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ORIGINAL RESEARCH

Remineralization potential of a novel varnish: an *in vitro* comparative evaluation

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Abstract

Despite fluoride's widespread use in preventing dental caries, it remains a significant oral disease with some drawbacks. Consequently, new preventative agents have emerged that can function independently of fluoride. Our aim is to demonstrate the efficacy of newly developed varnishes, 3% Rennou (theobromine calcium and phosphate) and 1% Rennou, in remineralizing initial caries. In our experiment, 40 human enamel samples were randomly allocated into four groups of 10 samples each as: Group 1 (G1): 5% NaF (Colgate PreviDent®), Group 2 (G2): 1% Rennou®, Group 3 (G3): 3% Rennou® and Group 4 (G4): Casein Phosphopeptide-Amorphous Calcium Phosphate + Fluoride (MI Varnish^{TM} GC). To produce an artificial carious lesion in the enamel, the samples were kept in a demineralizing solution for 72 hours. Samples underwent pH cycling for 6 days in order to induce remineralization. The means of the three measurements were compared, and the percentage of Surface Microhardness Recovery in (SMHR%) was calculated. Scanning Electron Microscopy (SEM) was used for qualitative assessment of surface changes. G1 had the highest SMHR% value, followed by G3, G2 and G4. The One-way ANOVA (Analysis of Variance) showed significant differences in the SMHR% values among the groups after six days of cycling ($p < 0.001$). In pairwise comparisons, groups did not show differences in means of SMHR% except for G1 and G4 ($p = 0.006$). In the SEM Images, after treatment within the NaF group, many flaky sediments were found on the enamel surface. Similarly, the maximum mineral gain was seen in the NaF and Rennou groups. SEM images of both varnish surfaces revealed a uniform layer interspersed with shapeless precipitates. All varnishes treated artificial enamel lesions to varying degrees. However, both concentrations of Rennou showed remineralization potential comparable to 5% NaF in acceptable statistical measurements. Thus, it could be used as a potentially effective preventive measure for pediatric patients.

Keywords

Theobromine; Rennou; SMHR; Vickers; Varnish; Remineralization

1. Introduction

Dental caries, also known as tooth decay, is a condition that arises from an imbalance in the process of losing and gaining minerals in the teeth [1]. This is a complex condition that can be caused by multiple factors and requires a complex treatment plan in which remineralization plays a vital role in preventing the progression of the disease and reversing early signs of mineral loss. Tooth decay can be prevented and reversed in its early stages by stopping the loss of minerals in the enamel and dentin, which can be achieved through the inhibition of biofilm formation and the use of protective factors in saliva [2, 3].

In recent years, researchers have focused on developing non-invasive methods for managing early tooth decay through remineralization to preserve the structure of the teeth [4, 5]. For this purpose, various commercially available remineralization agents including fluoride (F), theobromine and Casein Phosphopeptide-Amorphous Calcium Phosphate (CPP-ACP) have been developed. These substances release active ions that bond to the crystalline structures in the enamel and form new crystals or repair damaged ones [4, 6, 7].

Fluoride ions are highly efficient in preventing mineral loss in enamel by forming fluorapatite with calcium and phosphate ions that are generated during the enamel demineralization process caused by plaque bacteria. Low concentrations of fluoride solutions are available for daily use, such as toothpaste and mouthwash, while higher concentrations are used for professional treatments such as gels and varnishes [8]. F varnish improves the uptake of F^- ions into the dental structure by coating the teeth with an adhering film that lasts for up to 24 hours, the required duration before a patient can resume brushing. Calcium Fluoride, which is formed when F is deposited, acts as a reservoir for F^-ions that are gradually released. Fluoride's effect is therefore connected to its ability to prevent demineralization while also encouraging enamel remineralization [6].

