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Remineralizing effects of fluoride varnishes containing nanohydroxyapatite or casein phosphopeptide-amorphous calcium phosphate

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Abstract

Objective: This study compared the effects of sodium fluoride varnish (NaF), NaF varnish containing nanohydroxyapatite (nano-HA), and NaF varnish containing casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) on demineralized enamel.

Methods: Thirty premolars were split mesiodistally, resulting in 60 specimens. The crowns were immersed in a demineralizing solution (pH 4.4) for 96 hours to create incipient carious lesions, then randomly assigned to three groups (n = 20): Group 1: NaF varnish, Group 2: NaF varnish containing 10 wt% nano-HA, and Group 3: NaF varnish containing 10 wt% CPP-ACP. The mineral content of enamel was evaluated by measuring the calcium-to-phosphorus ratio (Ca/P) at baseline (T1), after demineralization (T2), and following remineralization (T3) using energy-dispersive X-ray spectroscopy, and the changes in mineral content between T2 and T3 (mineral absorption) were calculated. Data were analyzed with repeated measures and one-way ANOVA, followed by Bonferroni and Tukey tests ($\alpha = 0.05$).

Results: ANOVA revealed no significant differences in Ca/P ratio at T1 ($P=0.91$) or T2 ($P=0.88$) among groups, but a significant between-group difference was observed at T3 ($P<0.001$). The nano-HA-containing NaF group had a significantly higher Ca/P ratio than other groups at T3 ($P<0.05$). There was a significant difference in the percentage of mineral absorption among groups ($P<0.001$). Group 1 had the lowest and group 2 showed the highest percentage of mineral absorption, with significant differences observed among all groups ($P<0.05$).

Conclusions: NaF varnish containing nano-HA was significantly more effective than other groups in improving the mineral content of demineralized enamel.

Keywords: Casein phosphopeptide-amorphous, calcium phosphate, Dental caries, Fluoride varnishes, Hydroxyapatites, Sodium fluoride, Tooth remineralization

Introduction

Dental caries is the most prevalent chronic disease worldwide, imposing considerable financial, health, and emotional burdens. According to the Global Burden of Disease Study 2015 (GBD 2015) data, untreated caries in permanent teeth was the most prevalent condition among 313 evaluated diseases (1). Epidemiological studies indicate that 60% to 90% of school-aged children experience dental caries (1, 2). Untreated caries in children can lead to anxiety, sleep and nutrition problems, and increased risk of infection and hospitalization, potentially affecting growth, weight, and overall quality of life (3).

Dental caries is caused by an imbalance between demineralization and remineralization cycles in tooth structure. In the early, non-cavitated stage, incipient lesions can be reversed through the uptake of calcium, phosphate, and fluoride ions (4). Based on the concept of minimally invasive dentistry, early intervention of untreated caries is essential to avoid lesion progression and future complications. To support this approach, various remineralizing agents have been developed,

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including topical fluoride and calcium- and phosphate-based compounds (3).

The caries-preventive effect of fluoride was first documented in the 1930s, when epidemiologic and experimental studies demonstrated its ability to reduce dental caries (5). Fluoride varnishes were developed in the 1960s and 1970s to overcome the limitations of gels and mouthrinses by prolonging the contact time between fluoride and enamel through a viscous, resin-based vehicle (5, 6). When a fluoride varnish is applied, the high concentration of fluoride ions (F^-) interacts with calcium ions (Ca^{2+}) present in saliva and at the enamel surface, resulting in the precipitation of calcium fluoride (CaF_2-like) globules on the enamel. Under acidic conditions, these CaF_2 deposits partially dissolve, releasing fluoride ions (F^-). In the presence of calcium (Ca^{2+}) and phosphate (PO_4^{3-}) ions from saliva or other sources, the released fluoride promotes remineralization by enhancing the nucleation, precipitation, and incorporation of fluoridated hydroxyapatite into demineralized enamel (5, 7).

Casein phosphopeptide–amorphous calcium phosphate (CPP-ACP) contains amorphous calcium phosphate stabilized by casein phosphopeptides derived from milk protein. These peptides bind calcium and phosphate ions, maintaining their availability for enamel remineralization (8, 9). CPP-ACP enhances remineralization by increasing the concentration of calcium and phosphate ions in dental plaque and tooth surfaces. Recent studies indicate that combining CPP-ACP with fluoride may produce synergistic effects on enamel remineralization (10, 11).

