

Biomodifying Effect of Different Cross-Linking Agents on the Mechanical Properties of Dentin Collagen

Andreza Camurça Ferreira,¹ Talita Arrais Daniel Mendes,^{1,2} Marcelo Victor Sidou Lemos,² Déborah Maria Martins de Paula,¹ Karlos Eduardo Rodrigues Lima,¹ Luiz Filipe Barbosa Martins^{1,3}

¹Dentistry Course at the Centro Universitário Católica de Quixadá (Unicatólica), Quixadá, CE, Brazil

²Department of Restorative Dentistry, Universidade Federal do Ceará, Fortaleza, CE, Brazil

³Faculdade Paulo Picanço, Fortaleza, CE, Brazil

• **Conflicts of interest:** none declared.

ABSTRACT

Objective: to evaluate the biomodifying effect of cross-linking agents on the mechanical properties of the teeth. **Material and Methods:** this is an in vitro laboratory study, in which 18 bars of dentin were made from extracted, caries-free human third molars. The samples were demineralized in 10% phosphoric acid for 5 hours, randomly distributed in different groups and kept in their pre-treatment solutions: distilled water (AD), Proanthocyanidin at 6.5% (PAC6.5) and Cardanol at 6.5% (CAR6.5) for periods of 1 hour. Three-point bending tests were performed to obtain the modulus of elasticity (ME) and mass modification (MM). They were submitted to the Kolmogorov-Smirnoff test followed by the Kruskal-Wallis' test and the Tukey's post-test for mass modification, and Kruskal-Wallis' test after a Dunn's test for difference in the modulus of elasticity ($p < 0.05$). **Results:** the values selected in the ME show possible statistical changes in all groups tested, when compared to the negative control ($p < 0.001$), as well as the mass variation. Data were shown in the average values between the groups tested ($p = 0.012$). **Conclusion:** The use of PAC6.5 will improve the mechanical properties of the collection.

Keywords: Dentin; Collagen; Proanthocyanidins.

Introduction

In restorations with composite resins, adhesive systems are retained to the dentin in a micromechanical way, thus promoting the union of the restoration to the tooth. The interaction between adhesive and dentin manifests an interdiffusion zone called the hybrid layer (HL).^{1, 2, 3, 4}

HL has distinct forms of degradation, which represent the most fragile link of the union to dentin. Degradation can occur in a hydrolytic, enzymatic, and/or polymeric way. Hydrolytic degradation occurs through the binding of hydrophilic and ionic monomers, and due to this junction, the layer generated by adhesive systems is susceptible to water penetration and consequent degradation of the hybrid layer.⁵ Another way in which degradation can occur is through enzymatic digestion.^{6, 7} Metalloproteinases (MMPs) and cysteine cathepsins (CTPs) degrade exposed/demineralized collagen fibers (CF) from the carious process or by acid conditioning in adhesive procedures.^{8, 9, 10, 6, 7} Polymer degradation occurs by polymerization contraction generated by restorative materials in the absence of cross-links, generating gaps and causing degradation of the hybrid layer.⁵

The hybrid layer resulting from the union of dentin affected by caries, partially demineralized, is heterogeneous. Dentin affected by caries is hypomineralized, because of this area presents a greater number of pores and causes greater infiltration of water in the HL region. Thus, there is a large number of collagen fibrils unprotected and exposed to hydrolytic degradation, as well as endogenous enzymes (MMPs and CTPs). With this, research has aimed to

improve the mechanical properties of the hybrid layer and prevent the collagen fibers from degradation, such as dentin biomodification, through cross-linking agents (CLA) and inhibitors of MMPs and CTPs.^{1, 12, 13, 14, 6, 15, 16}

Collagen is responsible for collaborating with the structural unit of dental tissues. Dentin consists of 20% collagen, and some studies show that to obtain an ideal union with adhesive systems (AS) it is necessary that dentin is demineralized and the collagen fibers, externalized. With dentin demineralization, the collagen fibers may be degraded due to incomplete impregnation by the adhesives systems, leading to a less enduring restructuring process.^{17, 18, 19}

