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Novel resin-based material containing β-tricalcium phosphate nanoparticles for the reduction of dentin permeability

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ABSTRACT

Objectives: To synthesize and characterize a novel dentin adhesive containing Beta-Tricalcium Phosphate (β-TCP) nanoparticles and test its ability to reduce dentin permeability (dP).
Methods: Experimental adhesives were prepared by mixing Bis-GMA, TEGDMA, HEMA (50/25/25 wt.%), photo-initiators, and inhibitors. The following groups were tested: Experimental adhesives without β-TCP (Exp.); with 10 wt.% β-TCP (Exp.10 wt.% β-TCP); with 15 wt.% β-TCP (Exp.15 wt.% β-TCP), Scotchbond Multi-Purpose (SBMP) and Clearfil SE Protect Bond (CFPB). Degree of conversion (DC%, 10 and 20 s); Flexural Strength (FS), Knoop Hardness (KHN), and Cell Viability (OD%) tests were performed. dP was evaluated by hydraulic conductance, using human dentin disks (n=12), at three-time intervals: post-EDTA (T_0); post-treatment (T_1); and post-erosion/abrasion cycling (T_2). Data were statistically analyzed (α=0.05).
Results: For all groups, exposure time for 20 s presented a higher DC% than for 10 s. For FS, filled adhesives did not differ from unfilled and from CFPB. Experimental adhesives did not differ among them and showed lower KHN than the commercial products. Cell viability did not differ among adhesives, except Exp. 15 wt.%, which showed lower OD% than Exp., Exp. 10% and, CFPB. For dP, only Exp.10 and 15 wt.% β-TCP did not present difference between the times T_1 and T_2. After cycling, Exp.10 wt.% β-TCP presented lower permeability than Exp. and CFPB.
Conclusions: The incorporation of 10 wt.% β-TCP nanoparticles into the resin-based dental material did not affect its mechanical properties and biocompatibility, and promoted the greatest reduction in dentin permeability, sustaining this effect under erosive/abrasive challenges.
Clinical significance: A novel resin-based dental material containing β-TCP nanoparticles was able to reduce dentin permeability, maintaining its efficacy after erosive/abrasive challenges. The synthesized material did not affect dental pulp cell viability and might be promising for other conditions that require dental remineralization, such as tooth wear and dental caries.

1. Introduction

Dental hard tissues are mainly composed by calcium (Ca^{2+}), phosphate (PO_4^{3-}) and hydroxyl (OH^-) ions, which form hydroxyapatite (HA) crystals – $Ca_{10}(PO_4)_6(OH)_2$. These tissues can be demineralized as a result of a chemical dissolution promoted by acids of bacterial origin, related to dental caries [1], or erosive acids, associated with Erosive Tooth Wear (ETW) [2]. ETW is characterized by the progressive loss of tooth structure, resulting from the association between acids and mechanical forces, such as toothbrushing abrasion [2], and one of its consequences is dentin hypersensitivity (DH) [3]. As enamel undergoes continuous chemical/mechanical insults, the dentin layer underneath

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can be exposed, with widening of the dentinal tubules and increase of dentin permeability [4]. DH is a painful condition that impacts the quality of life [5] and has an increasing global prevalence [6]. Many strategies have been used to reduce DH, some of which include daily use of desensitizing toothpastes containing stannous fluoride, potassium, calcium sodium phosphosilicate and, arginine [7–9]. To increase dentin remineralization, the use of calcium and phosphate was also suggested [10], through diet [11], home-use healthcare products and in-office products [12], such as toothpastes, mouthwashes, gels and varnishes.

However, the low solubility of calcium and phosphate in the presence of fluoride limited their dental applications. Premature reactions allow calcium to bind to fluoride ions, forming calcium fluoride (CaF_2), which is poorly soluble in aqueous media. This implies less bioavailability and, consequently, less mineral deposition. In contrast, the increase in solubility also limits its action, as a quick ionic dissociation can disable the ability of these soluble minerals ions to diffuse into the tooth structure [13]. The solubility factor motivated the synthesis of different bioceramics systems, such as tricalcium phosphate (TCP), with a similar mineral phase as the teeth and bone [14], to offer calcium and phosphate (Ca/P) simultaneously, reducing limitations related to solubility.