Nanohydroxyapatite (nano-HA) is a biocompatible material that has recently attracted attention for enamel remineralization in dentistry. Its small particle size allows penetration into enamel porosities and the formation of a protective layer on the tooth surface. Compared with conventional hydroxyapatite, nano-HA has a greater surface area and higher reactivity, which may enhance its remineralizing ability. Several studies have shown that nano-HA can promote enamel remineralization. However, there is limited evidence regarding the effectiveness of nano-HA when combined with fluoride-based preventive treatments (12, 13). Previous studies assessing nano-hydroxyapatite have shown concentration-dependent remineralizing effects (14, 15). Huang et al. (15) investigated the effects of 1%, 5%, 10%, and 15% nano-hydroxyapatite on initial enamel lesions and reported that 10% achieved the highest recovery of surface microhardness, with no additional benefit observed at 15% under their in vitro pH-cycling conditions.

Although the remineralizing effects of fluoride varnishes are well established, few studies have compared the additional mineral uptake when standard NaF varnish is combined with nano-HA or CPP-ACP. Therefore, the present study aimed to compare the remineralizing effects of sodium fluoride varnish alone (NaF), NaF varnish containing nano-HA, and NaF varnish containing CPP-ACP on demineralized enamel.

Materials and methods

Study design and ethical consideration

This in vitro study was performed on sound human dental specimens obtained from intact premolars extracted for orthodontic reasons. The study protocol was approved by the ethics committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.RIDS.REC.1346.472).

Sample size calculation

The sample size was calculated as 20 specimens per group, assuming $\alpha = 0.05$, $\beta = 0.2$ (80% power), group means of 1.7, 1.8, and 1.9, and a common standard deviation (SD) of 0.2. These values were based on a pilot study with four specimens per group. Statistical analysis of the pilot data was performed using one-way ANOVA in IBM SPSS Statistics (Version 26.0; IBM Corp., Armonk, NY, USA).

Preparation of nano-HA powder

Nano-hydroxyapatite (nano-HA) was synthesized using the wet chemical precipitation method. Calcium nitrate tetrahydrate (35.42 g; Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 150 mL of distilled water to prepare the calcium solution, while diammonium hydrogen phosphate (11.88 g; Sigma-Aldrich) was dissolved in 90 mL of distilled water to prepare the phosphate solution. The precursor quantities were calculated to achieve a Ca/P molar ratio of 1.67, corresponding to the stoichiometric composition of hydroxyapatite (16).

Before the two precursor solutions were combined, 1 M sodium hydroxide (NaOH; Sigma-Aldrich) was added dropwise to each solution to raise the pH to approximately 11, which was monitored using a digital pH meter (Metrohm, Herisau, Switzerland). The calcium solution was then gradually added to the phosphate solution under continuous stirring, while the pH was

maintained between 10.5 and 11 by adding NaOH as needed.

The resulting suspension was centrifuged (Hettich, Tuttlingen, Germany), and the precipitate was incubated at 80 °C for 10 hours. The dried precipitate was then calcined in a furnace (Nabertherm, Lilienthal, Germany) at 800 °C for 1 hour to produce crystalline nano-HA powder. The resulting powder after drying and calcination was approximately 15 g, and the particle size of the nano-HA ranged from 50 to 200 nm.

Preparation of NaF varnish containing nano-HA or CPP-ACP

The base fluoride varnish used in this study was a 5% sodium fluoride (NaF) varnish (FluoroDose; Centrix, St. Paul, MN, USA). For group 1, this commercial NaF varnish was applied without modification.

For Group 2 (NaF varnish containing nano-HA), the nano-HA-enhanced varnish was prepared by thoroughly mixing 0.27 g of NaF varnish with 0.03 g of synthesized nano-hydroxyapatite powder, yielding a final nano-HA concentration of 10 wt%. The mixture was homogenized using a magnetic heater–stirrer (aLFA, Tehran, Iran) at 60 °C for 8 hours until a uniform suspension was obtained.

