

Original Research

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Comparative Evaluation of Surface Microhardness and Antibacterial Efficacy of Phosphorylated Chitosan, Silver Diamine Fluoride, and Sodium Fluoride Varnish: An In-Vitro Study

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ABSTRACT:

Background: Dental caries involves enamel mineral loss and cariogenic bacterial activity. Materials with both remineralizing and antibacterial properties may improve preventive care.

Aim: To compare phosphorylated chitosan (PCh), silver diamine fluoride (SDF), and sodium fluoride (NaF) varnish for enamel surface microhardness and antibacterial activity against Streptococcus mutans.

Materials and Methods: Forty sound extracted permanent teeth were divided equally into PCh, SDF, NaF varnish, and distilled-water groups. Vickers microhardness was measured at baseline, after demineralization, and after treatment. Antibacterial activity was assessed using agar-well diffusion with 10 observations per group. Data were analyzed using SPSS version 23.0. One-way ANOVA was used for intergroup comparisons, followed by post hoc analysis. Statistical significance was set at $p < 0.05$.

Results: Microhardness increased significantly with PCh, SDF, and NaF varnish ($p = 0.001$ each), but not with distilled water ($p = 0.923$). Percentage increases were 4.26%, 5.66%, and 6.05%, respectively, without a significant overall intergroup difference ($p = 0.552$). SDF showed the highest inhibition zone (15.95 ± 1.59 mm), followed by PCh (14.45 ± 1.91 mm) and NaF varnish (8.97 ± 4.38 mm). The antibacterial difference was

significant ($p < 0.001$).

Conclusion: PCh showed promising combined remineralizing and antibacterial properties, warranting further clinical investigation.

INTRODUCTION

Dental caries remains a major public health problem worldwide, affecting people of all ages and adversely influencing oral health, daily activities, and quality of life.¹ It is a dynamic process involving repeated cycles of demineralization and remineralization. When mineral loss exceeds mineral gain, progressive destruction of dental hard tissues occurs, affecting both primary and permanent teeth.² Early management therefore aims to preserve tooth structure, promote mineral recovery, and control the microbial factors responsible for disease progression.

Fluoride has long been used for caries prevention and management because it promotes remineralization, reduces enamel solubility, and limits further mineral loss. Topical fluoride preparations, including gels and varnishes, are widely used because they are inexpensive, easy to apply, and effective in managing early carious lesions.³ Sodium fluoride (NaF) varnish, commonly available at a concentration of 5%, provides prolonged contact between fluoride and the tooth surface. However, repeated applications may be required depending on the patient's caries risk.⁴

Silver diamine fluoride (SDF) is another established agent used to arrest carious lesions. Its effectiveness is attributed to the combined remineralizing action of fluoride and antimicrobial activity of silver.⁵ Alternative silver-based formulations, such as nano silver fluoride, have been investigated to overcome some limitations of conventional SDF.⁶ SDF can reduce *Streptococcus mutans* levels, although bacterial recolonization may occur after treatment. Despite its effectiveness, dark staining of carious tooth structure remains a major disadvantage, particularly in children and aesthetically sensitive areas.^{7,8}

Chitosan is a naturally derived polysaccharide obtained through the deacetylation of chitin, which is present in crustacean shells and fungal cell walls. Its biocompatibility, biodegradability, and antimicrobial properties have encouraged its use in biomedical and dental applications.⁹ Phosphorylated chitosan is a modified derivative containing phosphate groups that can interact with calcium ions. These interactions may facilitate mineral nucleation and support biomimetic remineralization of demineralized enamel.¹⁰ Its potential to combine mineral recovery with antimicrobial activity makes it a promising material for preventive dentistry.

Surface microhardness is an important indicator of enamel's mechanical integrity and mineral status. Demineralization reduces enamel hardness, while remineralization may improve its resistance to indentation. Microhardness testing therefore provides a useful method for assessing treatment-related changes in enamel.¹¹ *S. mutans* is also an important contributor to cariogenic biofilm formation and acid production.¹² Evaluating antibacterial activity alongside microhardness may therefore provide a more complete understanding of the potential benefits of caries-preventive agents.¹³

Although fluoride-based agents are widely used, interest in natural biomaterials has increased because of the need for effective alternatives with complementary antimicrobial properties. Direct comparison of phosphorylated chitosan with established fluoride-based agents may help clarify its potential role in caries management. Therefore, this in vitro study aimed to evaluate and compare the surface microhardness and antibacterial efficacy of phosphorylated chitosan, silver diamine fluoride, and sodium fluoride varnish using Vickers microhardness testing and agar-based microbial assessment.

