Combined morphological-compositional analysis of the interaction of collagen and chitosan based-materials with demineralized bovine dentin

Análise morfológica-composicional combinada da interação de materiais à base de colágeno e quitosana com dentina bovina desmineralizada

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ABSTRACT
Treatments to promote the biomineralization of dentin are the focus of much research with different materials and processes. Biomineralization is a process mediated by an organic matrix in which organic macromolecules act as models for the nucleation and growth of mineral crystals to form hierarchically ordered hybrid materials, such as bones or teeth. This study describes the application of novel materials based on collagen and chitosan aimed to protect the dentin from erosive conditions. The dentin morphology of the bovine teeth treated with these materials was visualized using scanning electron

microscopy (SEM). In additions, it was possible to confirm the data by analyzing the distribution of inorganic content of dentin by micro energy-dispersive X-ray fluorescence spectrometry (µ-EDXRF). The association of analytical techniques demonstrated greater surface coverage for the chitosan-fluoride followed by the hydrolyzed collagen. Both materials are promising for the application of dentin coverage in dentistry.

Keywords: Chitosan, collagen, fluoride, scanning electron microscopy, micro energy-dispersive X-ray fluorescence spectrometry.

RESUMO
Tratamentos para promover a biomineralização da dentina são o foco de muitas pesquisas com diferentes materiais e processos. A biomineralização é um processo mediado por uma matriz orgânica em que macromoléculas orgânicas atuam como modelos para a nucleação e crescimento de cristais minerais para formar materiais híbridos ordenados hierarquicamente, como ossos ou dentes. Este estudo descreve a aplicação de novos materiais à base de colágeno e quitosana com o objetivo de proteger a dentina das condições erosivas. A morfologia dentinária dos dentes bovinos tratados com esses materiais foi observada em microscopia eletrônica de varredura (MEV). Além disso, foi possível confirmar os dados analisando a distribuição do conteúdo inorgânico da dentina por micro fluorescência de raios X por energia dispersiva (µ-EDXRF). A associação das técnicas analíticas demonstrou maior cobertura superficial para o fluoreto de quitosana seguido do colágeno hidrolisado. Ambos os materiais são promissores para a aplicação de coberturas dentinárias em odontologia.

Palavras-chave: Quitosana, colágeno, flúor, microscopia eletrônica de varredura, micro fluorescência de raios X por energia dispersiva

1 INTRODUCTION

Treatments to promote the biomineralization of dentin are the focus of much research with different materials and processes. (CAO et al. 2015; DI FOGGIA et al. 2019; GAJJERAMAN et al. 2007; NIU et al. 2014). Biomineralization is a process mediated by an organic matrix in which organic macromolecules act as models for the nucleation and growth of mineral crystals to form hierarchically ordered hybrid materials, such as bones or teeth (DI FOGGIA et al. 2019).

The search for materials that have some affinity for dentin allows the development of new surface treatments that have the ability to protect dentin against mineral loss situations (demineralization) as well as to treat dentin by incorporating ions or particles after erosive processes (remineralization).

Some biomimetic strategies have been developed to achieve collagen remineralization, inspired by the behavior of the protein matrix in biomineralization processes (CAO et al. 2015; DI FOGGIA et al. 2019; GAJJERAMAN et al. 2007; KIM et al. 2010; NIU et al. 2014; TAY; PASHLEY, 2008). Among the biomineralization strategies we can highlight the treatment with phosphorylated chitosan (XU et al. 2011).

Studies using chitosan to treat dental erosion show promising results where the material has a significant protective effect (URURAHY et al. 2017). The association of chitosan with fluoride has also been tested with a bioadhesive of chitosan-fluoride microparticles that demonstrated dentin mineralization properties (KEEGAN et al. 2012).

Due to the role that the organic matrix plays on the nucleation and the growth of mineral particles and the high organic portion of dentin, collagen or collagen-containing products could be sources for possible mineralization or protection of dentin. In addition, the incorporation of chitosan in fluoride products can also be an option for protection or treatment of dentin. Therefore, this study demonstrated the possibility of the application of novel materials based in collagen and chitosan in demineralized bovine dentin.