For group 3 (NaF varnish containing CPP-ACP), a combined formulation of NaF varnish and CPP-ACP paste (MI Paste Plus™, GC America Inc., Alsip, IL, USA) was prepared by incorporating CPP-ACP at a final concentration of 10 wt%, using the same mixing protocol as for group 2.

All varnish mixtures were freshly prepared by a single operator immediately before use, and homogeneity was confirmed visually.

Sample preparation

Sound maxillary and mandibular premolars extracted within the past six months were collected. Inclusion criteria required teeth to be free of caries, fractures, previous restorations, or any visible structural defects. A total of 30 eligible premolars were selected after thorough cleaning to remove debris and residual tissues. The specimens were stored in 0.1% thymol (2-isopropyl-5-methylphenol; Sigma-Aldrich) at 4 °C until use.

The teeth were then sectioned mesiodistally into buccal and lingual halves with a diamond disc under continuous water cooling. Finally, the crowns were resected 2 mm below the cemento-enamel junction.

A small area at the center of the enamel surface was covered with paper-adhesive tape (3 × 3 mm). The surrounding regions were coated with two layers of acid-resistant varnish. Once the varnish had fully dried, the tape was removed, creating an exposed enamel window for further treatments. The specimens were air-dried for 24 hours, then individually placed in plastic containers and numbered from 1 to 60.

Induction of incipient carious lesions

To induce incipient caries, each specimen was immersed in 10 mL of demineralizing solution (pH 4.4) and incubated at 37 °C for 96 hours (5). The solution contained 2.2 mM calcium chloride ($CaCl_2$; Sigma-Aldrich), 2.2 mM sodium phosphate (NaH_2PO_4; Sigma-Aldrich), and 0.05 M acetic acid (CH_3COOH; Sigma-Aldrich). The pH was adjusted to 4.4 using sodium hydroxide (NaOH; Sigma-Aldrich). The solution was replaced daily to prevent the accumulation of demineralization products and to maintain a stable pH. After 96 hours, specimens were removed, rinsed thoroughly with distilled water, and air-dried for 24 hours.

Remineralizing protocol

The remineralization protocol consisted of the application of varnishes and a pH-cycling regimen. After caries induction, the specimens were randomly assigned to three groups (n = 20) according to the remineralizing agent applied:

- Group 1 (NaF varnish): Specimens were treated with a single application of 5% NaF varnish (FluoroDose; Centrix, St. Paul, MN, USA).
- Group 2 (NaF varnish containing nano-HA): specimens were treated with a nano-HA-containing NaF varnish (10 wt%), prepared as described above.
- Group 3 (NaF varnish containing CPP-ACP): Specimens were treated with a CPP-ACP-containing NaF varnish (10 wt%), prepared as described above.

A standardized volume of 0.02 mL of each remineralizing agent was applied to the enamel window using an insulin syringe (BD, Franklin Lakes, NJ, USA) to ensure complete coverage. The specimens were then immersed in distilled water for 4 hours to allow fluoride ion release and penetration into the enamel. Subsequently, the residual varnish was gently removed using a No. 15 scalpel blade (Swann-Morton, Sheffield, UK), following a previously described protocol (17).

Subsequently, specimens underwent a 7-day pH-cycling regimen (18) to reproduce the dynamic conditions of the oral environment, in which repeated fluctuations in pH lead to alternating phases of demineralization and remineralization. Each daily cycle consisted of 3 hours of immersion in the demineralizing solution described previously, followed by 21 hours of immersion in a remineralizing solution (pH 7). The remineralizing solution contained 1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4, and 0.15 M KCl (all from Sigma-Aldrich). After completing the pH cycles, specimens were rinsed with distilled water and air-dried for 24 hours.

Ca/P ratio measurement

The mineral content of enamel was assessed by determining the calcium-to-phosphorus (Ca/P) ratio at three time points: baseline (before demineralization; T1), after demineralization (T2), and after completion of the remineralization protocol (T3).

At each time point, specimens were examined using a scanning electron microscope (SEM; SU3500, Hitachi, Japan) equipped with an energy-dispersive X-ray spectroscopy (EDX) system (Octane Prime, EDAX; Ametek, USA) at an accelerating voltage of 15 kV. For each specimen, the enamel window was positioned perpendicular to the electron beam, and EDX spectra were acquired from the treated area. The EDX software was used to quantify calcium and phosphorus, and the Ca/P ratio was calculated for each specimen at T1, T2, and T3.