MATERIALS AND METHODS Study design and setting

This in vitro experimental study was conducted at the Department of Pediatric and Preventive Dentistry,

D.J. College of Dental Sciences & Research, and D.J. College of Pharmacy, Modinagar, Ghaziabad, in collaboration with Labxperts, Ghaziabad, and SD Testing & Calibration Laboratory, Indirapuram, Ghaziabad, Uttar Pradesh, India.

Sample size and grouping

Sample size was calculated using G*Power version 3.1.9.6, with an effect size of 0.521, 90% power, and a 5% significance level. The minimum sample size was 10 per group. Forty teeth were allocated equally to phosphorylated chitosan (PCh), silver diamine fluoride (SDF), sodium fluoride (NaF) varnish, and distilled water groups. For antibacterial assessment, 40 wells were

prepared in 10 agar plates, with one well for each material and normal saline as the control.

Enamel specimen preparation

Forty freshly extracted, sound permanent anterior and premolar teeth were obtained from the oral surgery department. Teeth with caries, fractured crowns, or enamel hypoplasia were excluded. Following ultrasonic debridement, the crowns were separated from the roots using a diamond disc. Enamel slabs were prepared from the buccal surfaces under continuous water irrigation and embedded in acrylic resin blocks.

Baseline surface microhardness was measured using a Micro-Vickers hardness tester under a 25 g-force load (approximately 0.245 N) for 5 seconds. Five evenly spaced indentations were made on each specimen, and the mean Vickers hardness number (VHN) was recorded. The surfaces were then polished sequentially with 400-, 800-, and 1000-grit emery papers. A 4 × 4 mm enamel window was exposed using adhesive tape, while the remaining surface was coated with acid-resistant nail varnish.

Demineralization and treatment

The demineralizing solution was prepared using 2.205 g calcium chloride and 2.041 g potassium dihydrogen phosphate in 5 L distilled water. Specimens were immersed for 3 hours, removed, and gently blot-dried. Post-demineralization microhardness was measured using the same testing parameters and five-indentation averaging method.

PCh gel was prepared by mixing 1 g chitosan powder, 5 g urea, and 10 mL phosphoric acid in 40 mL dimethylformamide. The mixture was heated at 120°C with continuous stirring for 1 hour. After cooling, it was filtered, washed with distilled water and ethanol, dried, and incorporated into a Carbopol-940 gel base to obtain a final concentration of 0.5%. The gel was stored at 2–8°C until use. Commercial e-Kids SDF solution and Copal-F NaF varnish were used for the other treatment groups (Figure 1).

Each assigned material was applied generously with a microbrush to cover the enamel window. After 6 hours, specimens were rinsed with distilled water and dried. This cycle was repeated seven times over 42 hours. Final surface microhardness was measured using the same parameters, and percentage surface microhardness change was calculated to assess remineralization.

Antibacterial assessment

A standard strain of *Streptococcus mutans* (MTCC 497) was obtained from Labxperts, Ghaziabad, in freeze-dried form and stored at 2–8°C. The culture was reactivated, streaked onto nutrient agar, and incubated at 37°C for 24 hours. Colony morphology and Gram staining were used for preliminary microbial confirmation. A suspension equivalent to 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL) was prepared from a 24-hour culture in nutrient broth.

The suspension was spread uniformly over agar plates using sterile cotton swabs. Four wells, each 6 mm in diameter and 4 mm deep, were prepared on every plate. The wells received 150 µL SDF, 150 µL NaF varnish, 0.15 g PCh gel, or 150 µL normal saline. Ten plates were prepared using the same procedure.

The plates were left at room temperature for 2 hours to facilitate diffusion and incubated at 37°C for 48 hours. Inhibition zones were examined at 24 and 48 hours. Zone diameters were measured at three points using a stainless-steel ruler and vernier caliper. The mean diameter was calculated, and the 6 mm well diameter was subtracted to obtain the final inhibition-zone value (Figure 2).