There has been a remarkable advance in the field of adhesive systems in later years, which has favored a complete remodeling in the evolution of restorative dentistry. The usual cavity preparations are traced based on dental anatomy and carried out in a standardized manner with unnecessarily large proportions, which over time is changing through less invasive restorative techniques, due to the contribution of the most recent adhesive materials.²⁰

Thus, to improve the durability of these restorations, there has been research on the cross-linking agents that would form bonds between amino acids present in the triple helix structure of collagen, generating a more rigid structure between the resin and dentin,²¹ besides acting by inhibiting CTPs and MMPs. There are two classes of cross-linking agents; synthetic and natural. Natural agents have gained a greater prominence in the literature. Among them are the Proanthocyanidins (PACs) that demonstrate advantages

in the improvement of properties such as tensile strength, adhesion and biodegradation, hardness, greater modulus of elasticity and reduction of demineralization.^{21, 22, 23, 24, 25, 26, 27}

Another natural component currently quite relevant are the components of the cashew nut bark liquid, as its constituents have favorable effects on demineralized dentin. The standing-out molecules in this component are cardol and cardanol, as they are able to cross-link and present a long carbon chain ending in polyphenols. These molecules increase resistance to biodegradation and improve adhesion to caries-affected dentin.^{28, 29}

Therefore, there are still not many significant studies that investigate how long these connections remain active, an essential point of data to predict the effectiveness of this type of dentin treatment. Therefore, the aim of this study is to evaluate the biomodifying effect among cross-linking agents with dentin collagen.

Materials and Methods

The project of research was approved by the Ethics Committee under the CAAE number 86044218.4.0000.5046. This is an *in vitro* laboratory study in which the factors under investigation were pretreatments in dentin with: distilled water (AD), PAC 6.5% (95% PACs, *Vitis vinifera*, Mega-Natural Gold, Madera, USA) [PAC6.5], and Cardanol 6.5% (Amendoas do Brasil LTDA, Fortaleza, Brazil) [CAR6.5] for 1 hour and submitted to three-point bending tests to obtain modulus of elasticity (ME) and mass modification (MM), with the aid of a universal testing machine and a high-precision analytical balance, respectively.

Cardanol synthesis

Cardanol was obtained from the main liquid of the industrial cashew nut supplied by Amendoas do Brasil LTDA (Fortaleza, Brazil) separated by column chromatography (silica gel 60) and characterized by gas chromatography / mass spectroscopy.

Preparation of specimens

We used 18 third permanent molars, free of caries, obtained through a teeth donation term signed by patients who attended the surgery clinic of the Catholic University Center of Quixadá. The teeth were manually cleaned with periodontal cures in order to remove organic and inorganic remnants, and stored in saturated solution of thymol 1%, at a temperature of 4°C, until the time of use (up to 60 days).

The teeth were selected to make dentin bars with dimensions 1.7 X 0.5 X 6.0 mm, that were crafted with the aid of a metallographic cutter in low rotation under constant refrigeration (Isomet 4000 Buehler, Lake Bluff, USA). Then, the specimens were completely demineralized, in 10% phosphoric acid solution, during 5 hours, at room

temperature and in constant agitation. The dentin bars were randomly distributed in groups (n=10) and kept in their respective solutions (distilled water, PAC 6.5%, and Cardanol 6.5%) for one hour.²⁷

Modulus of Elasticity

The modulus of elasticity (ME) of demineralized dentin bars was evaluated at the beginning and after immersion in their respective solutions. This data was determined in a three-point bending test with a 5.0 N load cell built on a universal testing machine (Instron, Canton, Massachusetts, USA) with a velocity of 0.5 mm/min. The data were expressed in mega pascal (MPa), and the increase in ME was calculated between the ratio of the final value (after the biomodification of the dentin in the respective groups) and the initial values (“baseline”).²⁷