TCP is a polymorphic system, existing in α and β phases, with a Ca/P ratio and solubility close to that of HA [15]. Recent studies highlighted β-TCP (β-$Ca_3(PO_4)_2$) as a promising system for tooth remineralization [16,17]. With the advent of nanomedicine and nanotechnology, β-TCP particles were synthesized in nanometric dimensions, increasing their potential to reduce dentin surface loss when functionalized with fluoride in an experimental solution [16]. Additionally, nanoparticles have a higher surface area, which has motivated their incorporation into resin-based materials, as they provide benefits for the bioactivity of the restorative material [18]. Concerning DH management, the use of resin-based dental materials is considered a good approach, as they can form a mechanical barrier on the tooth surface and seal the dentin [19], reducing dentin permeability. This ability has stimulated dental adhesives modification through incorporation of fillers, such as encapsulated arginine and calcium carbonate [20], and titanium tetrafluoride - TiF_4 [21]. However, these materials did not provide a long-lasting effect compared to commercial agents, since they do not resist the continuous erosive/abrasive challenges [20].

Therefore, the synthesis of a resin-based material containing β-TCP nanoparticles remains attractive, because even if the material is removed from the dentin surface, it is expected that the nanoparticles would easily penetrate through dentinal tubules, providing a physical obliteration and a chemical stimulus for the formation of a mineralized matrix. Hence, the purpose of this study was to synthesize an experimental adhesive containing β-TCP nanoparticles; perform the mechanical and biological characterization; and evaluate the material's potential in reducing dentin permeability and providing a long-term effect. The null hypotheses tested were that the experimental material with β-TCP nanoparticles would not differ from commercially available adhesives in (1) mechanical properties, (2) cell viability, and (3) dentin permeability.

2. Materials and methods

2.1. Study design

The present study had two phases. The first phase aimed to synthesize and characterize the resin-based dental material and had one experimental factor, "resin-based dental materials – adhesives" at 5 levels: (1) experimental adhesive without β-TCP; (2) experimental adhesive with 10 wt.% β-TCP; (3) experimental adhesive with 15 wt.% β-TCP; (4) Scotchbond Multi-Purpose, 3M ESPE (SBMP), and (5) adhesive with fluoride - Clearfil SE Protect Bond, Kuraray Dental (CFPB). These materials had the following mechanical/biological properties evaluated: 1. degree of conversion (expressed in DC%); 2. flexural strength (in MPa); 3. Knoop hardness (KHN) and, 4. cytotoxicity (expressed as optical densitiy percentage - OD% of cell viability).

The second phase evaluated the ability of the adhesives tested in phase one to reduce dentin permeability and to resist the erosive/abrasive challenges *in vitro*. In this phase, two experimental factors were considered: the adhesives aforementioned, which were also compared with a negative control (no-treatment), and; time, at 3 levels (T_0: post-EDTA – simulation of dentin hypersensitivity, T_1: post-treatment - 24 h after the application of the adhesive on dentin surface, and T_2: post-erosive/abrasive cycling). The response variable was dentin permeability (expressed as %Lp). All materials used are presented in Table 1.

2.2. Synthesis and characterization of the experimental adhesive

2.2.1. Synthesis

The resin-based material was prepared by mixing the following monomers: Bisphenol A glycerolate dimethacrylate - BisGMA (50 wt.%, CAS No: 1565-94-2, Sigma-Aldrich Co, St Louis, MO, USA), Triethylene glycol dimethacrylate - TEGDMA (25 wt.%, CAS No: 109-16-0, Sigma-Aldrich Co, St Louis, MO, USA) and 2-hydroxyethyl methacrylate - HEMA (25 wt.%, CAS No: 868-77-9, Sigma-Aldrich Co, St Louis, MO, USA), in a dark room, at 22°C, under overnight mixing (350 rpm, Multistirrer Digital®, Velp Scientifica). Photo-initiator system camphorquinone (0.4 wt.%, CAS No: 10373-78-1, Sigma-Aldrich Co, St Louis, MO, USA) and 2-(Dimethylamino) ethyl methacrylate (0.8 wt.%, CAS No: 2867-47-2, Sigma-Aldrich Co, St Louis, MO, USA) were also incorporated [22]. Beta - Tricalcium Phosphate nanoparticles (β-TCP) [16] were added to the experimental resin-based material in two different concentrations: 10 wt.% and 15 wt.%. All experimental adhesives were kept under vacuum pressure (30 min at 25 inHg) after mixing to remove any air bubbles formed during the synthesis.

