Original paper

Effect of an experimental chitosan/casein gel on demineralized enamel under a cariogenic challenge

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Abstract

Background. Dental caries is considered one of the most common oral health diseases.

Objectives. The aim of the study was to evaluate the effects of an experimental chitosan/casein gel on enamel demineralization/remineralization in an environment with a high cariogenic challenge.

Material and methods. Thirty-six specimens of bovine enamel (4 mm × 3 mm × 2 mm) were ground flat and polished. Then, the specimens were immersed in acetate buffer for 43 h with half of the surface protected (serving as control) and the other half exposed. All demineralized surfaces were randomly assigned into 3 groups ($n = 12$ per group) according to the type of treatment (G1 – control, G2 – 1.5% chitosan gel with 1.5% casein, and G3 – 1.5% chitosan gel without casein), and the corresponding treatment was applied once a week for 3 weeks. The specimens were also subjected to pH cycles of demineralization/remineralization and the treatments were performed 3 times at 7-day intervals for a total of 21 days. Surface images were obtained for the analysis of initial roughness and, after the cariogenic challenge, new images were obtained to evaluate the final roughness, volume loss and wear profile using laser confocal microscopy. After the analyses, the specimens were cut and the depth of demineralization was measured. The data were analyzed using the Kruskal–Wallis analysis of variance (ANOVA) and the Tukey's test.

Results. While the chitosan gel with casein showed a similar loss to the control group ($p > 0.05$), both gels resulted in similar volume loss ($p > 0.05$). There were no statistical differences regarding the wear profile, surface roughness and depth of demineralization between the groups ($p > 0.05$).

Conclusions. The chitosan gel reduced volume loss of the demineralized enamel without significantly impacting the surface smoothness.

Keywords: chitosan, dental caries, tooth remineralization, caseins, confocal microscopy

Introduction

Dental caries is considered one of the most prevalent chronic diseases in adults and children worldwide.[1] A multifactorial etiology of dental caries involves specific microorganisms, fermentable carbohydrates, the dental surface, characteristics inherent to the host, and time of exposure. All of these factors play a role in the dynamic processes of demineralization and remineralization that occur on the dental surface.[1] If demineralization occurs continuously, the lesion may progress from an initial decalcification to a white spot lesion until an irreversible loss of the mineral structure occurs, which can be visualized in the form of a cavity lesion.[2,3]

If found at an early stage, lesions can be reversed noninvasively through the use of remineralizing agents.[4] Thus, research on the use of remineralizing agents is important as it can be helpful in preventing cavity formation. Remineralizing agents should meet some requirements,[5] including being safe for human use, displaying efficacy of bioactive compounds, promoting rapid precipitation in partially demineralized teeth, becoming a stable and resistant phase against the attack of bacteria and other acidic agents, remaining active on both the surface and subsurface of the lesion, and having the ability to diffuse through the biofilm and lesion. As of today, there has not been an agent that meets all of the aforementioned characteristics.

Several studies have shown that the formation of white spot lesions is directly attributable to the accumulation and prolonged retention of visible bacterial biofilm,[6] as well as the presence of *Abiotrophia defective* spp., *Actinomyces*, *Actinobaculum*, *Aggregatibacter*, *Bergeyella*, *Campylobacter gracilis* spp., *Cardiobacterium hominis* spp., Clostridiales, *Corynebacterium*, *Fusobacterium*, *Gemella*, *Granulicatella*, *Haemophilus parainfluenzae*, *Kingella*, *Lautropia mirabilis* spp., *Leptotrichia*, *Neisseria*, *Porphyromonas*, *Prevotella melaninogenica* spp., *Rothia*, *Streptococcus*, *Nanosynbacter lyticus* spp., and *Veillonella*,[7–9] among others. Traditionally, the topical application of fluoride has been the most commonly used method to prevent enamel demineralization[6] and promote a surface less susceptible to acid degradation.[10] However, most of the current fluoride regimens depend on patient adherence and collaboration, and those who would benefit most from the application of supplemental fluoride due to poor oral hygiene are also the least likely to comply with this treatment.[11] For this reason, finding alternative treatments has been a major challenge in the field of remineralization research. However, interesting results have been obtained using arginine, xylitol gums, self-assembling peptides, enamel matrix proteins, crystalline calcium phosphates, polyphosphate systems, unstable calcium phosphates, and stable calcium phosphates, such as casein derivatives.[12–19]