Mass modification

The masses were measured before (M1) and after (M2) the biomodification process of the demineralized dentin bars, with an analytical scale (Shimadzu, Kyoto, Japan) with five decimal places of accuracy (0.00001g). The samples were then dehydrated in a vacuum desiccator for 24 hours at room temperature. The evaluation of mass change (MM%) was determined as the percentage of gain or loss of each sample based on the following formula: $MM\% = ((M2 \times 100)/M1) - 100$. In which M1 is the demineralized dentin matrix mass before dentin biomodification and M2 is the biomodified dentin matrix mass.^{27th}

Data analysis

Data analysis was carried out through the SigmaPlot Software 14.0 program (SYSTAT Software, Point Richmond, CA, USA). The Kolmogorov-Smirnoff test was applied to test the normal distribution of the data, in which a parametric nature was observed; then, an ANOVA test followed by a Tukey post-test were carried out for mass variation as well as a Dunn’s test for the flexural modulus of elasticity. A significance level of 5% was established.

Results

We can assess that the for the modulus of elasticity groups the PAC6.5 (9.1 ± 2.3) presented a statistically significant result ($p < 0.001$) when compared to the CAR6.5 (2.3 ± 0.9). The PAC6.5 also had a higher mechanical property than others. The distilled water group was statistically different from CAR6.5 and PAC6.5, presenting an inferior gain in modulus of elasticity when compared to the others (Table 1).

For the evaluation of mass modification there was a statistically significant difference ($p < 0.05$). We can observe that the PAC6.5 (100.8 ± 4.8) showed a greater variation in mass, however, it was statistically similar to the CAR6.5 (73.8

± 4.5). The distilled water group did however obtain the lowest gain when compared to the others (p=0.012) (Table 2).

Table 1. Modulus of elasticity (Mpa) [mean and standard deviation] of dentin specimens pretreated in the respective groups for 1 hour.

Experimental groups	1 hour immersion
Distilled water	1.1 ± 0.6 C
PAC6.5	9.1 ± 2.3 A
CAR6.5	2.3 ± 0.9 B

Different letters show statistical differences (p<0.05).

Table 2. Mean mass variation % (± mean and standard deviation), after demineralization of specimens and use of respective treatment groups, for 1 hour.

Experimental groups	1 hour immersion
Distilled water	67.6 ± 6.3 B
PAC6.5	100.8 ± 4.8 A
CAR6.5	73.8 ± 4.5 A

Different letters show statistical differences (p<0.05).

Discussion

It was noted how important it is to restructure, remineralize and strengthen the collagen structure. As such, new cross-links are being used, in order to reduce degradation in the collagenous interface and improve the adhesion of resinous materials without causing degradation of collagen fibrils, thus inhibiting the enzymes MMPs and CTPs, and preventing the flow of water.^{30, 31}

According to the results of this study, the effectiveness of the treatment groups CAR6.5% and PAC6.5% was noted, as they showed an increase in the modulus of elasticity and mass gain. Breschi *et al*³¹ and Delgado *et al*³² mentioned the use of synthetic cross-links (carbodiimide and glutaraldehyde), in which the durability of adhesion of resin materials with dentin was noted. The study however reported that there is difficulty in generating cross-links when using carbodiimide, and reported the ability of natural cross-links, such as PACs, to react with the enzymes that degrade collagen.

Over time, studies showing the reduction of collagen degradation, as well as the degradation of hybrid layer components, have indicated important allies in the preservation of the adhesive interface.²⁵ As such, the present study demonstrated that the PACs stood out in their results, showing the effectiveness of these cross-linking agents through a greater amount of collagen.

The PACs are extracted from grapeseed and are classified as a collagen cross-linking agents of natural origin, presenting polyphenols in abundance and a molecular chain rich in condensed tannins^{22, 29}, able to form various chemical bonds, such as covalent and hydrogen bonds.^{23, 24, 27} This agent has, throughout several studies, presented several advantages in

the previous treatment of dentin. Some of these are increasing the mechanical properties of collagen fibers unprotected by acid conditioning and consequently the hybrid layer, besides providing better bond strength.^{22, 23} This demonstrates the quality of this agent and justifies the reduction of degradation through the formation of cross-links. These data corroborate the present study; in our bending test for modulus of elasticity (Table 1), the PAC was superior to the other groups in the gain of mechanical properties of collagen.