2.2.2. Degree of conversion

Disc-shaped specimens (7 mm × 0.30 mm, n = 3) were prepared in a dark room and, light-cured with two different exposure times (on the top only): 10 s, as recommended by both manufacturers from the commercial adhesives; and 20 s. They were cured with a light-emitting diode curing system (Bluephase Style, Ivoclar Vivadent) with a minimum output intensity of 1,100 mW/cm^2 checked with a calibrated radiometer (Cure Rite Visible Curing Light Meter, DENTSPLY Caulk, Milford, DE, USA) and stored for 24 h, under a dark and dry closet. Three specimens per group were used as in a previous study [23].

Each specimen was read at three points with the equipment Fourier Transform Infrared Spectroscopy (FTIR; Jasco 4100, Jasco Corp., Tokyo, Japan), using a diamond crystal plate (1.8 mm diameter) and analyzed under Spectra Manager Software (Version 2.10.01., JASCO Corporation). Also, uncured (unpolymerized) adhesive was read in triplicate. The following parameters were used: attenuated total reflection device (ATR-MIRacle, PikeTechnologies, Madison, WI) in the absorbance mode (at an 8 cm^{-1} resolution and a mirror speed of 2.8 mm/s). The waves were analyzed in a range of 1750 and 1550 cm^{-1}. For the aliphatic carbon-to-carbon (C=C), a peak absorption of 1638 cm^{-1} (baseline between 1650 and 1622) was used; For the aromatic C=C, 1608 cm^{-1} (baseline between 1590 and 1623) was used [24,25]. The area of the aromatic peaks was calculated by the average of both absorbances. DC (expressed in %) was calculated according to the following equation [26]:

$$DC\ (\%) = 1 - \frac{cured\ (area\ under\ aliphatic\ C=C\ /\ area\ under\ aromatic\ C=C)}{uncured\ (area\ under\ aliphatic\ C=C\ /\ area\ under\ aromatic\ C=c)} x100$$

2.2.3. Flexural strength

Bar-shaped specimens (25 mm in length × 2 mm in width × 2 mm in thickness; n = 5/group) were prepared according to ISO4049:2009 [27]. Each specimen was light cured on the top and on the bottom surfaces, for 20 s, with five overlapping irradiation zones. The specimens were prepared using the same light-emitting diode curing system afore mentioned and stored in distilled water at 37°C for 24 h. All sample surfaces were gently polished with an 8" silicon carbide disc (PSA #600 grit, LOT# 211026).

Specimens were tested using a three-point bending apparatus (MTS Sintech Renew 1123, MTS Systems Corporation, Eden Prairie, MN, USA) at a crosshead speed of 1 mm per minute and a load cell of 2.5 kN, model #4501028. The Flexural Strength (in MPa) was provided by the Software, according to the following equation:

$$FS = \frac{3Fl}{2bh^2}$$

Where F represents the maximum load, expressed in Newton (N); l is the distance between the supports (20 mm); b is the width of the specimen (in mm), and h is the thickness of the specimen (in mm).

2.2.4. Hardness

Disk-shaped specimens (10 mm in diameter × 1 mm in thickness, n = 5) were light-cured on the top and on the bottom surfaces, for 20 s. Specimens were flatened with silicon carbide discs (grit number #1200), polished with a polishing cloth (Imperial Cloth, Leco Corporation, St. Joseph, MI, USA) and, wet with Polycrystalline Diamond Suspension 9 μm (MetaDi™ BUEHLER, Lake Bluff, IL, USA). Five specimens per group were used as in a previous study [23].

Specimens were tested in a hardness tester (LM 248AT, Leco, St. Joseph, MI, USA) using a Knoop diamond indenter, 1000 gF of load, and 15 s of dwell time. Prior to the readings of each group, the machine was calibrated using a standardized device. Five readings were performed for each specimen. After each indentation, the diagonal length was measured (length 10/0.25), and the machine provided the Knoop Hardness Number (KHN) [22].