CPP-ACP is a milk protein derivative that has been shown to have anti-cariogenic effects. It works by stabilizing high concentrations of calcium and phosphate ions with F^- ions at the surface of the teeth by binding to the pellicle and plaque [9]. Amorphous calcium phosphate (ACP) applies calcium ions and phosphate ions separately, allowing ACP or amorphous calcium fluoride phosphate (F-ACP) to form in the mouth [10]. Research has shown that CPP-ACP can aid in preventing mineral loss in the enamel and promoting remineralization by utilizing calcium and phosphate ions. These ions help to stabilize ACP within dental plaque at neutral or alkaline pH levels, thus maintaining enamel supersaturation [8, 9].

Several animal studies have shown that chocolate may have anti-cariogenic effects [7, 11]. Theobromine, which is an alkaloid derived from cocoa beans, is a natural component of chocolate and has been shown to enhance the remineralization properties of fluoride and calcium-containing products [12]. *In vitro* experiments have demonstrated that theobromine can reduce the dissolution of hydroxyapatite and create deposits on the enamel surface [12, 13]. Additionally, it has the ability to diminish tooth sensitivity and improve the rehardening of enamel lesions [12].

The increase in the number of enamel remineralization products available commercially can be perplexing for operators, as there is a lack of research to recommend the most suitable one. Therefore, this study intends to investigate the impacts of two commonly used varnish materials, CPP-ACP + Fluoride Varnish and Sodium Fluoride (NaF) Varnish, which are identified as bioactive (including fluorides and CPP-ACP), as well as a new experimental varnish containing theobromine.

2. Materials & methods

2.1 Sample size

According to the power analysis, the sample size calculation for the study conducted in an independent 4-group experiment setup was based on the basic features of the *F* test with the G-power (SPSS IBM SPSS Statistics version 22.0 (SPSS Inc. Chicago, IL, USA) 17.50.18 package program, Type 1 error rate was 0.05 (Confidence level 95%), test power was 80% (Type 2 error 20%), with an effect size of 0.38, the total sample size was calculated as 40 for 4 groups.

2.2 Specimen preparation

For the study, 40 newly extracted human third molars from both the maxillary and mandibular regions of humans were chosen. Any teeth with noticeable indications of caries, restorations, hypoplastic lesions, stains, cracks or white spot lesions were excluded from the study. The teeth were thoroughly cleaned to remove any debris, calculus, or soft tissue from the surface. Then, using a slow-speed diamond disc, the teeth were sectioned 1 mm beneath the cementoenamel junction, and the crowns were retained for the study, while the roots were disposed of. The specimens were kept in a 0.1% thymol solution until the experimental procedure was initiated. The methodology is depicted in flow chart in a step-wise manner (Fig. 1). The enamel blocks underwent grinding using a rotary electric polisher (Ecomet 250, Buehler, Lake Bluff, IL, USA) with a rotary head (Buehler Automet 250, Buehler, Lake Bluff, USA) and aluminum oxide abrasive paper (Arotec S/A Ind. e Com., Cotia, Brazil) that had varying levels of coarseness (600, 800, 1000, 1200). To create a 5 mm $\times$ 5 mm exposed enamel window at the center of the sample surface, adhesive tape was utilized. To protect the sample from acid attack, a consistent layer of nail varnish was applied. Following the drying of the samples, an explorer was used to remove the adhesive tape from the tooth surface, exposing a rectangular region on the enamel surface.

2.3 Preparation of demineralising and remineralising solutions

The remineralization solution used in this study was saturated with calcium and phosphate ions at a fixed pH level of 7.0. On the other hand, the demineralizing solution had an acidic buffer with a pH of 4.4, which approximates the mineral ion composition and supersaturation of saliva. The composition of the solutions used in this study was similar to the ones used by Ten Cate *et al.* [14].