The CAR is considered a natural phenol with a large carbon chain ending in polyphenols, and because it has lower molecular weight than other natural agents it can generate a greater increase in mass variation and modulus of elasticity. This agent is extracted from cashew nut liquid, and brings advantages under demineralized dentin, inhibition of the MMP and CTP enzymes and antioxidant capacity.²⁸

The treatments performed in the collagen matrix showed that this agent had influence under demineralized dentin. We noticed that in all groups, the dentin matrix presented better values with the treatment groups, when compared to the control group, further proving the efficacy of the PAC in improving the mechanical properties of the dentin matrix. Castellan *et al*.²³ agreed with this assessment. It is worth noting that Moreira *et al*.²⁹ observed an opposite result; the study reported better results with the CAR6.5 group, which was justified by the fact that this agent presented a greater increase in the rigidity of demineralized dentin, increased resistance to degradation and did not alter the staining of the specimens, which happened with the use of PACs.

The PAC group showed efficacy in the 1- hour span of application,^{21, 22, 27} and thus its use in clinical practice is very significant, considering its improvement in the biomechanical qualities of collagen, which makes its use in restorative dentistry more explicit. We therefore take into account that the established clinical application timespan is not feasible. Liu *et al*²⁶ showed that the one-minute clinical application of the PAC is effective in the modulus of elasticity, which shows its performance in diversified periods of activity.

When it comes to mass variation, it can be observed that there was statistical difference between the groups. Both PAC and CAR showed a superior gain after biomodification, proving that cross-links were formed with type I collagen present in the dentin matrix. These results are in agreement with the studies by Macedo *et al*,²² Castellan *et al*,²³ Bedran-Russo *et al*,²⁴ Dos Santos *et al*,²¹ Broyles *et al*,²⁵ Liu *et al*,²⁶ Aguiar *et al*²⁷ and Moreira *et al*.²⁸

The interaction between biomodification and its natural agents can be justified by the presence of different types of polyphenols, which generate covalent and non-covalent bonds with collagen, due to the presence of carboxillary groups in the molecular chain of natural agents, which depend on their composition and concentration to generate satisfactory



effects. Results of this research were better as it presented satisfactory modulus of elasticity, showing that there were greater bonds with collagen, thus reducing degradation. What this and other studies (such as Aguiar *et al.*) proved is that there are several possibilities of chemical bonds with collagen, generating better effectiveness for PAC due its exacerbated antioxidant property, thus generating a greater effectiveness of collagen bonds.

In addition, it has already been proven by Epasinghe *et al.*³³ that the treatment of dentin collagen with PAC 6.5% is effective in the formation of amorphous calcium complexes that can significantly increase the modulus of elasticity in collagen, and the mass increase in the present study is justified. It is not known for certain the mechanism of action

of CAR with collagen, but it has already been shown to be very effective in gaining mass with shorter application time.²⁹ It does however need to have its mechanism of interactivity with collagen clarified and established.

According to the limitations of this study, it is necessary to conduct further studies around the CAR to discover for certain its mechanism of action and improve its activity as a cross-linking agent.

Conclusion

PAC is more effective in gaining mechanical resistance of collagen; however, CAR may also be a promising biomodifying agent for dentin. Both PAC and CAR are effective in mass gain.