Statistical analysis and ethics

Data were entered into Microsoft Excel 2007 and analyzed using SPSS version 23.0. Descriptive statistics were expressed as means and standard deviations. Intergroup comparisons were performed using one-way ANOVA, followed by post hoc analysis. Statistical significance was set at $p < 0.05$. Ethical approval was obtained from the institutional ethics committee (DJC/IEC/12/2023).



Figure 1. Specimen preparation and surface microhardness evaluation. (A) Extracted permanent anterior and premolar teeth. (B) Sectioning of teeth using a diamond disc. (C) Preparation of enamel slabs from the buccal surfaces. (D) Enamel specimens embedded in acrylic resin. (E) Preparation of the standardized enamel window. (F) Application of the test agent to the enamel surface. (G) Demineralizing solution. (H) Prepared phosphorylated chitosan (PCh) gel. (I) Sodium fluoride (NaF) varnish and silver diamine fluoride (SDF) used in the study. (J) Application of PCh gel to the enamel specimen.

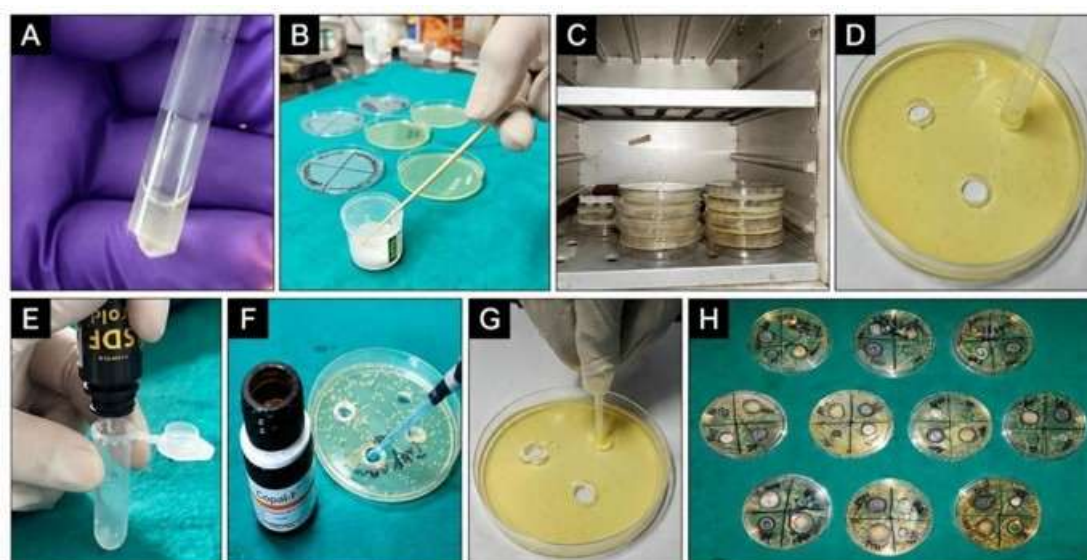


Figure 2. Agar-well diffusion assay for antibacterial activity against *Streptococcus mutans*. (A) Freeze-dried bacterial strain of *S. mutans*. (B) Preparation of bacterial culture on nutrient agar plates.

(C) Incubation of inoculated agar plates. (D) Preparation of wells in the agar medium. (E) Transfer of silver diamine fluoride (SDF) into a sterile microtube. (F) Application of sodium fluoride (NaF) varnish into the agar wells. (G) Application of phosphorylated chitosan (PCh) gel into the agar wells. (H) Representative agar plates showing zones of inhibition around the test agents after 48 hours of incubation at 37°C.

RESULTS

A total of 40 enamel specimens were evaluated for surface microhardness, with 10 specimens in each group. Antibacterial activity was assessed using 40 wells distributed across 10 agar plates, providing 10 observations per treatment.

Baseline surface microhardness ranged from 330.75 ± 17.85 VHN in the phosphorylated chitosan group to 356.31 ± 11.39 VHN in the sodium fluoride varnish group. Following demineralization, microhardness decreased in all four groups. After remineralization, the mean values increased from

298.71 ± 25.71 to 315.26 ± 25.13 VHN with phosphorylated chitosan, from 295.96 ± 38.55 to 312.07

± 30.17 VHN with silver diamine fluoride, and from 297.07 ± 13.98 to 315.11 ± 17.45 VHN with sodium fluoride varnish. These within-group improvements were statistically significant ($p = 0.001$ for each). The distilled-water group showed only a small, nonsignificant increase ($p = 0.923$) (TABLE 1).