Preventive therapies have been refined to curb demineralization, favor remineralization, and, therefore, halt lesions of active caries.[12] An example of one such preventive therapy is casein, a milk-derived phosphoprotein, which has shown a beneficial effect in preventing and inhibiting the onset of the disease. Casein phosphopeptide (CPP) can bind to phosphate and calcium ions in the dental structure.[20,21] It is thought that this compound is able to intervene in the dynamics of the demineralization/remineralization process, reducing demineralization and increasing the opposite process during an acid challenge of the dental surface.[22] The bioavailability of these remineralizing components inhibits acidic attacks on the tooth surface, thereby enhancing the dynamic remineralization process.[22]

Chitosan is a biopolymer with a high nitrogen content that can function as a carrier to transport calcium and phosphate ions during the biomineralization process.[23] Chitosan has been widely used in the medical field and, although relatively new to dentistry, the potential it holds is promising.[24] On top of promoting the remineralization process, it can also bind to the surface of *Streptococcus mutans*, reducing the availability of these bacteria to bind to others and to colonize the dental surface. Consequently, the formation of dental plaque biofilm is significantly reduced.[25] Moreover, chitosan is positively charged, which allows it to adhere to negatively charged surfaces, such as demineralized enamel, and form a protective film against acidic attacks.[24]

Our study aimed to evaluate the effects of an experimental chitosan/casein gel on enamel demineralization/remineralization in an environment with a high cariogenic challenge. To our knowledge, this is one of the first studies to evaluate enamel demineralization/remineralization with a combination of 1.5% chitosan and 1.5% casein. The null hypotheses included the following: (i) wear profile and surface roughness would not vary after treatment with a combination of chitosan and casein; (ii) time would not influence the enamel roughness analysis after the experimental treatment.

Material and methods

Experimental design

The study involved the experimental treatment with chitosan gel on demineralized enamel in 3 groups (G1 – control, G2 – 1.5% chitosan gel with 1.5% casein, and G3 – 1.5% chitosan gel without casein). The experimental units consisted of 36 enamel specimens obtained from the buccal surfaces of bovine incisors (n = 12 per group). The response variables were volume loss, surface wear and surface roughness. They were evaluated using 3D confocal laser scanning microscope (OLS 4000 LEXT; Olympus Inc., Waltham, USA), and depth of the demineralization lesion, which was measured by means of optical microscopy (Axiostar Plus; Carl Zeiss, Oberkochen, Germany).

Sample preparation

Bovine incisors were freshly extracted and stored in a 0.1% thymol solution (pH = 7.0) at 4°C. The incisors were cleaned with a scaler and water/pumice slurry in dental prophylaxis cups. Teeth with hypoplastic stains, extensive cracks or marked wear were discarded. The teeth were sectioned using an IsoMet™ Low Speed Saw (Isomet 1000; Buehler, Lake Bluff, USA) with a water-cooled diamond disc (Extec Corp., Enfield, USA) in order to obtain enamel slabs measuring 4 mm high × 3 mm wide × 2 mm thick.[26]

The surfaces of the specimens were ground flat, finished and polished using a polishing machine (APL-4; Arotec Indústria e Comércio, Cotia, Brazil) with 400-, 600- and 1,200-grit sandpaper and felt disks impregnated with 0.3-µm and 0.05-µm alumina (Arotec Indústria e Comércio). The dentin surface was also corrected to remove unevenness across the samples. Next, the specimens were immersed in an ultrasonic bath with deionized water (Ultrasonic Cleaner T-1449-D; Odontobrás Indústria e Comércio de Equipamentos Médicos Odontológicos LTDA, Ribeirão Preto, Brazil) for 10 min to remove the polishing debris.[26]

All specimens were covered with a composite resin (3M™ Filtek™ Z350; 3M, St. Paul, USA) to protect all surfaces and only part of the external surface (3 mm) was not covered with resin.[26] This surface was divided in half, with half of the enamel surface (control area) covered with composite resin (without adhesive application) and the other half exposed to a cariogenic challenge.

