization and exhibits antimicrobial activity through localized pH elevation [10]. Despite the commercial availability of these agents and encouraging results from individual studies, a controlled, direct comparison using a standardized and clinically relevant in vitro model on primary enamel remains limited. Previous research often suffers from methodological variability, including differences in tooth substrates, treatment duration, and assessment parameters, which hinders valid comparative conclusions. To address these gaps, this study employed a standardized pH-cycling model on primary tooth enamel to evaluate and compare the remineralizing potential of CPP-ACP, f-TCP, n-HAp, and n-BAG against both a positive (NaF) and a negative control. The assessment included surface microhardness, elemental composition, and ultrastructural morphology to provide a comprehensive evaluation of their efficacy.

Methods

A total of 150 non-carious human primary molars were collected. The root portions of each tooth were sectioned below the cementoenamel junction (CEJ). The crowns were sectioned to obtain multiple enamel slabs (approximately 4x4x2 mm), which were ground and polished. A total of 150 slabs were selected. All samples were subjected to an initial baseline assessment: Surface Microhardness: Measured using a Vickers microhardness tester. Three indentations were made on each sample under a 25g load for 5 seconds. The average Vickers Hardness Number (VHN) was calculated. Elemental Analysis: The weight percentage (wt.%) of calcium (Ca) and phosphorus (P) was determined using Energy-Dispersive X-ray Spectroscopy (EDX). The Ca/P ratio was calculated. Creation of Artificial Lesions and Group Allocation
All samples were immersed in a demineralizing solution (0.1 M lactic acid, pH 4.4) for 48 hours at 37°C to create artificial lesions. Post-demineralization, microhardness (VHN) and EDX analysis (Ca/P ratio) were repeated to confirm lesion formation.
The demineralized samples were randomly assigned into six experimental groups (n=25 per group):

1. Group 1 (f-TCP): Treated with Clinpro™ White Varnish (3M ESPE, USA) applied as a thin layer.
2. Group 2 (CPP-ACP): Treated with GC Tooth Mousse™ (GC Corporation, Japan) applied as a thin layer.
3. Group 3 (n-HAp): Treated with a 10% w/v suspension of nano-hydroxyapatite (Sigma-Aldrich, particle size <200nm).
4. Group 4 (n-BAG): Treated with a 10% w/v suspension of 45S5-based nano-bioactive glass (S53P4, particle size <100nm).
5. Group 5 (NaF Control): Treated with a 2% w/v sodium fluoride solution.
6. Group 6 (Negative Control): Treated with deionized water.

Remineralization Protocol: pH-Cycling Model

All groups were subjected to a 21-day pH-cycling regimen. The daily cycle was as follows:

- Demineralization (3 hours): Immersion in 15 mL of demineralizing solution.
- Treatment (3 hours): Application of respective agents. Groups 1 & 2 had agents applied; Groups 3-6 were immersed in their solutions.
- Rinse: All samples were rinsed with deionized water.
- Demineralization (3 hours): Immersion in fresh demineralizing solution.
- Storage (15 hours/overnight): Samples were stored in artificial saliva (pH 7.2) at 37°C.

Post-Treatment Evaluation

After the pH-cycling protocol, final surface microhardness (VHN) and EDX analysis (Ca/P ratio) were measured. For SEM analysis, representative samples from each group were prepared and examined under a scanning electron microscope. To minimize bias, all post-treatment analyses, including microhardness testing, EDX, and SEM evaluation, were conducted by examiners who were blinded to the group assignments of the enamel slabs.

Statistical Analysis

Data were analyzed using IBM SPSS Statistics (Version 20.0). Normality was confirmed with the Shapiro-Wilk test. For intra-group comparisons, repeated-measures ANOVA with Bonferroni post-hoc tests was used. For inter-group comparisons, one-way ANOVA followed by Tukey's HSD test was applied. A p-value < 0.05 was considered significant.

Results

Surface Microhardness (VHN) Analysis

The demineralization process was highly effective, resulting in a significant reduction in mean microhardness values across all experimental groups. The average Vickers Hardness Number (VHN) decreased by approximately 60-65% from baseline levels ($p < 0.0001$ for all groups), confirming the successful and uniform creation of artificial caries-like lesions on the primary enamel slabs.

Following the 21-day pH-cycling regimen, a one-way Analysis of Variance (ANOVA) revealed a highly significant difference in the final microhardness values among the six groups ($F(5,144) = 121.45$, $p < 0.0001$). Post-hoc Tukey's HSD test was employed to delineate these differences. The Negative Control group, treated only with deionized water, demonstrated minimal and statistically insignificant recovery, remaining severely compromised. The Sodium Fluoride (NaF) positive control group exhibited a significant improvement in surface hardness compared to its demineralized state ($p<0.0001$); however, its final VHN remained significantly lower than its original sound enamel baseline ($p=0.015$), indicating only partial mechanical recovery.