2.2.5. Cytotoxicity

Disc-shaped specimens (6 mm × 1.00 mm, n = 3) were light-cured on the top and on the bottom, as previously described, and stored for 24 h at 37 °C [28]. They were submitted to an ultraviolet light (UV) disinfection for 1h (30 min on each side), in a cell culture biosafety cabinet and then immersed individually in falcon tubes with 5 mL Basal Media - DMEM (Dulbecco`s Modified Eagle Medium, 1g/L D-Glucose, L-Glutamine and 110 mg/L Sodium Pyruvate, LOT 2322974), in a ratio of 1:4 [20]. The falcon tubes containing the discs and the media were mixed for 24h at 37°C, using an incubator shaker at 100 rpm. Aliquots were collected from each falcon and stored under – 20°C for 48h.

Dental pulp cells (commercially purchased) were cultured in T75 tissue culture flasks. After growth, flasks of sub-confluent cells were

Table 1
Experimental groups.

Groups	Material/LOT	Composition	Application method
Exp.	Experimental adhesive without β-TCP	Bisphenol A Glycidyl Methacrylate - BisGMA (50 wt.%), Triethylene glycol dimethacrylate -TEGDMA (25 wt.%), 2-hydroxyethyl methacrylate - HEMA (25 wt.%), Camphorquinone and 2-(Dimethylamino)ethyl methacrylate	ETCHING: Applied on dentin surface, left for 15 s, rinsed for 15 s and dried for 5 s. PRIMER: applied with a disposable brush tip and gently dried for 5 s. Same primer from group SBMP. ADHESIVE: applied on the entire surface with a disposable brush tip and light-cured for 20 s.
Exp.10 wt. % β-TCP	Experimental adhesive with 10 wt.% β-TCP	Bisphenol A Glycidyl Methacrylate - BisGMA (50%), Triethylene glycol dimethacrylate -TEGDMA (25%), 2-hydroxyethyl methacrylate - HEMA (25%), Camphorquinone, 2-(Dimethylamino)ethyl methacrylate and 10 wt.% β-TCP	ETCHING: Applied on dentin surface, left for 15 s, rinsed for 15 s and dried for 5 s. PRIMER: applied with a disposable brush tip and gently dried for 5 s. Same primer from group SBMP. ADHESIVE: applied on the entire surface with a disposable brush tip and light-cured for 20 s.
Exp.15 wt. % β-TCP	Experimental adhesive with 15 wt.% β-TCP	Bisphenol A Glycidyl Methacrylate - BisGMA (50%), Triethylene glycol dimethacrylate -TEGDMA (25%), 2-hydroxyethyl methacrylate - HEMA (25%), Camphorquinone, 2-(Dimethylamino)ethyl methacrylate and 15 wt.% β-TCP	ETCHING: Applied on dentin surface, left for 15 s, rinsed for 15 s and dried for 5 s. PRIMER: applied with a disposable brush tip and gently dried for 5 s. Same primer from group SBMP. ADHESIVE: applied on the entire surface with a disposable brush tip and light-cured for 20 s.
SBMP	Adper™ Scotchbond™ Multi-Purpose Adhesive, 3M ESPE / NE34896	Not provided by the manufacturer.	ETCHING: Applied on dentin surface, left for 15 s, rinsed for 15 s and dried for 5 s. PRIMER: applied with a disposable brush tip and gently dried for 5 s. ADHESIVE: applied on the entire surface with a disposable brush tip and light-cured for 20 s.
CFPB	Clearfil™ SE Protect Bond, Kuraray Dental / 6M0130	PRIMER (self-etching-primer): 10-Methacryloyloxydecyl dihydrogen phosphate (MDP); 12-Methacryloyloxdodecylpyridinium bromide (MDPB); 2-Hydroxiethyl methacrylate (HEMA); Hydrophilic dimethacrylate; Water. BOND (fluoride-bonding agent): 10-Methacryloyloxydecyl dihydrogen phosphate (MDP); Bisphenol A diglycidylmethacrylate (Bis-GMA); 2-hydroxyethyl methacrylate (HEMA); Hydrophobic dimethacrylate; dl-Camphorquinone; N,N-Diethanol-p-toluidine; Silanated colloidal silica; Surface treated sodium fluoride.	PRIMER: applied with a disposable brush tip, left for 20 s and evaporation of volatile ingredients with mild air stream. BOND: applied on the entire surface with a disposable brush tip and gently spread with a air flow to create a uniform bond film. Light-cured for 20 s.
N-CT	Negative control	Distilled water	Specimens were immersed in distilled water for 15 s.

collected. Cells were trypsinized and transferred to a 15 ml conical containing DMEM with 10 % FBS, aiming to neutralize the Trypsin-EDTA effect. They were centrifuged and counted using a Countess Cell Counter (ThermoFisher) and transferred to a 96-well plate. A cell density of ≅ 10,000 cells per well was used. The morphology of the cells was checked under a light microscope. 5μl of aliquots (material + DMEM) were pipetted into each well containing the dental pulp cells.