Initial cariogenic challenge

The artificial caries lesions (white spot formations) were induced by immersing the specimens in 1 L of 50 mM acetate buffer solution (1.28 $mmol^{-1}$ $Ca(NO_3)_2 \cdot 4H_2O$, 0.74 $mmol/L^{-1}$ $NaH_2PO_4 \cdot 2H_2O$, 0.03 µgF, pH 5.0) for 43 h, according to the protocol described by Queiroz et al.[27] After the cariogenic challenge, the specimens were analyzed using a confocal laser scanning microscope for initial measurements and topographic images.

Surface treatment

After the cariogenic challenge,[27] the specimens were stored in artificial saliva[28] for 24 h and randomly assigned to 3 groups. The corresponding experimental treatments were applied: G1 – no treatment, G2 – 1.5% chitosan gel (75–85% deacetylated; Sigma-Aldrich Brasil Ltda., Barueri, Brazil) with 1.5% casein (cat. No. C3400, casein from bovine milk; Sigma-Aldrich Brasil Ltda.) (composition: distilled water, 1.0% glacial acetic acid, 1.5% chitosan powder, casein powder; pH 5.0), and G3 – 1.5% chitosan gel without casein (75–85% deacetylated, Sigma-Aldrich Brasil Ltda.) (composition: distilled water, 1.0% glacial acetic acid, 1.5% chitosan powder; pH 5.0). For groups G2 and G3, the gels were actively applied to the enamel surface with a microbrush (Microbrush® Regular 2.0 mm; Vigodent SA Indústria e Comércio, Rio de Janeiro, Brazil) for 3 min, followed by 2 min of further contact before removal with jets of deionized water.

After each treatment, the specimens were stored in artificial saliva for 6 h.[28] The treatments were performed 3 times at 7-day intervals for a total of 21 days. Between treatments, a pH cycle was carried out in a demineralizing/remineralizing solution[27] for the entire 21-day period.

pH cycling

Twenty-four hours after the end of treatment, the specimens were placed in a demineralizing solution (DES) for 4 h. Then, they were moved to a remineralizing solution (RE) for 20 h[27] at a temperature of 37°C to simulate a high caries risk situation. After each 7-day interval, the specimens were washed with deionized water and re-treated according to each group protocol. After treatment, the specimens were washed as outlined above and the pH cycle was restarted.

The DES consisted of 2.0 $mmol/L^{-1}$ $Ca(NO_3)_2 \cdot 4H_2O$, 2.0 $mmol/L^{-1}$ $NaH_2PO_4 \cdot 2H_2O$, 0.075 $mmol/L^{-1}$ acetate buffer, and 0.02 ppm F (pH 4.7).[27] The RE consisted of 1.5 $mmol/L^{-1}$ $Ca(NO_3)_2 \cdot 4H_2O$, 0.9 $mmol/L^{-1}$ $NaH_2PO_4 \cdot 2H_2O$, 0.150 $mmol/L^{-1}$ KCl, 0.1 mol/L^{-1} Tris, and 0.03 ppm F (pH 7.0).[27] The solutions were applied at a volume ratio per area of 6.25 mL/mm^2 (50 mL) and 3.12 mm^2 (24 mL), respectively. The demineralizing and remineralizing solutions were replaced every 4 days. After 21 days, the specimens were kept in the RE for 24 h, completing the experimental period in 22 days.[17] After that, the specimens were analyzed using a confocal laser scanning microscope to obtain images of the enamel surface.

Volume loss, surface roughness and wear profile analysis

Images were captured using a ×5 objective lens, obtaining a ×107 total optical zoom. After the acquisition of the images, volume loss (µm) was calculated using the difference between the surface ($µm^3$) of the plane of the reference area (control) and the structure lost below its plane. Surface roughness analysis (Sa-µm) was carried out on both the control and demineralized areas to compare the different patterns of surface texture after treatment. For the wear profile analysis (Rv-µm), the control and demineralized areas were calculated using the length of the wear line (in µm) between them (Fig. 1). The same specimens were used for morphological evaluation captured at the end of the pH cycle in relation to the control area, with a ×100 objective lens and a ×2137 optical zoom.