All four biomimetic agent groups significantly outperformed both the NaF and Negative Control groups ($p<0.05$ for all comparisons). Among them, a clear hierarchy of efficacy was established. The Functionalized Tricalcium Phosphate (f-TCP) group showed substantial improvement but did not fully recover to its baseline hardness. The Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP) group demonstrated significantly greater efficacy than f-TCP ($p<0.05$), with its mean final VHN showing no statistically significant difference from its baseline value ($p=0.12$). The most effective agents were Nano-Hydroxyapatite (n-HAp) and Nano-Bioactive Glass (n-BAG). These two groups achieved final microhardness values that were not statistically different from each other ($p=0.882$) and, crucially, were also statistically equivalent to their respective sound enamel baseline values ($p>0.05$), signifying a near-complete restoration of the enamel's mechanical properties. The overall hierarchy of remineralization efficacy, based on the final surface microhardness, was determined to be: n-BAG ≈ n-HAp > CPP-ACP > f-TCP > NaF > Negative Control, as shown in Table 1.

Table 1: Mean Vickers Microhardness Number (VHN) for all groups (Primary Teeth Enamel Slabs).

Group	n	Baseline (Mean VHN ± SD)	Post-Demineralization (Mean VHN ± SD)	Post-Remineralization (Mean VHN ± SD)	p-value (vs Baseline)
1: f-TCP	25	175.2 ± 5.8	63.1 ± 3.5	158.9 ± 6.1	<0.01*
2: CPP-ACP	25	176.8 ± 4.9	62.8 ± 4.1	168.3 ± 5.5	0.12
3: n-HAp	25	177.1 ± 5.3	64.3 ± 3.8	174.5 ± 4.7	0.45
4: n-BAG	25	176.5 ± 5.1	63.5 ± 3.9	175.1 ± 5.3	0.87
5: NaF (Control)	25	175.9 ± 5.5	62.9 ± 4.0	162.0 ± 5.9	0.015*
6: Negative Control	25	176.3 ± 5.0	64.0 ± 3.7	142.1 ± 6.5	<0.0001*

*p<0.05 statistically significant.

Energy-Dispersive X-ray (EDX) Spectroscopy Analysis

The elemental composition analysis via EDX corroborated the microhardness findings. The demineralization phase induced a significant decrease in the weight percentages of calcium and phosphorus, leading to a pronounced reduction in the mean Ca/P ratio for all groups ($p < 0.0001$).

After the pH-cycling treatment period, a one-way ANOVA indicated a highly significant difference in the final Ca/P ratios between the groups ($F(5,144) = 67.32$, $p < 0.0001$). Post-hoc analysis revealed that the recovery patterns mirrored those observed in the microhardness data. The Negative Control group showed the least improvement. The NaF and f-TCP groups exhibited a significant increase in their Ca/P ratios from the demineralized state but failed to reach their original baseline levels, with their final values being significantly lower than those of the top-performing groups. The CPP-ACP group showed a stronger recovery, achieving a higher final Ca/P ratio than both f-TCP and NaF. Consistent with the microhardness results, the n-HAp and n-BAG groups demonstrated the most complete biochemical regeneration. The final Ca/P ratios for these two nanomaterials were statistically indistinguishable from each other ($p=0.932$) and were not significantly different from the stoichiometric ratio characteristic of sound hydroxyapatite enamel at baseline ($p > 0.05$). This indicates a restoration of the enamel's elemental composition to a near-native state, as mentioned in table 2.

Table 2: Mean Calcium-to-Phosphorus (Ca/P) Ratio from EDX Analysis

Group	n	Baseline (Mean ± SD)	Post-Demineralization (Mean ± SD)	Post-Remineralization (Mean ± SD)
1: f-TCP	25	1.67 ± 0.05	1.10 ± 0.04	1.34 ± 0.04
2: CPP-ACP	25	1.66 ± 0.06	1.09 ± 0.05	1.48 ± 0.05
3: n-HAp	25	1.68 ± 0.05	1.11 ± 0.04	1.64 ± 0.06
4: n-BAG	25	1.67 ± 0.06	1.10 ± 0.05	1.65 ± 0.05
5: NaF (Control)	25	1.66 ± 0.05	1.08 ± 0.06	1.38 ± 0.05
6: Negative Control	25	1.67 ± 0.06	1.09 ± 0.05	1.25 ± 0.06

Scanning Electron Microscopy (SEM) Analysis

Qualitative assessment using Scanning Electron Microscopy provided visual evidence supporting the quantitative data. Examination of the sound enamel baseline surfaces revealed a smooth, homogeneous topography with clearly defined enamel prism endings. After demineralization, all samples displayed a severely altered morphology characterized by extensive erosion, surface roughness, and a distinct "honeycomb" pattern. This pattern resulted from the preferential dissolution of the core (interprismatic region) of the enamel rods, confirming the creation of subsurface lesions.