To assess the metabolic activity of cells, the water-soluble tetrazolium salts assay (WST-1) test was conducted by using a Cell Proliferation Reagent kit WST-1 (VWR Chemicals, Ohio, USA, LOT 62844900). The solution was pipetted to each well plate, according to the manufacturer's instructions. The plates were stored under 37 °C and analyzed after 4 h. A microplate reader (iMark^{TM} BIO-RAD) was used to measure the Optical Density by absorbance mode at 450 nm. The test was performed in triplicate as required by ISO 10993-5:2009 [29]. Each plate had a column as a control, where no treatment was used, only the cells and the basal media, which were considered maximum cell viability (100 %).

2.3. Dentin permeability

2.3.1. Sample size calculation

The present study was submitted and approved by the Institutional Review Board (IRB Protocol #16543), since human permanent molars were used. A pilot study was conducted to define the number of specimens required for dentin permeability test. Three human dentin discs were used for the Negative Control (No-Treatment) and Positive Control (Clearfil SE Protect Bond) groups. The ANOVA Sample Size Test of SigmaPlot 13.0 software was used (Systat Sotware Inc., Chicago, IL, USA), considering a power of 80%, a significance level of 5% and six experimental groups. Data of the main outcome (dentin permeability after cycling) were used for sample size calculation. A difference in means of 40.530 and standard deviation of 17.780, among positive and negative controls, resulted in six specimens per group. For standardization purposes, and based on a previous study with the same methodology [30], twelve specimens per group were used.

2.3.2. Specimen preparation

All collected teeth were evaluated to check the absence of cracks, caries, or restorations and then autoclaved, immersed in distilled water, for 20 min [20]. Teeth were mounted in a precision saw machine (ISOMET^{TM} 1000, BUEHLER, Lake Bluff, IL, USA), and two parallel sections were made in the crowns using a double-sided diamond disc (Diamond Wafering Blade 0.3 mm thickness, Series 15LC Diamond, No. 11-4255, BUHLER, USA). Enamel surface and pulp horns were removed, and dentin discs with 1.5 mm thickness were obtained. Dentin discs were polished in a manual polishing machine (Spectrum System^{TM} 1000, Leco) using silicon carbide discs with #320 grit (Leco Corporation, Lakeview Ave., St. Joseph, MI) to obtain discs with 1.0 mm thickness (verified with a digital caliper – Mitutoyo Corp, Kanagawa, Japan). The occlusal surface was identified, and specimens were stored in relative humidity at 4 ºC.

2.3.3. Dentin hypersensitivity simulation

Specimens were sonicated in a solution of 17% EDTA (EthyleneDiamineTetraAcetic acid, Pulpdent®, Watertown, MA, U.S.A., LOT 181221) for 5 min to open dentinal tubules and to simulate dentin hypersensitive. Then, specimens were rinsed in distilled water and stored under the same conditions until the first permeability analysis [30].

2.3.4. Dentin permeability analysis

Dentin permeability was evaluated by hydraulic conductance test, using a permeability machine (THD03d - Odeme Equipamentos Médicos e Odontológicos Ltda, Luzerna, Brazil) connected to a Nitrogen tank. While the system performed a pressure of 5 PSI (1 PSI = 703.07 mmH_2O) through the dentin disc specimen (occlusal surface facing upward) [20,21], an air bubble moved through a microcapillary. The air bubble linear displacement (mm) in 3 min was measured 3 times. The hydraulic conductivity of dentin (Lp, in μl/min.cmH O.cm^2), was calculated by the equipment-specific software (Analysis 4.1, Odeme), as follows:

$$Lp = \frac{Q}{P \times Asup}$$

Codes: Q is the Filtration Index (μl/min), P is the difference of hydrostatic pressure through dentin (cm H O) and Asup is the exposed dentin surface area (0.05817 cm^2).