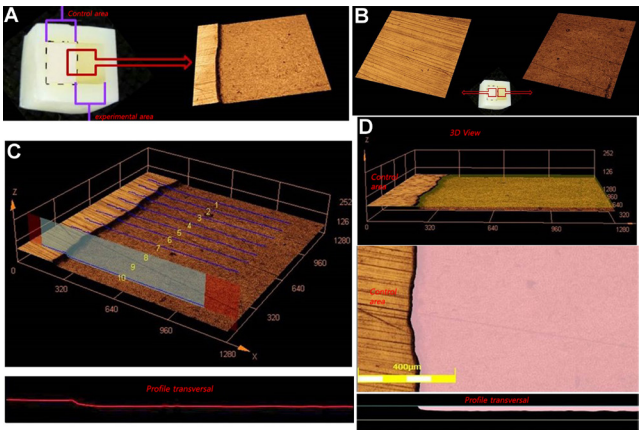

Fig. 1. Confocal laser scanning microscope images and analyses

A – specimen with control area and experimental area after the removal of protection; B – surface roughness of control and experimental area; C – 3D images of wear profile analysis (10 reads of the image, with the control area as a reference point); D – volume loss analysis in 3D images; volume analysis is marked in pink (observed in transversal profile).

Depth of demineralization

After the images were captured using the laser confocal scanning microscope, the specimens were individually embedded into an acrylic resin and longitudinally sectioned using a low-speed water-cooled diamond saw (Isomet 1000; Buehler) to obtain 0.5-mm thick slices. Next, the slices were polished with 600- and 1,200-grit aluminum oxide paper to achieve a thickness of 150 µm. Each slice was then placed in an ultrasonic cleaner with distilled water for 10 min. In the end, 2 sections were obtained per specimen and taken for the analysis under an optical microscope (Axiostar Plus; Carl Zeiss). Images were obtained with ×40 magnification and captured using a digital camera coupled to the microscope. Measurements of the demineralization depth (µm) of the initial white spot lesion and the white spot lesion after the demineralizing pH cycle were obtained using AxioVision® software LE v. 4.3 (Carl Zeiss).

Statistical analysis

The data was analyzed for normality and homogeneity, and data distribution was non-normal only in the case of surface roughness. Therefore, the Kruskal–Wallis test was performed. For wear profile, volume loss and depth of demineralization, one-way analysis of variance (ANOVA) was used. For surface roughness, an ANOVA was employed to compare the different time points of the treatment (white spot, after treatment, after cycling). For differentiation of the means, the Tukey's test was performed for all variables ($p < 0.05$).

Results

The analysis of volume loss revealed that the experimental chitosan gel without casein group was similar to the experimental chitosan gel with casein ($p > 0.05$). The 2 groups significantly differed from the control group, which showed a higher volume loss (Fig. 2A–C). For the wear profile and surface roughness, all groups showed similar values ($p > 0.05$; Table 1).

For the surface roughness analysis at different time points (Fig. 3), a statistically significant difference was observed between the initial and final evaluation for all groups ($p < 0.05$), with a significant increase in values (Table 2).

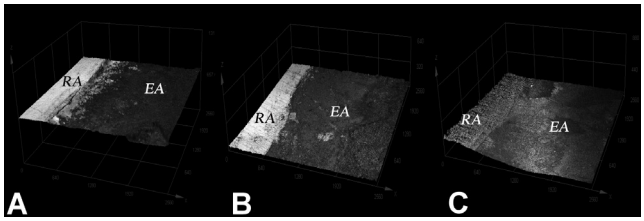

Fig. 2. Wear profile between the reference area (RA) and the experimental area (EA) among the groups

A – control; B – experimental chitosan gel with casein; C – experimental chitosan gel without casein.