The percentage change in mineral content between T2 and T3 (% of mineral gain) was calculated for each specimen to represent enamel remineralization.

Statistical analysis

Data were analyzed using IBM SPSS Statistics (Version 26.0; IBM Corp., Armonk, NY, USA). The Kolmogorov–Smirnov test was used to assess the normality of the data distribution ($P > 0.05$).

Changes in Ca/P ratio over time within each group were assessed using repeated-measures ANOVA, followed by a Bonferroni multiple comparisons test. Differences among groups in Ca/P ratio and percentage of remineralization were analyzed using one-way ANOVA, with Tukey's post hoc test for pairwise comparisons. A P value < 0.05 was considered statistically significant.

Results

Table 1 presents the mean and standard deviation (SD) of the Ca/P ratio at baseline (T1), after demineralization (T2), and after remineralization (T3) in the study groups.

Repeated-measures ANOVA showed a significant effect of time on the Ca/P ratio in all groups ($P < 0.001$). Subsequent pairwise comparisons using the Bonferroni test showed that the Ca/P ratio was significantly lower at T2 than at T1 and T3 in all groups ($P < 0.05$), whereas no significant difference was observed between T1 and T3 ($P > 0.05$).

One-way ANOVA showed no significant differences among the groups at T1 ($P = 0.91$) or T2 ($P = 0.88$) time points, but a significant between-group difference was observed at T3 ($P<0.001$). Pairwise comparisons using Tukey's HSD test indicated no significant difference between group 1 (NaF) and group 3 (NaF containing CPP-ACP) at the T3 time point ($P > 0.05$). However, group 2 (NaF-containing nano-HA) exhibited a significantly higher Ca/P ratio than both group 1 and group 3 ($P < 0.05$; Table 1).

Table 1. Means and standard deviations (SD) of the Ca/P ratio at baseline (T1), after demineralization (T2), and after remineralization (T3) in the study groups

Groups	Definition	Baseline (T1)	After demineralization (T2)	After remineralization (T3)	P-value
		Mean ± SD	Mean ± SD	Mean ± SD	
Group 1	NaF varnish	1.86 ± 0.08A	1.77 ± 0.07B	1.81 ± 0.07Aa	<0.001
Group 2	NaF varnish containing nano-HA	1.86 ± 0.09A	1.76 ± 0.08B	1.93 ±0.10Ab	<0.001
Group 3	NaF varnish containing CPP-ACP	1.85 ± 0.08A	1.75 ± 0.08B	1.83 ± 0.08Aa	<0.001
P-value		0.91	0.88	<0.001	

NaF: Sodium fluoride; HA: Hydroxyapatite; CPP-ACP: Casein phosphopeptide-amorphous calcium phosphate
*Different superscript uppercase letters indicate statistically significant differences between time points within each group at $P < 0.05$, whereas different superscript lowercase letters indicate statistically significant differences between groups at $P < 0.05$.