The demineralizing solution was adjusted to a pH of 4.4 using 1 M Potassium Hydroxide (KOH) and contained 2.2 mM Calcium Chloride ($CaCl_2$), 2.2 mM Sodium Dihydrogen Phosphate (NaH_2PO_4) and 0.05 mM acetic acid. The remineralising solution contained 1.5 mM $CaCl_2$, 0.9 mM NaH_2PO_4, and 0.15 mM Potassium Chloride (KCl) and had a pH of 7.0.

2.4 Lesion formation

To produce an artificial carious lesion in the enamel, the samples were kept in a demineralizing solution (20 mL) for 72 hours. Subsequently, the surface microhardness values were evaluated using a Vicker's microhardness testing machine (Shimadzu HMV-2 Microhardness tester (Shimadzu, Japan)), in the similar manner as the baseline surface microhardness assessment [15].

2.5 Test and control varnishes

In this single-blinded study, the manufacturer applied the remineralising chemicals and then coded all the specimens using sealed envelopes in order to conceal the allocation of the various groups. After collecting the data, the manufacturer decoded the samples while the lead investigator was unaware of which readings were taken. Table 1 lists the varnishes that were examined.

2.6 The pH cycling model

The dynamic pH-cycling protocol involves subjecting dental substrates to a series of demineralization and remineralization cycles, designed to simulate the mineral loss and gain processes involved in caries formation [14]. The blocks underwent pH cycling for 6 days in order to induce remineralization [16]. After the varnish had been applied, the blocks were submerged in a remineralising solution (1.5 mM $CaCl_2$, 0.9