Table 1. Mean and standard deviation of the volume loss [µm], wear profile [Rv-µm] and surface roughness [Sa-µm] of the different groups

Group	Volume loss	Wear profile	Surface roughness
G1 – control	15.15 ±9.79^{B}	4.10 ±1.26^{C}	0.96 ±0.39^{C}
G2 – chitosan/casein gel	10.66 ±9.21^{AB}	3.66 ±1.14^{C}	1.05 ±0.37^{C}
G3 – chitosan/casein-free gel	3.88 ±3.80^{A}	3.24 ±1.68^{C}	0.87 ±0.39^{C}

The values in the columns have been compared. The same capital letters indicate statistical similarity ($p > 0.05$).

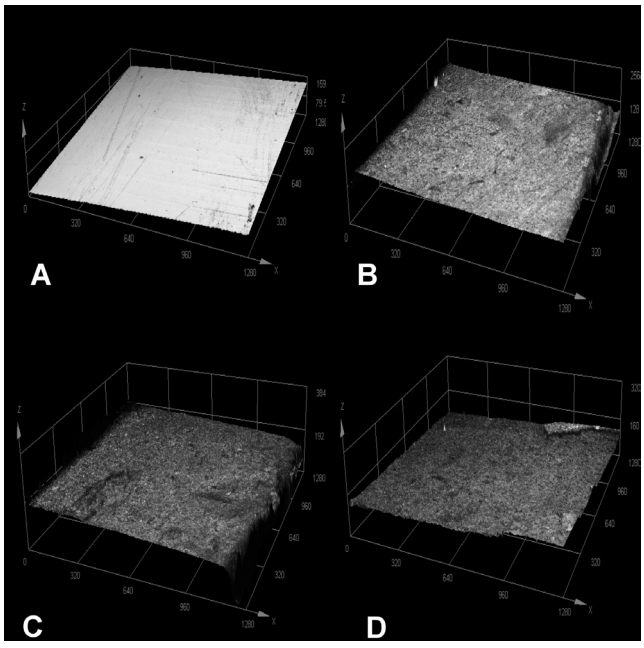

Fig. 3. Topography of the different groups after the pH cycle

A – surface roughness before the pH cycle/surface treatment; B – control; C – experimental chitosan gel with casein; D – experimental chitosan gel without casein.

Table 2. Mean and standard deviation of surface roughness [Sa-µm] at different time points

Time point	G1	G2	G3
White spot	0.59 ±0.39^{AB}	0.53 ±0.14^{AB}	0.52 ±0.26^{A}
After treatment	0.40 ±0.35^{A}	0.45 ±0.21^{A}	0.44 ±0.19^{A}
After cycling	0.96 ±0.39^{B}	1.05 ±0.37^{B}	0.87 ±0.39^{B}

The same capital letters indicate statistical similarity ($p > 0.05$). G1 – control; G2 – 1.5% chitosan gel with 1.5% casein; G3 – 1.5% chitosan gel without casein.

The 3 groups presented similar behavior concerning demineralization depth (Table 3 and Fig. 4). No experimental treatment was able to inhibit the process. In the comparison between the white spot and treated areas, the data showed that for all groups there was no significant increase in the demineralization depth of the treated areas.

Table 3. Mean and standard deviation of the demineralization depth of the white spot lesion, treated area (experimental) and the difference between them (in µm)

Area	G1	G2	G3
White spot lesion	0.09 ±0.03^{A}	0.08 ±0.03^{A}	0.08 ±0.03^{A}
After pH cycling	0.12 ±0.03^{B}	0.11 ±0.04^{B}	0.13 ±0.03^{B}
Difference	0.04 ±0.02^{C}	0.03 ±0.02^{C}	0.04 ±0.02^{C}

The same capital letters indicate statistical similarity ($p > 0.05$).