References

- Nakabayashi N, Nakamura M, Yasuda N. Hybrid Layer as a Dentin-Bonding Mechanism. *J Esthet Dent.* 1991;3(4):133-138.
- Van-Meerbeek B, Dhem A, Goret-Nicaise M, Braem M, Lambrechts P, VanHerle G. Comparative SEM and TEM examination of the ultrastructure of the resin-dentin interdiffusion zone. *J Dent Res.* 1993;72(2):495-501.
- Baratieri LN, Monteiro-Jr S, Melo TS, Ferreira KB, Hilgert LA, Schlichting LH *et al.* Odontologia restauradora: fundamentos e técnicas. v. 1. São Paulo: Editora Santos; 2014.
- Niu L, Zhang W, Pashley DH, Breschi L, Mao J, Chen J *et al.* Biomimetic remineralization of dentin. *Dent Mater.* 2014;30(1):77-96.
- Oliveira LV, Prado M, Menezes LR, Dias CT, Paulillo LAMS, Pereira GDS. Influência da camada híbrida na resistência à microtração de sistemas adesivos após armazenamento. *Rev Bras Odontol.* 2015;71(2):163-3.
- Bedran-Russo AK, Pauli GM, Chen SN, McAlpine J, Castellan CS, Phansalkar RS *et al.* Dentin biomodification: strategies, renewable resources and clinical applications. *Dent mater.* 2014;30(1):62-76.
- Mazzoni A, Tjaderhane L, Chechi V, Di Lenarda R, Salo T, Tay FR *et al.* Role of dentin MMPs in caries progression and bond stability. *J Dent Res.* 2015;94(2):241-251.
- Pashley DH, Tay FR, Yao C, Hashimoto H, Breschi L, Carvalho RM *et al.* Collagen degradation by host-derived enzymes during aging. *J Dent Res.* 2004;83(3):216-221.
- Breschi L, Mazzoni A, Ruggeri A, Cadenaro M, Di Lenarda R, Dorigo ES. Dental adhesion review: aging and stability of the bonded interface. *Dent mater.* 2008;24(1):90-101.
- Toledano M, Sauro S, Cabello I, Watson T, Osório R. A Zn-doped etch-and-rinse adhesive may improve the mechanical properties and the integrity at the bonded-dentin interface. *Dent Mater.* 2013;29(8):e142-e152.
- Nakajima M, Sano H, Urabe I, Tagami J, Pashley DH *et al.* Bond strengths of single-bottle dentin adhesives to caries-affected dentin. *Oper Dent.* 2000;25(1):2-10.
- Haj-Ali R, Walker M, Williams K, Wang Y, Spencer P. Histomorphologic Characterization of Noncarious and Caries-Affected Dentin/Adhesive Interfaces. *J Prosthodont.* 2006;15(2):82-88.
- Wang Y, Spencer P, Walker MP. Chemical profile of adhesive/carries-affected dentin interfaces using Raman microspectroscopy. *J Biomed Mater Res A.* 2007;81(2):279-286.
- Tay FR, Pashley DH. Biomimetic remineralization of resin-bonded acid-etched dentin. *J Dent Res.* 2009;88(8):719-724.
- Lenzi TL, Tedesco TK, Soares FZM, Loguercio AD, Rocha RO. Chlorhexidine application for bond strength preservation in artificially-created caries-affected primary dentin. *I J Adhes.* 2014;54:51-56.
- Lemos MVS, Araújo-Neto VG, Mendes TAD, de Paula DM, Moura MEM, Lomonaco D *et al.* Efeito de Diferentes Agentes Naturais Biomodificadores na Adesão À Dentina Hígida e Afetada por Cárie. *J Health Sci.* 2018;19(5):65.
- Donassollo TA, Lima FG, Rodrigues-Junior SA, Coelho-de-Souza FH, Demarco FF. Adesão aos substratos dentários e seus principais aspectos: uma revisão da literatura. *Stomatos.* 2010;16(31).
- Da Silva TF, Penna ALB. Colágeno: Características químicas e propriedades funcionais. *Rev Inst Lutz.* 2012;71(3):530-539.
- De Sousa JHP, Moro AFV. Solventes do Primer: revisão de literatura. *Ver Bras Odontol.* 2014;71(1):80-84.
- Arinelli AMD, Pereira KF, Prado NAS, Rabello TB. Sistemas adesivos atuais. *Rev Bras Odontol.* 2016;73(3):242-246.
- Dos Santos PH, Karol S, Bedran-Russo AK. Long-term nano-mechanical properties of biomodified dentin-resin interface components. *J Biomech.* 2011;44(9):1691-1694.