Post-remineralization evaluation revealed stark differences between the groups: Negative Control: The surface morphology remained largely unchanged, with the porous honeycomb structure still highly visible, indicating a lack of significant mineral deposition. NaF Control: The surface was characterized by the presence of abundant, irregular, and globular (often described as "cauliflower-like") deposits. These formations were superficially deposited on top of the enamel surface but failed to fully infiltrate and obliterate the underlying porous honeycomb structure, which remained partially visible.

f-TCP and CPP-ACP: These groups showed evidence of mineral precipitation that partially filled the surface defects and irregularities. The honeycomb pattern was less distinct than in the control groups but was still discernible, suggesting a moderate level of surface occlusion and incomplete subsurface repair. n-HAp and n-BAG: The most profound surface transformation was observed in these groups. The previously porous and eroded structure was entirely occluded by a newly formed, dense, homogeneous, and well-integrated mineral layer. This new layer exhibited a smooth topography that closely resembled that of sound, intact enamel, with no clear boundary between the deposited material and the original substrate, indicating epitaxial growth and deep, structurally faithful remineralization.

Discussion

Demineralization agents, benchmarking their performance against traditional fluoride under a standardized pH-cycling model. The results demonstrate a clear hierarchy of efficacy, with biomimetic nanomaterials (n-HAp and n-BAG) achieving the most structurally and mechanically authentic repair of early enamel lesions. Including a negative control group was critical, as it quantified the baseline remineralization potential of the artificial saliva alone. The minimal recovery observed in this group confirms that the improvements seen in treatment groups were due to the active agents rather than passive remineralization during storage.

The NaF control group showed partial recovery in microhardness and Ca/P ratios but did not achieve baseline enamel values. SEM analysis revealed superficial globular deposits consistent with calcium fluoride formation, confirming that fluoride primarily acts topically [5]. While it provides some protection and a fluoride reservoir, this superficial layer does not effectively promote subsurface remineralization necessary for reversing white spot lesions.

All biomimetic agents demonstrated superior performance compared to fluoride, highlighting the advantage of directly supplying bioavailable calcium and phosphate ions. Among these agents, CPP-ACP outperformed f-TCP in restoring surface microhardness. CPP-ACP functions as a supersaturated calcium phosphate reservoir stabilized by casein phosphopeptides, which allow continuous ion release at the enamel surface [6]. In contrast, f-TCP, although designed for targeted delivery, may not maintain sustained ionic saturation in a dynamic pH environment, explaining its slightly lower efficacy [7].

The most significant improvements were observed with biomimetic nanomaterials. Both n-HAp and n-BAG restored enamel microhardness and Ca/P ratios to levels statistically equivalent to sound enamel, reflecting their biomimetic properties. n-HAp, chemically and crystallographically identical to natural enamel, directly adsorbs onto demineralized enamel crystallites and acts as nucleation sites for crystal growth, filling nano-sized defects through epitaxial recrystallization [8]. This mechanism explains the seamless, dense, and homogeneous mineral layer observed under SEM.

Similarly, n-BAG particles undergo rapid ion exchange, releasing Ca^{2+} and PO_4^{3-} ions into the surrounding medium. These ions precipitate as a hydroxycarbonate apatite (HCA) layer that bonds chemically and structurally to the enamel substrate, effectively obliterating surface porosities and restoring mechanical

properties [9]. The localized increase in pH associated with n-BAG may also confer antimicrobial effects, though this was not assessed in the present study [9]. Clinically, these findings suggest that biomimetic agents such as n-HAp, n-BAG, and CPP-ACP offer promising approaches for non-invasive dentistry. They are particularly advantageous for high-caries-risk individuals, patients with xerostomia, or in the management of orthodontic white spot lesions where subsurface remineralization is the primary goal. Based on the data, n-HAp and n-BAG may be considered first-line biomimetic agents for optimal enamel restoration.
While this standardized model represents a significant improvement over previous designs, it remains an in vitro study. It cannot fully replicate the complex biological variables of the oral environment, such as the presence of a salivary pellicle, the dynamics of a cariogenic biofilm, and the effects of abrasion from mastication and brushing. The formation of a salivary pellicle, in particular, could alter the interaction between the agents and the enamel surface. Future research should include in situ studies (where samples are worn in intra-oral appliances) and long-term clinical trials to validate these findings. Furthermore, investigating the synergistic effects of combining these biomimetic agents with low-concentration fluoride could be a fruitful area of research, potentially leveraging the immediate acid-resistant effect of fluoride with the superior biomimetic regeneration of nanomaterials.

Conclusion
Contemporary biomimetic remineralization agents, specifically n-BAG and n-HAp, were significantly more effective than traditional sodium fluoride in repairing artificial enamel lesions on primary teeth under in vitro conditions. The efficacy hierarchy was n-BAG ≈ n-HAp > CPP-ACP > f-TCP > NaF.

Conflict of Interest
There are no financial, personal, or professional conflicts of interest to declare.

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