2 MATERIALS AND METHODS

2.1 SAMPLES

Ethical approval for this study was granted by the local Ethics Committee (approval number: A03/CEUA2019, Ethics Committee of the Universidade do Vale do Paraíba, Univap, Brazil). Sample preparation followed the protocol of our group previously tested (SOARES et al. 2020). The exposed dentin from five bovine teeth had the surface pre-treated by etching with 37% phosphoric acid (CONDAC 37, FGM, Joinville, SC, Brazil) for 15 s and cleaned with distilled water for 30 s and dried with absorbent paper to simulate an erosion-demineralized surface and to increase dentin surface energy (NAHORNY et al. 2017).

The following five novel materials were prepared and applied to each dentin sample surface: Hydrolyzed collagen (fibers) (HF_Col), non-hydrolyzed collagen (powder) (NH_Col), hydrolyzed collagen (powder) (H_Col), chitosan (powder) (Chi) and a combination of chitosan powder and acidulated phosphate fluoride gel (Chi_F). The materials were weighed and diluted in distilled water.

These solutions were applied by immersing the dentin blocks in plastic vials containing 3 ml of the respective solutions. This application was made on dentin for 5 hours, under agitation on a magnetic stirrer (75 rpm, Model 752A, FISATOM), as a surface treatment. At the end of this treatment step, the samples were characterized by means of combined morphological-compositional analysis.

2.2 COMBINED MORPHOLOGICAL-COMPOSITIONAL ANALYSIS

The compositional analysis was performed using the micro energy-dispersive X-ray fluorescence spectrometry system (μ-EDXRF, model μ-EDX 1300, Shimadzu, Kyoto, Japan). The elemental distribution area maps (n = 5) were obtained with the following parameters: 20 × 20 points; steps, 20 μm; voltage, 15 kV; incident beam diameter, 50 μm. The data obtained and the images resulting from the area mappings were processed in the system software (SOARES et al. 2019).

For the morphological analysis, the dentin samples were dehydrated using a graded series of ethanol (30%, 50%, 70%, 95%, and 100%) for 10 minutes at each stage. Then they were dried and metallized with a gold layer and examined by SEM (EVO-MA10, Carl STM Zeiss, Oberkochen, Germany) (NAHORNY et al. 2017; SOARES et al. 2020).