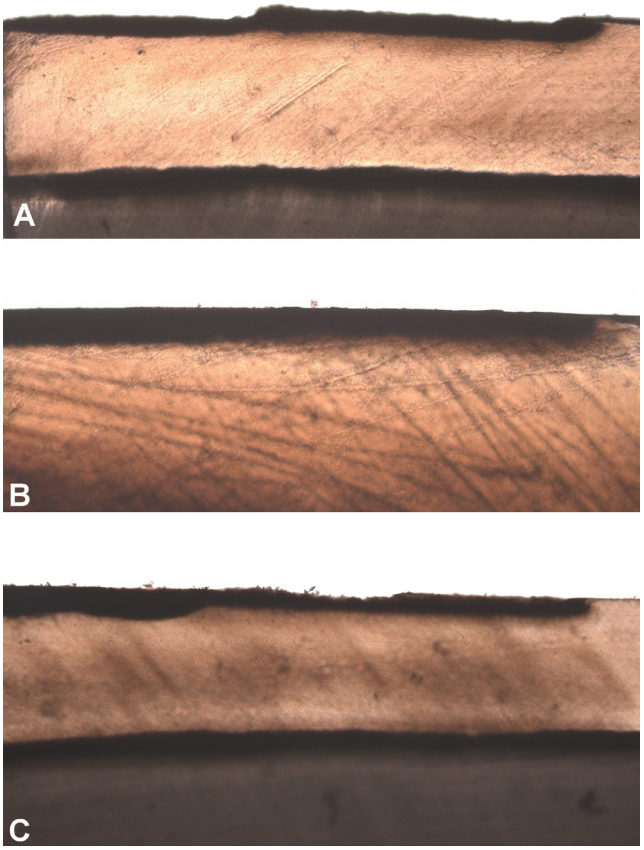

Fig. 4. Depth demineralization lesion

A – control; B – experimental chitosan gel with casein; C – experimental chitosan gel without casein.

Discussion

Dental caries is the result of an imbalance in the dynamics of the enamel remineralization and demineralization processes, mainly due to a low oral pH after the release of acid by facultative anaerobic bacteria.[6,7] To simulate this condition, the current study subjected dental enamel to an intense cariogenic challenge that provided a high risk for caries. Accentuated enamel loss, including the formation of cavities, was observed in some specimens.

The substrate used in the present study was bovine teeth enamel. This methodology was based on previous studies that employed this substrate for studies of minimally invasive processes in enamel.[29] Moreover, its use is supported by recent studies, which verified that the microstructure of bovine enamel is very similar to human enamel,[30] making its use feasible for research in this area. Additionally, it has been observed that the use of this substrate in the microbial caries model showed similar behavior to human teeth.[31]

To suspend or minimize the structural loss in high-risk caries, an experimental chitosan/casein treatment was employed, as literature has shown that casein, a milk protein, is similar in function to proteins responsible for the biomineralization of the tooth.[20,32] Casein phosphopeptide, in particular, can bind to surfaces such as plaque, soft tissue and dentin, as well as provide a reservoir of bio-available calcium and phosphate in the saliva and on the surface of dental structure.[20,32] Under oral acid conditions, CPP considerably increases the solubility of calcium phosphate by stabilizing amorphous calcium phosphate (ACP) through the presence of phosphorylated serine groups in its structure.[33] The process of remineralization of the subsurface occurs through the dissociation of the CPP-ACP complex and slows the release of calcium and phosphate ions as the pH in the plaque decreases producing a supersaturation state that reduces the demineralization process.[33,34] The large calcium reservoir within the plaque delays the diffusion of free calcium ions and provides a source of calcium for remineralization, thus restricting mineral loss during a cariogenic episode.[22,33,35] Incorporating casein peptides into enamel plaques increases the enamel's calcium and phosphate content, thereby depressing its demineralization and improving remineralization.[22,32,33] Since the effectiveness of casein has been demonstrated by several studies,[34,36,37] it was suggested to combine the remineralizing property of casein with the multi-beneficial properties of chitosan.

The experimental gels were produced on a chitosan base (with or without casein) and prevented the continuity of the mineral loss in the enamel specimens and caused little volume loss in this group, without interfering with demineralization depth. Therefore, the first null hypothesis was accepted. This result can be explained since chitosan, besides possessing a strong positive charge at a low pH, can absorb other structures with a negative zeta potential, such as enamel,[38] thus protecting the organic layer against cariogenic challenges.[38,39]