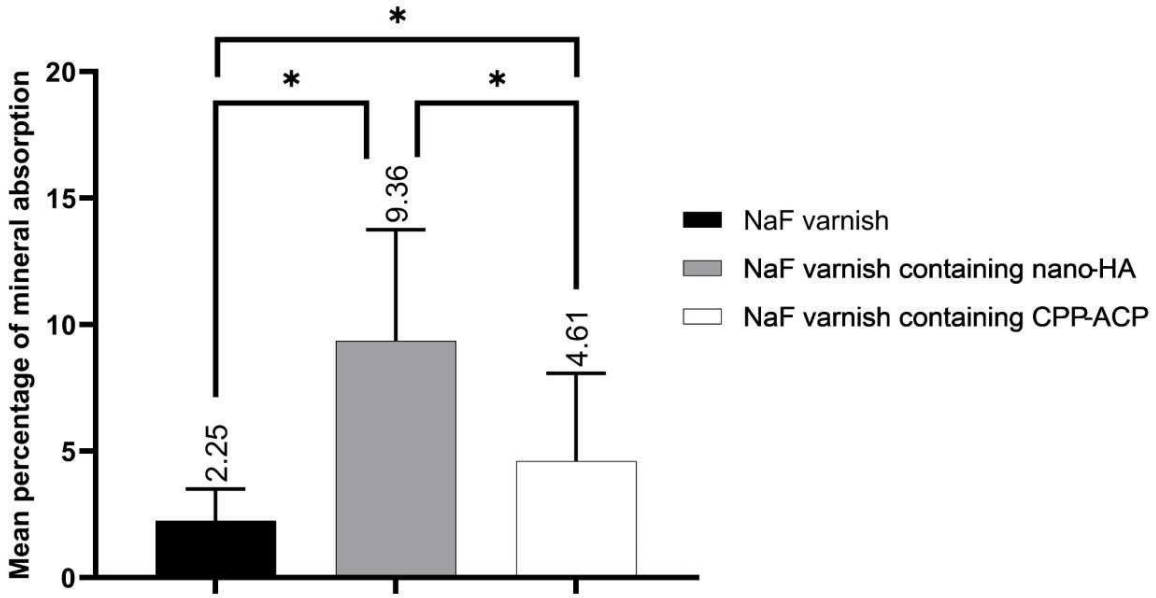

Figure 1. Comparison of the mean percentage of mineral absorption in the three groups. An asterisk (*) indicates a

Figure 1 illustrates the percentage of mineral absorption in the study groups. One-way ANOVA revealed a statistically significant difference in the percentage of mineral absorption among the groups ($P < 0.001$). Post hoc comparisons using Tukey's HSD test showed that group 1 had the lowest and group 2 the highest mean percentage of mineral absorption, with statistically significant differences among all three groups ($P < 0.05$).

Discussion

This study evaluated the remineralizing effects of NaF varnish, NaF varnish containing nano-hydroxyapatite (nano-HA), and NaF varnish containing casein phosphopeptide–amorphous calcium phosphate (CPP-ACP) on demineralized enamel. The mineral content of the examined enamel surface was assessed using energy-dispersive X-ray spectroscopy (EDX), which provides elemental analysis of surface elements. Changes in calcium (Ca), phosphorus (P), and particularly the calcium-to-phosphorus ratio (Ca/P) are commonly used in laboratory studies as indirect indicators of enamel demineralization and remineralization, as they reflect mineral loss or gain within the enamel structure (10, 19-22). In sound hydroxyapatite crystals, the Ca/P ratio is approximately 1.67, and deviations from this value may indicate mineral loss or gain in enamel (23, 24). During demineralization, the Ca/P ratio decreases as calcium is lost and enamel becomes porous, whereas remineralization increases this ratio due to mineral deposition and regeneration of hydroxyapatite crystals (25).

In the present study, the Ca/P ratio decreased significantly in all three groups after demineralization (T2). Following the remineralization protocol (T3), the Ca/P ratio increased significantly in all groups, reaching values comparable to baseline (T1) measurements. This indicates that all remineralizing agents were effective for treating incipient carious lesions.

No significant differences were observed in the Ca/P ratio among groups at baseline or after demineralization, confirming successful randomization and uniform demineralization across all groups. However, a significant between-group difference was observed after remineralization (T3). The NaF varnish containing nano-hydroxyapatite showed the highest Ca/P ratio, which was significantly greater than that of both the NaF group and the CPP-ACP-containing NaF group. Although the mean Ca/P ratio in the CPP-ACP-containing NaF group was slightly higher than in the NaF group at T3, this difference was not statistically significant.

In this study, significant differences were observed in the percentage of mineral absorption (the change in Ca/P ratio between demineralization and remineralization stages) among the groups. The NaF varnish containing nano-hydroxyapatite showed the highest percentage of mineral absorption, followed by the CPP-ACP-containing NaF group, while the NaF group exhibited the lowest values. The differences in mineral absorption were statistically significant between all groups. These findings suggest that incorporating either CPP-ACP or nano-HA into NaF varnish enhances its remineralizing effect. Furthermore, nano-HA–modified NaF varnish demonstrated greater efficacy than CPP-

ACP–modified NaF varnish in the management of demineralized enamel.