3 RESULTS

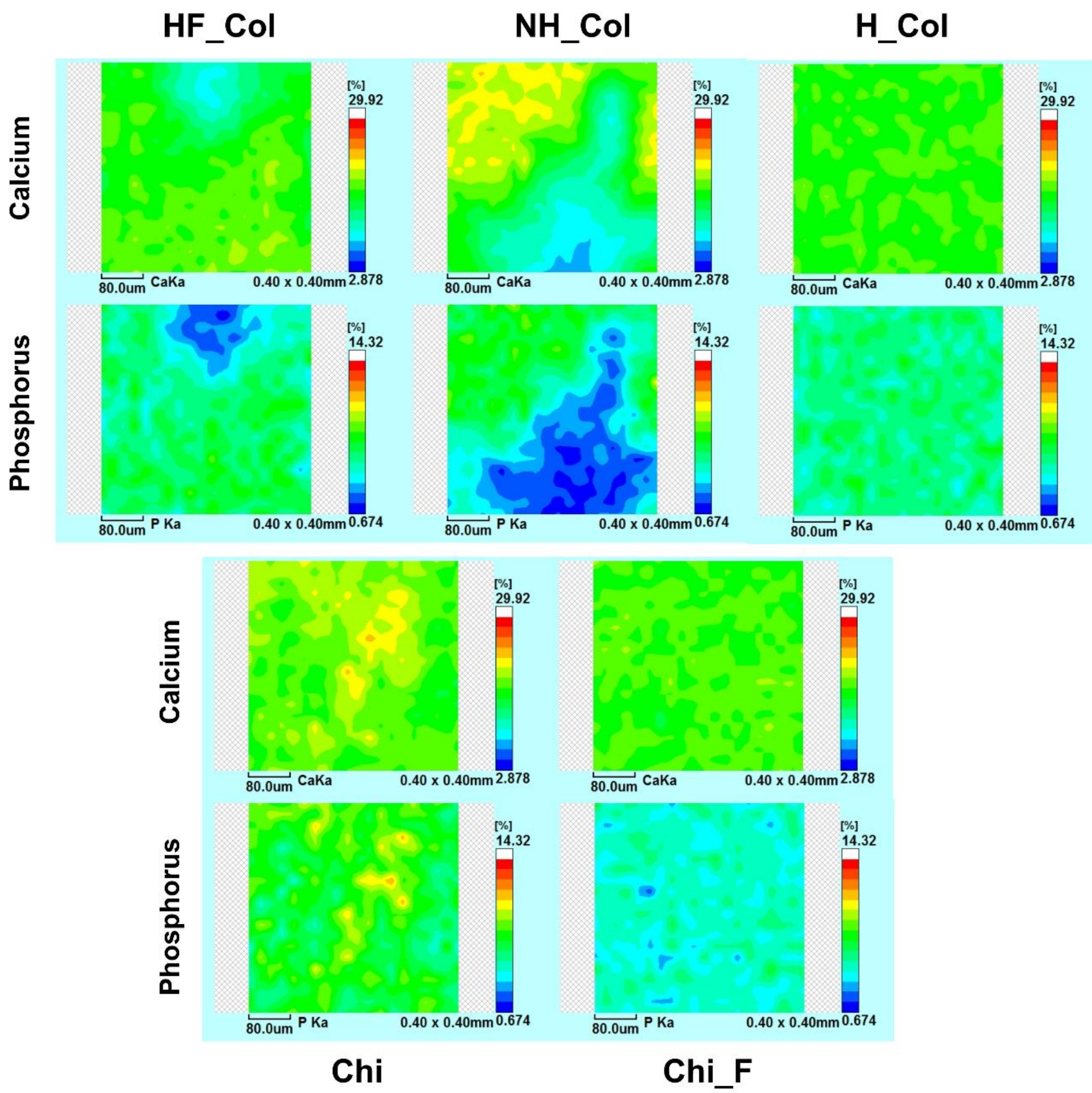
The data obtained from the dentin surface area mapping indicate that the covering with H_Col and Chi_F materials resulted in the lowest standard deviation and variance coefficient in the concentration of both calcium (Ca) and phosphorus (P) elements (Table 1). The homogeneity of Ca and P dentin content was found in the order Chi_F > H_Col > Chi > HF_Col > NH_Col.

Table 1. Results obtained by μ-EDXRF area mapping, after treating the dentin surface with biomaterials. Maximum, minimum, mean, standard deviation (S.D.) and coefficient of variation (C.V.) from the percentages by weight (% -weight) of calcium (Ca) and phosphorus (P) on the dentin surface.

Calcium	**Maximum**	**Minimum**	**Mean**	**S.D.**	**C.V.**
HF_Col	19.47	9.10	15.61	2.06	13.18
NH_Col	21.76	6.61	14.94	3.92	26.21
H_Col	18.70	14.38	16.21	0.56	3.44
Chi	22.16	13.92	17.59	1.30	7.40
Chi_F	19.68	13.71	16.56	0.61	3.68
Phosphorus	**Maximum**	**Minimum**	**Mean**	**S.D.**	**C.V.**
HF_Col	7.99	0.67	5.20	1.18	22.63
NH_Col	9.72	0.76	4.28	1.90	44.40
H_Col	7.46	3.51	5.31	0.54	10.17
Chi	10.80	3.81	6.88	0.98	14.22
Chi_F	6.94	2.01	4.38	0.48	11.04

The images of the chemical distribution of the elements calcium and phosphorus on the dentin surface after the treatments illustrated different patterns of interaction with the dentin for the collagen groups and the chitosan groups (Fig. 1). The gradient in the intensity of the color scale indicates variations in the Ca content so that places with high mineral content are represented in yellow and orange colors, while places with low mineral content are shown in shades of green and blue.

Figure 1. Representative images of calcium (Ca) and phosphorus (P) distribution at the dentin surface obtained by μ-EDXRF microanalysis from treatments: HF_Col, NH_Col, H_Col, Chi and Chi_F. The different colors represent the intensities of the components Ca and P and their distribution on the dentin surface.