The highest percentage change was observed with sodium fluoride varnish ($6.05 \pm 6.21\%$), followed by silver diamine fluoride ($5.66 \pm 12.77\%$) and phosphorylated chitosan ($4.26 \pm 11.23\%$). Distilled water showed the lowest change ($0.40 \pm 7.13\%$). However, the overall intergroup difference was not statistically significant ($F = 0.710$, $p = 0.552$) (TABLE 2).

Silver diamine fluoride produced the largest mean zone of inhibition against *Streptococcus mutans* (15.95 ± 1.59 mm), followed by phosphorylated chitosan (14.45 ± 1.91 mm) and sodium fluoride varnish (8.97 ± 4.38 mm). Normal saline showed no inhibition. The overall difference among groups was statistically significant ($F = 82.268$, $p < 0.001$) (TABLE 3).

Post hoc analysis showed no significant difference between phosphorylated chitosan and silver diamine fluoride ($p = 0.191$). Both agents demonstrated significantly greater inhibition than sodium fluoride varnish and saline, while sodium fluoride varnish also showed significantly greater inhibition than saline ($p < 0.001$ for all comparisons).

Table 1. Surface microhardness before demineralization, after demineralization, and after remineralization

Treatment group	Baseline (T1), VHN	After demineralization (T2), VHN	After remineralization (T3), VHN	p-value
Phosphorylated chitosan	330.75 ± 17.85	298.71 ± 25.71	315.26 ± 25.13	0.001
Silver diamine fluoride	333.17 ± 27.27	295.96 ± 38.55	312.07 ± 30.17	0.001
Sodium fluoride varnish	356.31 ± 11.39	297.07 ± 13.98	315.11 ± 17.45	0.001
Distilled water	345.41 ± 5.09	330.71 ± 17.97	331.86 ± 23.18	0.923
<i>Values are mean $\pm$ SD; n = 10 per group. p-value represents the within-group comparison between T2 and T3. VHN: Vickers hardness number.</i>				

Table 2. Intergroup comparison of percentage change in surface microhardness

Treatment group	Percentage change, mean $\pm$ SD	F-value	p-value
Phosphorylated chitosan	4.26 $\pm$ 11.23 ^a	0.710	0.552
Silver diamine fluoride	5.66 $\pm$ 12.77 ^a		
Sodium fluoride varnish	6.05 $\pm$ 6.21 ^a		
Distilled water	0.40 $\pm$ 7.13 ^b		
<i>Values are mean $\pm$ SD; n = 10 per group. One-way ANOVA was followed by post hoc analysis. Different superscript letters indicate significant differences between groups (p < 0.05); groups sharing the same letter are not significantly different.</i>			

Table 3. Intergroup comparison of antibacterial activity against *Streptococcus mutans*

Treatment group	n	Zone of inhibition (mm), mean $\pm$ SD	95% CI	F-value	p-value
Phosphorylated chitosan	10	14.45 ± 1.91^a	13.09–15.81	82.268	<0.001
Silver diamine fluoride	10	15.95 ± 1.59^a	14.81–17.09		

Sodium fluoride varnish	10	8.97 ± 4.38 ^b	5.84–12.10
Normal saline	10	0.00 ± 0.00 ^c	0.00–0.00

Values are mean ± SD. One-way ANOVA was followed by post hoc pairwise comparisons. Different superscript letters indicate statistically significant differences between groups ($p < 0.05$); groups sharing the same letter are not significantly different. CI: confidence interval.

DISCUSSION

The present study evaluated the remineralizing and antibacterial effects of phosphorylated chitosan (PCh), silver diamine fluoride (SDF), and sodium fluoride (NaF) varnish. Demineralization reduced enamel surface microhardness in all groups. Following treatment, significant within-group improvements were observed with PCh, SDF, and NaF varnish, whereas distilled water produced only a small, nonsignificant change. NaF showed the highest numerical percentage increase, followed by SDF and PCh. However, the overall intergroup difference was not statistically significant ($p = 0.552$). Therefore, the findings demonstrate improvement with all three active agents but do not establish the superiority of any one material for microhardness recovery.

These findings are consistent with **Mohamed and Ashraf (2023)**³, who directly compared PCh, SDF, and NaF varnish and reported favourable remineralizing effects with all three materials. **Noaman et al. (2020)**¹⁴ also found that chitosan promoted remineralization of early enamel lesions over time. Their study used DIAGNOdent rather than surface microhardness, so the findings provide supporting evidence rather than a direct quantitative comparison.