In an oral environment, the caries process produces lactic and acetic acids that infiltrate the interprismatic spaces, which results in a continuous decrease in oral pH (range 5.0–5.5) that consequently leads to mineral loss by a binding of the positive hydrogen ions (H^+) of the acid to the negative phosphate (PO_4^{3-}) and hydroxyl ions (OH) of the enamel structure that transformed in water as well as HPO_4^{2-}, until the moment of saturation.[40] The chitosan amino groups can decrease mineral loss by apprehending the acid's hydrogen ions and creating a positively charged protective layer that prevents the diffusion of hydrogen ions into the mineral surface.[3,38,41] Chitosan disrupts the H^+ ions of the acid solution, inhibiting the demineralization process and increasing the pH of the solution.[42] The chitosan-based layer formed by the reaction of these biopolymer molecules with the enamel surface leads to coverage of at least a few nanometers[43] of a ubiquitous chitosan layer.[44] The variation in the sample surface may explain the high standard deviation in volume loss found in this study. This is corroborated by Ruan et al.[45] who demonstrated the efficacy of an amelogenin-containing chitosan hydrogel as a biomaterial for the biomimetic reconstruction of the enamel structure. The employment of nano-complexes of phosphorylated chitosan (Pchi-ACP)[41] and ACP on enamel lesions had a remineralization effect significantly higher than that of fluoride.[46] In addition, chitosan in a fluoride-containing varnish significantly inhibited the enamel demineralization process.[47] As mentioned above, chitosan can be widely used, regardless of the material type, to minimize dental demineralization.

However, contrary to expectations, the results of the present study did not demonstrate significant differences in volume loss or in the wear profile of enamel in the experimental chitosan group with casein when compared to the chitosan group without casein. The promising effects of casein on the enamel demineralization process were not demonstrated in this study. On the other hand, it was observed that casein reduced the therapeutic effect of chitosan, increasing the intensity of the mineral loss due to the demineralization process, and produced greater volume loss, similar to the control group. It is possible that casein could have lost affinity for enamel under the severe acidic conditions,[48] and may have affected the performance of the chitosan. As far as we know, few studies have examined the efficacy of chitosan associated with casein in enamel remineralization under the conditions similar to this study. Therefore, it is difficult to make any comparisons. One study found that casein was not effective in remineralizing early enamel caries at the subsurface level.[49] Moreover, casein demonstrated a lower efficacy than fluoride in remineralizing early enamel caries at the surface level.[50–52] Although CPP-ACP was able to remineralize the early caries lesion, low hardness values were found in those studies, suggesting that the remineralization process was not efficient enough to increase the hardness values. Chitosan added to the CPP-ACP (GC Tooth Mousse) had no additional effect on tooth enamel remineralization.[53]

Although the demineralization depth in the remaining tissue was the same, structural loss and cavity formation were different, with the chitosan gel without casein exhibiting better surface integrity. However, no difference was observed among the control, chitosan gel with and without casein groups regarding the surface roughness. The only difference was found in the first and final treatment; that is, after the cycling and conclusion of the treatment, thus rejecting the second hypothesis of this study. This result is probably due to the active application of the agents with the microbrush, which favors a smoother surface of the demineralized enamel. As the demineralizing/remineralizing process and mineral loss were continuous, this procedure probably favored a rougher surface, regardless of the treatment performed. These results will lead to further investigations of chitosan gel formulations, as there is still a promising potential for the use of chitosan-based materials in the remineralization process of white spot lesions.

It is known that the demineralization process promotes mineral loss of the hydroxyapatite crystals, leading to the appearance of white spot lesions due to a loss of the optical properties of the hard tissues.[21] Thus, if a treatment is not effective, demineralization is greater, as shown in this study, where the 3 groups showed a similar increase in the demineralization levels, and only the experimental treatment of chitosan gel without casein presented a satisfactory result regarding lower enamel structure loss. Due to the lack of studies on the interaction between both materials, it is necessary to conduct more studies focused on the improvement and cohesion of materials with a preventive effect, testing new components that add value to their clinical properties. Additionally, necessary reformulations can be performed to improve materials' properties against the enamel demineralization process.

Conclusions

Chitosan seems to be the major component responsible for reducing the volume loss of demineralized enamel, yet it did provide a slight alteration in surface smoothness. In this study, no additive effect of casein on the demineralized enamel surface has been demonstrated.

Ethics approval and consent to participate

Not applicable.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

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