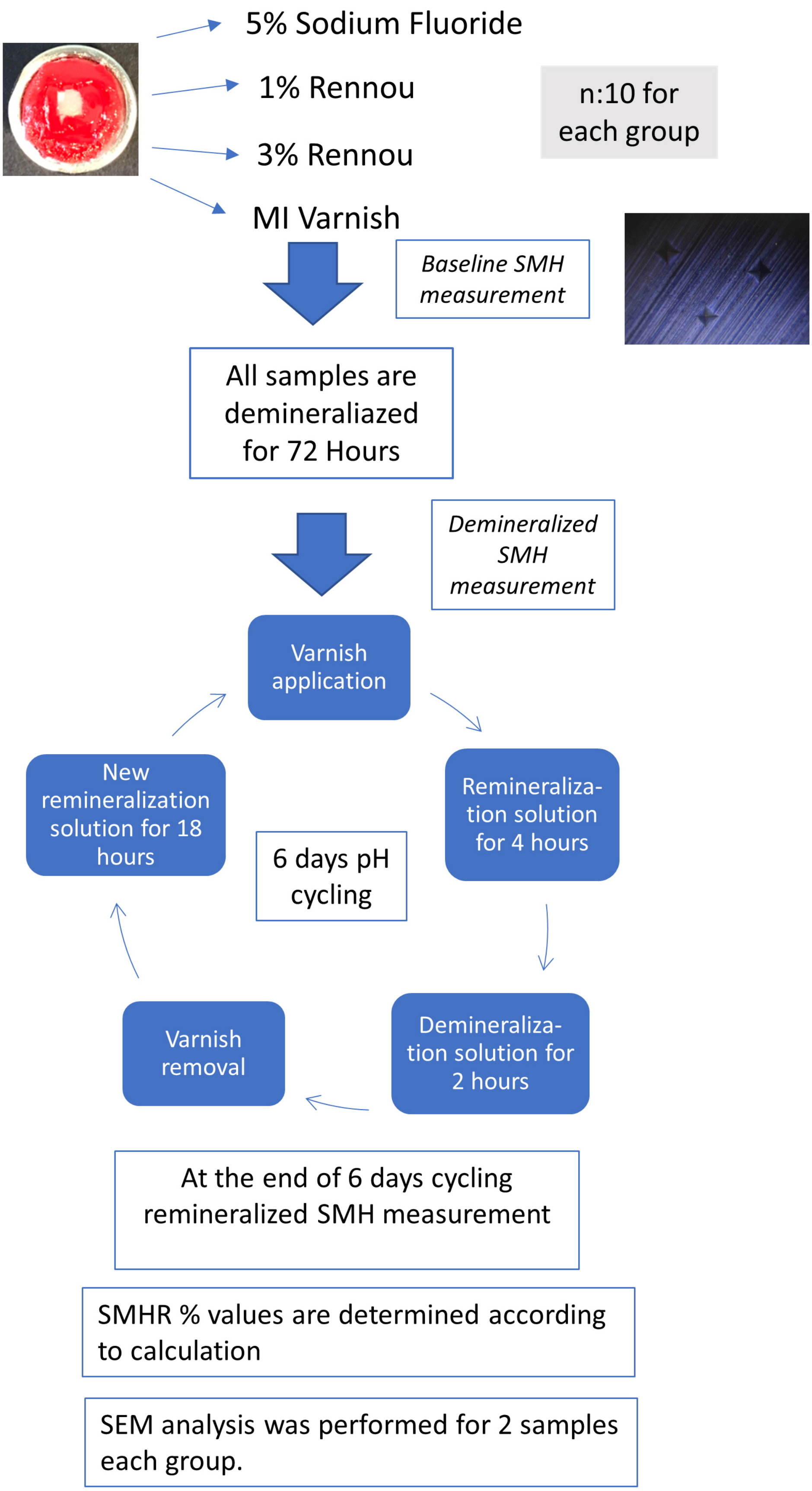


FIGURE 1. The flow chart of the experiment. Each third molar roots were sectioned, and the crowns were mounted in metal rings. After dividing groups, artificial enamel caries lesions were created using pH cycling with demineralization and remineralization solutions. At the end of the 6th day, SMHR% values were calculated. SMHR%: percentage of recovery in surface microhardness; SEM: Scanning electron microscopy; SMH: Surface Microhardness; pH: Potential of Hydrogen.

TABLE 1. Varnishes, ingredients and manufacturers.

Test Varnish	Ingredient	Manufacturer	Other Noteworthy Ingredients
Group 1 Control	5% NaF	Colgate PreviDent®	rosin, ethanol, xylitol, peppermint oil, sodium fluoride
Group 2	1% Rennou	Theodent®	rosin, ethanol, xylitol, peppermint oil theobromine, calcium and phosphate
Group 3	3% Rennou	Theodent®	rosin, ethanol, xylitol, peppermint oil theobromine, calcium and phosphate
Group 4	CPP-ACP + Fluoride	GC Corp MI Varnish^{TM}	CPP-ACP, 5% NaF (22,600 ppm), ethanol, mint flavor

NaF: Sodium Fluoride; CPP-ACP: Casein Phosphopeptides-Amorphous Calcium Phosphate.

mM NaH_2PO_4 and 0.15 mM KCl, which was at pH 7.0) for 4 hours, followed by 2 hours of demineralization (2.2 mM $CaCl_2$, 2.2 mM NaH_2PO_4 and 0.05 mM acetic acid, with the pH adjusted with 1 mM KOH to be 4.4). The blocks were relocated to a fresh remineralising solution for 18 hours after the varnishes were removed with a scalpel blade and acetone. Each cycle began with a fresh coat of varnish being applied [17]. After the pH cycling process was completed, the surface microhardness (SMH) of all enamel samples was evaluated using a Vickers hardness tester.

2.7 Surface microhardness measurement

Hardness was determined by the Vickers hardness test with Shimadzu HMV-2 tester under a load of 200 g for 15 seconds. Three areas on the midline surface of each specimen were subjected to impressions at each of the three separate times (baseline, after demineralization, and after the respective treatments). The percentage of surface microhardness recovery (SMHR%) is then determined by comparing the means of the three measurements.