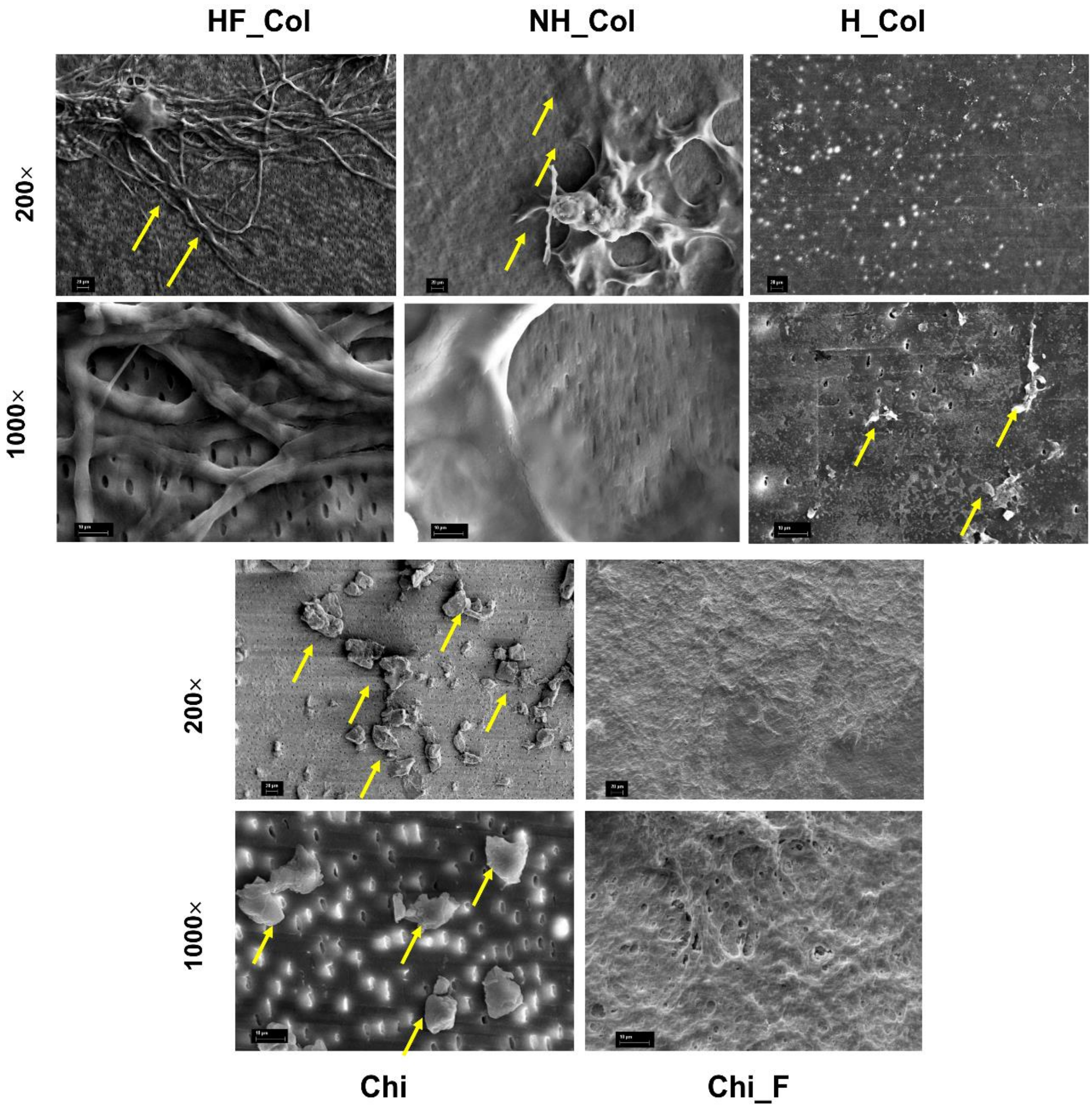


Among the collagen treatments, the one that showed the most homogeneous distribution was the dentin treated with hydrolyzed collagen (Fig. 1). For groups treated with chitosan, the Chi_F treatment showed the best material distribution on the surface (Fig. 1).