- Macedo GV, Yamauchi M, Bedran-Russo AK. Effects of chemical cross-linkers on caries-affected dentin bonding. *J Dent Res.* 2009;88(12):1096-1100.
- Castellan CS, Pereira PN, Grande RHM, Bedran-Russo AK. Mechanical characterization of proanthocyanidin-dentin matrix interaction. *Dent Mater.* 2010;26(10):968-973.
- Bedran-Russo AKB, Castellan CS, Shinohara MS, Hassan L, Antunes A. Characterization of biomodified dentin matrices for potential preventive and reparative therapies. *Acta Biomater.* 2011;7(4):1735-1741.
- Broyles AC, Pavan S, Bedran-Russo AK. Effect of Dentin Surface Modification on the Microtensile Bond Strength of Self-Adhesive Resin Cements. *J Prosthodont.* 2013;22(1):59-62.
- Liu Yi, Chen M, Yao X, Xu C, Zhang Y, Yong W. Enhancement in dentin collagen's biological stability after proanthocyanidins treatment in clinically relevant time periods. *Dent Mater.* 2013;29(4):485-492.
- Aguiar TR, Vidal CMP, Phansalkar RS, Todorova I, Napolitano JG, McAlpine JB *et al.* Dentin Biomodification Potential Depends on Polyphenol Source. *J Dent Res.* 2014;93(4):417-422.
- Ogliari FA, Silva EO, Lima GS, Madruga FC, Henn S, Bueno M *et al.* Synthesis of phosphate monomers and bonding to dentin: esterification methods and use of phosphorus pentoxide. *J Dent.* 2008;36(3):171-177.
- Moreira MA, Souza NO, Sousa RS, Freitas DQ, Lemos MV, De Paula DM *et al.* Efficacy of new natural biomodification agents from Anacardiaceae extracts on dentin collagen cross-linking. *Dent Mater.* 2017;33(10):1103-1109.
- Castellan CS, Bedran-Russo AK, Antunes A, Pereira PNR. Effect of dentin biomodification using naturally derived collagen cross-linkers: one-year bond strength study. *Int J Dent.* 2013;2013.
- Breschi L, Maravic T, Cunha SR, Comba A, Cadenaro M, Tjaderhane L *et al.* Dentin bonding systems: From dentin collagen structure to bond preservation and clinical applications. *Dent Mater.* 2017;34(1):78-96.
- Delgado CC, Scheffel DLS, Scheffel RH, Pashley D, Hebling J. Redução da atividade proteolítica da dentina após curtos períodos de aplicação de proantocianidina. *Rev Odontol UNESP.* 2015;44(6):355-359.
- Epasinghe DJ, Kwan S, Chu D, Lei MM, Burrow MF, Yiu CKY. Synergistic effects of proanthocyanidin, tri-calcium phosphate and fluoride on artificial root caries and dentine collagen. *Mater Sci Eng C Mater Biol Appl.* 2017;73:293-299.



Mini Curriculum and Author's Contribution

1. Andreza Camurça Ferreira - DDS. Contribution: Effective scientific and intellectual participation in the study and textual writing. ORCID: 0000-0001-9492-915X
 2. Talita Arrais Daniel Mendes – DDS; PhD. Contribution: Preparation and draft of the manuscript; critical review and final approval. ORCID: 0000-0003-3519-3618.
 3. Marcelo Victor Sidou Lemos – DDS; PhD. Contribution: conception and design of the project and critical review. ORCID: 0000-0002-2993-534X.
 4. Déborah Maria Martins de Paula - DDS. Contribution: Effective scientific and intellectual participation in the study. ORCID: 0000-0003-3531-9823.
 5. Karlos Eduardo Rodrigues Lima – Dentistry Student. Contribution: Effective scientific and intellectual participation in the study. ORCID: 0000-0003-3127-9772
 6. Luiz Filipe Barbosa Martins – DDS; PhD . Contribution: orientation, critical review and final approval. ORCID: 0000-0002-2123-2850.
-

Submitted: 06/12/2020 / Accepted for publication: 07/05/2020

Corresponding author

Talita Arrais Daniel Mendes

E-mail: talita_arrais@hotmail.com