2.8 SEM examination

Random selection of two sample specimens from each group was performed for assessment with a scanning electron microscope (SEM) to examine and contrast the surface changes. The SEM was utilized to analyze the morphological differences among the treated samples, and images were captured at 1000× and 2500× magnification. The surface morphology of enamel lesions and adjacent areas was examined, with texture, roughness, and the presence of irregularities or structural anomalies being assessed. Through these criteria, SEM images were evaluated by two researchers, with the aim of achieving a comprehensive understanding of enamel remineralization dynamics and ensuring the validity and robustness of our findings.

2.9 Statisticial analysis

The data collected was analyzed by employing statistical software (SPSS IBM SPSS Statistics version 22.0 (SPSS Inc. Chicago, IL, USA) and performing ANOVA. Paired *t*-tests and *post-hoc* Tukey tests were executed to conduct multiple comparisons between groups. A *p*-value of less than 0.05 was determined to be statistically significant for the entire evaluation.

3. Results

The SMH values for each group are displayed in Table 2. Hardness values at baseline and after demineralization showed a normal distribution between groups. There was no difference in the baseline SMH values for all groups ($p = 0.381$). However, the mean demineralization and remineralization SMH values for all groups showed difference ($p < 0.001$ and $p < 0.001$, respectively). Also, SMHR% was calculated for all the experimental groups. Table 2 shows that G1 has the

TABLE 2. Descriptive SMH values of all groups.

	Baseline	Demineralization	Remineralization	SMHR%	$p^{**,a}$
G1-5% NaF-Control	216 ± 37	137 ± 33	408 ± 49	377 ± 156	<0.001
G2-1% Rennou	218 ± 20	119 ± 24	379 ± 18	278 ± 62	<0.001
G3-3% Rennou	204 ± 23	108 ± 15	370 ± 44	285 ± 80	<0.001
G4-CPP-ACP + Fluoride	210 ± 20	105 ± 21	318 ± 35	206 ± 45	<0.001
$p^{*,a}$	0.624	0.027	<0.001	0.004	

Values were given as Mean ± SD.
**Baseline, demineralization and remineralization SMH means were compared with One-Way ANOVA.*
***Demineralization and remineralization of SMH values were compared with paired t test.*
$^{a}p < 0.05$ *was considered statistically significant.*
SMHR%: Percentage of Recovery in Surface Microhardness; NaF: Sodium Fluoride; CPP-ACP: Casein Phosphopeptides-Amorphous Calcium Phosphate.

highest SMHR% value, followed by G3, G2, G4. The One-way ANOVA shows that there are significant differences in the SMHR% values among the groups after six days of cycling ($p < 0.001$). Multiple comparisons of SMHR% between the experimental groups and control are presented in Table 3. Groups don't show differences in means of SMHR% except for G1 and G4 ($p = 0.006$).

The SEM analysis revealed that the use of various remineralization agents on pre-existing enamel lesions resulted in different surface morphologies (Fig. 2). After treatment within group NaF (G1), many flaky sediments were found on the enamel surface. Similarly, the maximum mineral gain was seen in NaF and Rennou groups. The SEM images of both varnishes revealed a uniform layer with dispersed amorphous precipitates. A qualitative comparison between the two treatments revealed that the 5% NaF (G1), and 3% Rennou (G3) varnishes produce similar large crystals and less porosity. Additionally, the high-resolution imaging revealed that demineralized enamel, when exposed to the 5% NaF (G1) or 3% Rennou (G3) varnishes systems, with an apparent increase in crystal density and size. In the CPP-ACP + Fluoride Varnish (G4) group, the enamel rods and prismatic structure are indistinguishable. However, the areas of calcified deposits are more visible and appear to be concentrated around the porous imperfections. At a higher magnification of 2500×, it is possible to observe areas of mineralized deposits that are noticeably scattered throughout the porous defects.