For the HF_Col group, collagen fibers adhered to the surface, partially covering the dentinal tubules in a heterogeneous disposition (arrows, Fig. 2). The application of the NH_Col material resulted in the deposition of a collagen layer more densely adhered and with greater obliteration of the entrance of the dentinal tubules (arrows, Fig. 2) than in the HF_Col group (Fig. 2). The micrograph of the dentin surface in the H_Col group shows the entrance of the dentinal tubules partially obstructed by collagen deposits (arrows, Fig. 2) covering the surface more evenly than the other collagen materials.

In the Chi group, the chitosan irregularly covered the dentin with deposits that partially obstructed the entry of dentinal tubules (arrows, Fig. 2). In the Chi_F group, the material completely covered the dentin surface without any exposure of dentin tubules (Fig. 2).

Figure 2. Representative SEM micrographs illustrating differences in the morphological pattern of dentin surface produced by the collagen and chitosan materials (magnification: 200× and 1000×). Sample treated with HF_Col, NH_Col, H_Col, Chi and Chi_F. The yellow arrows indicate collagen or chitosan deposits in the respective groups. Magnification bar: 20 µm in A-C, and 10 µm in D-F.



4 DISCUSSION

Dentin remineralization is a procedure with clinical relevance in the treatment of caries, dentin hypersensitivity and dental erosion (DI FOGGIA et al. 2019). Understanding how the components present in dentin interact with biomaterials is important to select and produce compounds containing materials that have a more direct interaction by ionic and chemical affinity with the components of dentin.

In the present study, µ-EDXRF and SEM demonstrated the effect of surface coverage on dentin by the materials (partial or total) with the greater layer uniformity obtained for Chi_F and H_Col (in order: Chi_F > H_Col > Chi > HF_Col > NH_Col).

The groups of collagen in fibers (HF_Col) and non-hydrolyzed collagen (NH_Col) did not showed enough interaction with dentin to form a protective layer as demonstrated in the chemical distribution images obtained by the µ-EDXRF area mappings (Fig. 1) and in the SEM micrographs (Fig. 2).

The affinity/interaction of hydrolyzed collagen (H_Col) and chitosan (associated or not with fluoride) with the dentin surface was demonstrated by the µ-EDXRF results (Table 1, Fig. 1). SEM micrographs corroborate and confirm the results. The micrographs showed that the positive effect of the surface coverage layer was more effective in the H_Col (Fig. 2) and Chi_F materials (Fig. 2). The chemical etching by the use of phosphoric acid in gel form is justified to enable control of the application area (DOS SANTOS FILHO et al., 2021), and to create a demineralized surface simulating a clinical condition. This etching exposed the dentin collagen and probably created a favorable substrate to adhesion of tested materials.

The application of these novel materials in dentin resulted in the deposition of a layer with a homogeneous profile (Fig. 1). The SEM micrographs confirmed these results where the obliteration of the dentin tubules was observed (Fig. 2). The combined morphological-compositional analysis evidenced the possible use of the association between chitosan powder and acidulated phosphate fluoride gel to produce a novel material with significant clinical relevance in protecting the dental structure.

The results obtained by the H_Col group are also relevant, both for the aspect of obtaining a homogeneous surface layer (Figs. 1 and 2) and for the aspect of the chemical composition of the material and possible interaction and affinity with the dentin collagen itself. The possibility of conducting area mappings on the dentin surface allows understanding how the material behaves in a given location with the advantage of greater precision of details and if the material produces a heterogeneous particle distribution on the surface as in the case of HF_Col and NH_Col (Fig. 1).

The association of combined morphological-compositional analysis was advantageous to study the interaction of materials with the dentin surface. The applied analytical tools allowed us to investigate the chemical composition associated with the morphology of the particles and their distribution along the surface, indicating as the best material options the hydrolyzed collagen and chitosan associated with fluoride.

5 CONCLUSION

The highest surface coverage was observed in the Chi_F group followed by the H_Col group. The materials based on collagen and chitosan are promising for the application of dentin coverage in dentistry.

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