In the present study, the addition of CPP-ACP to NaF varnish was effective in enhancing the percentage of mineral absorption in demineralized enamel as compared to NaF varnish alone. This improvement is likely related to the ability of CPP-ACP to provide bioavailable calcium and phosphate ions, thereby supporting enamel remineralization (26, 27). Previous studies have shown that CPP-ACP can increase calcium and phosphate levels in saliva, enhance remineralization, buffer plaque acids, and reduce hydroxyapatite dissolution (28, 29). In addition, it may limit the adhesion and growth of Streptococcus species (30). By releasing loosely bound calcium and phosphate ions, CPP-ACP can help fill subsurface defects in enamel lesions (31).

Nano-hydroxyapatite (nano-HA) closely resembles the mineral component of enamel and serves as a source of calcium and phosphate ions that are essential for remineralization. These ions can penetrate the porous structure of demineralized enamel and promote mineral deposition on the enamel surface. In the present study, the Ca/P ratio in the nano-HA-containing varnish group was significantly higher at T3 compared with the other groups. Furthermore, the percentage of mineral gain was highest in NaF varnish containing nano-hydroxyapatite. This finding is in agreement with previous studies (32, 33) and suggests that incorporating nano-HA into fluoride varnish may enhance mineral deposition and improve the remineralization of demineralized enamel.

The greater remineralizing effect observed with NaF varnish containing nano-HA may be attributed to the ability of nano-HA crystals to form an apatite-like layer on the enamel surface, which covers and protects demineralized areas. Because nano-HA has a structure similar to the natural hydroxyapatite crystals of enamel, it can integrate effectively with the enamel surface and act as a biomimetic scaffold for mineral deposition (25).

The outcomes of this study are in agreement with the results of Sharma et al. (34), who evaluated fluoride-free nano-HA paste and fluoride-free CPP-ACP paste, and reported that nano-HA showed greater remineralization potential than CPP-ACP. de Carvalho et al. (35) reported that nano-hydroxyapatite paste exhibited greater remineralizing effects than fluoride varnish or CPP-ACP paste after the cariogenic challenge. This was attributed to the formation of a protective layer with globular deposits on the surface (35). Although the formulations and methodologies differed from those used in the present study, these findings are consistent with the higher remineralization observed for the nano-HA–containing varnish.

The outcomes of this study contrast with those of Khan et al. (36), who compared nano-HA varnish, nano-silver fluoride (NSF) varnish, and NaF varnish in preventing caries lesions and found that nano-HA varnish was less effective than both NSF and NaF varnishes at 12-month follow-up. This difference may arise from their clinical focus on caries arrestment/prevention rather than in vitro remineralization of artificial lesions. Majithia et al (5) reported similar outcomes for NaF varnish + CPP-ACP, NaF varnish + ACP, and NaF varnish alone. They evaluated remineralization using Vickers surface microhardness testing and SEM-EDX analysis of the Ca/P ratio; both methods showed that all varnish-treated groups outperformed the no-varnish control, with no significant intergroup differences among varnishes.

This study has several limitations. First, remineralization was assessed only through elemental analysis, without complementary evaluations such as surface microhardness testing or SEM analysis. Second, as an in vitro study, the oral environment could not be fully replicated. Factors such as saliva, the acquired pellicle, and masticatory forces may affect the retention and effectiveness of varnishes. Therefore, caution is warranted when extrapolating these results to clinical conditions. Future research should include other quantitative or qualitative methods, such as surface microhardness testing and SEM analysis, to more comprehensively evaluate the effectiveness of NaF varnishes in managing carious lesions. Moreover, in situ and clinical studies are needed to confirm these findings under conditions that more closely simulate the oral environment.

Conclusions

Application of 5% NaF varnish, NaF varnish containing 10 wt% nano-HA, or NaF varnish containing 10 wt% CPP-ACP induces remineralization of incipient carious lesions. However, the NaF varnish containing nano-HA demonstrated significantly greater remineralizing effectiveness than other groups, and may be suggested for the treatment of incipient carious lesions.

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Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

F.M. and S.S.M.J. developed and supervised the project, helped in data analysis, and edited the manuscript. R.R., F.S., and S.S.M.J.collected and analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Ethical considerations

The study protocol was approved by the ethics committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.RIDS.REC.1346.472).

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