4. Discussion

Preventive strategies for dental caries in children and adolescents can stop the early stages of decay and even help to repair the damaged surface of the teeth. By using preventive materials, it's possible to slow down or prevent cavity development, preserving the integrity of the teeth. There is evidence that specifically designed treatment plans can repair initial enamel lesions and make them more resistant to acid [5]. Currently, various products that contain calcium, phosphate, and bioavailable fluoride are commonly utilized for remineralising enamel structure in the form of toothpastes, mouth rinses, gels, and varnishes [18].

Our study results indicate that 5% NaF usage leads to higher microhardness values compared to the other varnishes. Fluoride varnishes have traditionally been used for professional fluoride application. The process of depositing F^- ions in the enamel forms a calcium fluoride reservoir that releases F^- ions gradually. The fluoride mechanism of action involves inhibiting demineralization and promoting enamel remineralization [19]. When fluoride varnish is applied, the released F^- ions interact more strongly with enamel, leading to less mineral loss, reduced demineralization of the enamel surface, and decreased carious lesion depth [20]. Fluoride varnishes have also been found to be effective in reducing or stopping white spot lesions [21].

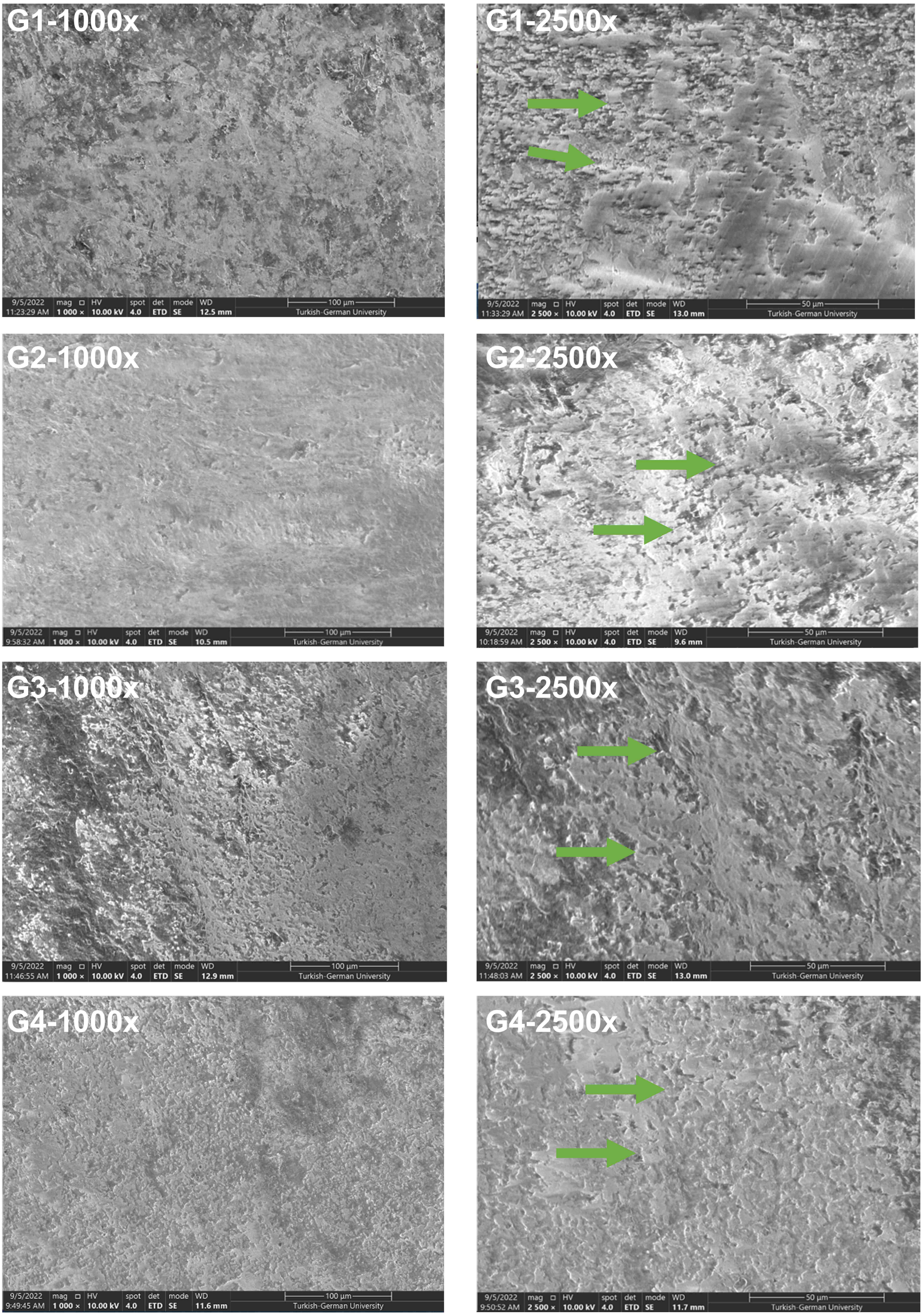