The potential remineralizing action of PCh may be related to its interaction with mineral ions and demineralized tooth surfaces. **Niu et al. (2021)**¹⁵ reported that phosphorylated chitosan/amorphous calcium phosphate complexes enhanced calcium release and promoted apatite formation in demineralized dentin. Similarly, **Wahied et al. (2023)**¹⁶ observed greater Vickers hardness improvement when chitosan nanoparticles were combined with calcium-phosphate materials. These studies support the mineral-promoting potential of chitosan derivatives, although their formulations and substrates differ from the PCh gel used in the present study.

SDF and NaF varnish also produced significant microhardness recovery. **Lee et al. (2021)**¹⁷ reported improved microhardness in SDF-treated artificial carious lesions, while **Kaur et al. (2023)**¹⁸ found favourable preservation of enamel microhardness with SDF and fluoride varnish in primary and permanent teeth. **El-Desouky et al. (2022)**¹² demonstrated similar preventive effects of nano silver fluoride and NaF varnish against enamel demineralization. **Deulkar et al. (2023)**⁴ likewise reported remineralization with NaF and nano silver fluoride varnishes. Differences in fluoride formulation, tooth substrate, exposure duration, and experimental conditions may account for variations in the magnitude of improvement reported across studies.

The antibacterial results showed a more distinct pattern. SDF produced the largest mean inhibition zone against *Streptococcus mutans* (15.95 ± 1.59 mm), followed by PCh (14.45 ± 1.91 mm) and NaF varnish (8.97 ± 4.38 mm). Saline showed no inhibition. SDF and PCh did not differ significantly from each other ($p = 0.191$), whereas both produced significantly greater inhibition than NaF varnish and saline. These findings suggest that PCh and SDF had stronger antibacterial activity than conventional NaF varnish under the present assay conditions.

The findings agree with **Rodrigues et al. (2022)**¹, who reported reduced biofilm metabolic activity following SDF application in an in vitro caries model. **Sharma et al. (2023)**⁷ also demonstrated significant reductions in viable *S. mutans* with silver-based agents in a monospecies biofilm model. More directly, **Alessa et al. (2024)**¹⁹ used agar-well diffusion and reported larger inhibition zones with SDF than with fluoride varnish, while the control showed no antibacterial activity. **Hassan et al. (2024)**²⁰ similarly found greater bacterial reduction with SDF than with NaF varnish. **Ismail et al. (2025)**²¹ reported the strongest antibacterial effect with SDF and the least activity with NaF among the tested varnishes. Together, these studies support the antibacterial advantage of SDF observed in the present investigation.

PCh also demonstrated marked antibacterial activity, with no statistically significant difference from SDF. **Arora et al. (2023)**²² highlighted the antimicrobial properties and versatility of chitosan in dental biomaterials. **Piati GC et al. (2024)**²³ reported antimicrobial and antibiofilm activity against *S. mutans* when phosphorylated chitosan was incorporated into a modified glass ionomer system. Although these findings support the potential of PCh-containing materials, they cannot be directly equated

with the activity of the gel evaluated in the present study.

The findings should be interpreted within the limitations of the experimental design. Surface microhardness provides an indirect measure of mineral recovery and does not establish complete remineralization or restoration of the original enamel structure. Similarly, agar-well diffusion reflects growth inhibition under specific laboratory conditions and is influenced by material diffusion, concentration, and formulation. It does not directly measure biofilm eradication or clinical antibacterial effectiveness. The small sample size and short observation period further limit generalizability. Future studies using standardized biofilm models, quantitative mineral analysis, longer observation periods, and clinical evaluation are required to establish the effectiveness and practical role of PCh in caries management.

CONCLUSION

Phosphorylated chitosan, silver diamine fluoride, and sodium fluoride varnish significantly improved enamel microhardness after demineralization, with no significant overall difference in percentage microhardness change. SDF showed the highest antibacterial activity against *Streptococcus mutans*, followed closely by phosphorylated chitosan, with no significant difference between them. Sodium fluoride varnish showed lower antibacterial activity. Phosphorylated chitosan therefore appears to be a promising material with combined remineralizing and antibacterial properties, although further in vivo and clinical studies are required.

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