The current study demonstrated that there were no significant differences in surface microhardness values among all the groups, thus supporting our hypothesis that Rennou varnishes have a similar protective effect against enamel demineralization as CPP-ACP + Fluoride Varnish and F Varnish. Since Rennou-containing varnish has not been used so far, there is no article in the literature that we can compare. However, there are some publications about toothpastes containing theobramine in Rennou. Theobromine is an efficient remineralising agent and a good substitute for other remineralising agents, according to numerous research [11, 12]. According to Premnath *et al.* [22], theobromine-containing dentifrice successfully remineralises enamel lesions. Farooq *et al.* [23] demonstrated that toothpaste with 0.2 weight % theobromine and 4 weight % fuoridated-bioactive glass can be recommended for frequent usage, particularly for individuals who are at high risk of caries and erosion. According to Pribadi *et al.* [24], the surface hardness of enamel appears to be noticeably stronger following exposure to theobromine cacao rind extract than in the fluoride group. Nasution *et al.* [25] conducted a study which showed that fluoride had a more significant effect on remineralization compared to theobromine. Another study by Premnath *et al.* [22] found that the potential for remineralization was lower with theobromine compared to a dentifrice containing fluoride, and there was no noticeable difference between the two groups. Nakamoto *et al.* [26] suggested that both theobromine and fluoride may contribute to an increase in apatite crystal size, leading to an improvement in enamel surface microhardness.

The qualitative evaluation of demineralization and surface properties can be effectively carried out by SEM analysis, which is a commonly used method [27]. Previous research has employed both SEM image analysis and microhardness determination to assess the demineralization resistance of fluoride-releasing materials on demineralized enamel [28]. In the present study, SEM images were used along with surface microhardness measurements to evaluate the efficacy of different groups in reducing demineralization of enamel. The

TABLE 3. Intergroup pairwise comparisons of SMHR%[a].

	G1-	G2-	G3-	G4-
G1-5% NaF-Control		1.000	1.000	0.006
G2-1% Rennou	1.000		1.000	0.222
G3-3% Rennou	1.000	1.000		0.777
G4-CPP-ACP + Fluoride	0.006	0.222	0.777	

[a]Significance values (p) have been adjusted by the Bonferroni correction.
$p < 0.05$ was considered statistically significant.
NaF: Sodium Fluoride; CPP-ACP: Casein Phosphopeptides-Amorphous Calcium Phosphate.



F I G U R E 2. (Scanning Electron Microscope) SEM Images of four groups after pH cycle. The surface of all test groups (G1, G2, G3 and G4) presented either scattered amorphous crystals or particles, or lines of remineralization along the prismatic borders (images with 1000× magnification and 2500× magnification). The areas shown with green arrows represent shapeless precipitates in each group.

results demonstrated that the group had the smoothest surface compared to the other groups, suggesting that it had a lower level of demineralization. Oshiro *et al*. [29], again using the same method *in vitro*, examined the results of applying paste containing 1% CPP-ACP twice a day on bovine enamel and dentin tissue, this time by comparing SEM images. As a result of this study, it was reported that while demineralization was evident in the surface enamel layer in the images of the control group, there was only very slight porosity in the enamel in the group in which CPP-ACP paste was applied, and demineralization was prevented [29]. In a study conducted by Tuloglu *et al*. [30] in 2017, Duraphat Varnish, Clinpro White Varnish, MI Varnish reported that topical F applications to the intact enamel surface reduce the bond strength by reducing the effectiveness of the acid because it creates a physical barrier before pickling. They state that it is due to the fact that the varnish in the SEM images infiltrates the enamel prisms and prevents the adhesive from penetrating into the areas it needs to reach [30]. In an *in vitro* study conducted by Nayak *et al*. [31] in 2017, they investigated the remineralization effects of NaF solution, NaF gel, NaF varnish, functionalized-tricalciumphosphate (fTCP) varnish and CPP-Amorphous Calcium Fluoride Phosphate (ACFP) topographically by SEM. In the SEM imaging of this study, investigators reported that they observed amorphous, globular and crystalline Calcium Fluoride (CaF_2) precipitates in all treatment groups. Researchers observed that there were differences between the treated and untreated groups [31]. It was also observed that different sizes of globular structures were formed on the lesion surfaces in the SEM examinations of the NaF solution applied group [32]. However, in another experiment, F varnish and F solution were applied to demineralized enamel surfaces and the samples were examined by SEM. There was no specific appearance that the enamel surface was covered with varnish residues in the F varnish applied group, and F was applied in the solution applied group, which was associated with the fact that the F varnish form was more sticky than the solution form [33]. Also in a study where DIAGNOdent and SEM were used to control the remineralization of CPP-ACP and CPP-ACFP in the mine, the highest remineralization was found in CPP-ACFP compared to outside of DIAGNOdent, and the lowest remineralization was found in the control. In SEM imaging examination, the mineral blades were mostly observed in CPP-ACFP, followed by CPP-ACP and control settings [34].

The limitations of this *in vitro* study include difficulty to precisely simulate the biological aspects of caries and the multitude of intraoral conditions that contribute to dental caries, the role of enzymes is not accounted for. Since solutions are composed of inorganic ions only, the effects of salivary proteins, pellicle and plaque on mineralisation inhibition are not taken into consideration. Other confounding factors involve the possibility of experimental errors and dissimilarities in the micro-structure of the enamel between specimens. Also, a positive control group is preferable over a negative control group because it allows researchers to verify that the experiment is functioning correctly and that any observed results are genuinely due to the experimental variables rather than uncontrolled variables. pH cycle-based studies require the application of varnishes in a narrow timeline. Normally, varnish applications are conducted usually in 6 monthly basis. So, this may not represent the clinical scenario which makes this study of small clinical relevance.

5. Conclusions

Compared to the control group, all the remineralising agents used in this study demonstrated the potential to promote remineralization of enamel surfaces by improving their surface microhardness *in vitro*. Although NaF showed slightly superior SMH values, there was no statistically significant difference between the two treatment modalities. However, further research is necessary to gain a deeper understanding of the abilities of these materials.

AVAILABILITY OF DATA AND MATERIALS

The data presented in this study are available on reasonable request from the corresponding author.

AUTHOR CONTRIBUTIONS

ANPG—conceptualization, data curation, formal analysis, investigation, methodology, validation and writing-original draft. EK—investigation, conceptualization, methodology, resources, supervision, validation, and writing-review and editing. KS—conceptualization, resources, visualization, and writing-review and editing. BK—methodology, supervision, validation, and writing-review and editing.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The present *in vitro* study was approved by the ethics committee with decision number of 2021-06. All procedures performed in the study were in accordance with the ethical standards of the University of Siena and with the 1964 Helsinki declaration and its later amendments.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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