The Filtration Index (Q) was calculated as follows:

$$Q = \frac{Vp \times D}{L \times T}$$

Codes: Vp is the standardized volume (in μl), D is the displacement of the bubble in the capillary tube (in mm), L is the length of capillary tube (100 mm), and T is the test time (in min).

After EDTA (T_0), the reading was considered the baseline (100 %). Based on the initial values, specimens (n=12) were randomly allocated to the experimental groups (Table 1). The percentage of Lp after treatment (% Lp_1) and after erosion/abrasion cycle (% Lp_2) were calculated as follows: % Lp_1 = (Lp_1 x 100)/Lp_0; % Lp_2 = (Lp_2 x 100)/Lp_0.

2.4. Application of the treatments

Treatments were applied as described in Table 1. Light-curing time was standardized at 20 s for all groups, based on the results of the Degree of Conversion test. After treatment application, dentin discs were stored in artificial saliva for 24 h, to allow complete polymerization of the material before the second dentin permeability analysis (T_1).

2.5. Erosive/Abrasive cycle

After treatment application, dentin discs were submitted to an *in vitro* erosive/abrasive cycling model, to simulate a patient with a high risk of erosive tooth wear and to verify if the treatments applied could resist the chemical and mechanical insults present *in vivo*. The cycle consisted of a daily immersion in 0.3 % citric acid solution (natural pH), for 2 min; followed by immersion in artificial saliva (pH adjusted to 7.0) for 60 min. This cycle was repeated 4 times a day for five days [31].

After the first and the last immersions in citric acid, specimens were exposed for 30 min to artificial saliva and then submitted to tooth brushing simulation, as follows: 45 brushing strokes, for 15 s under 1.5N load. Specimens were immersed in the slurry for a total of 2 min. The slurry consisted of a suspension prepared daily, by mixing fluoridated toothpaste (Colgate Triple Action, with Sodium Fluoride 0.24 %) with artificial saliva, in a ratio of 1:3 (w/w). The abrasive challenge was performed using a custom made automatic brushing machine (V-8 Cross Brushing Machine), and soft toothbrushes, Oral-B 40 (Procter & Gamble, Cincinnati, OH, USA) [31]. Artificial saliva was prepared by mixing 0.213 g/L $CaCl_2$*$2H_2O$; 0.738 g/L KH_2PO_4; 1.114 g/L KCl; 0.381 g/L NaCl and 12 g/L of Tris buffer, pH = 7 [32]. After each brushing cycle, specimens were again immersed in artificial saliva for 30 min. At the end of the 5 days of cycling, dentin permeability was again evaluated (T_2).

2.6. Data analysis

All data were analyzed for normal distribution (Shapiro-Wilk test) and homoscedasticity (Brown-Forsythe test). Data from DC% were analyzed by Two Way Analysis of Variance (factors: "exposure time" and "experimental adhesive") and Tukey tests. Data from FS and Hardness were analyzed by One Way Analysis of Variance and Tukey tests, and Cytotoxicity data by Kruskal-Wallis and Dunn`s Method. For dp, Two-Way Repeated Measures ANOVA and the Tukey tests were used. A significance level of 5 % was considered and tests were performed with the statistical program SigmaPlot (version 14, Systat Software Inc., San Jose, CA, USA).

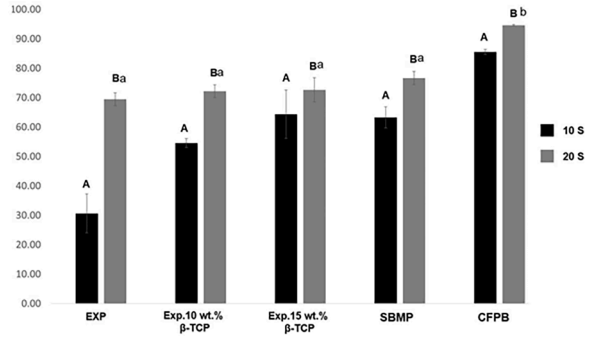

Fig. 1. Means and standard deviations of DC% for each group, according to the time tested. Different capital letters indicate statistically significant differences among exposure times ($p < 0.05$), for each group. Different lower letters indicate statistically significant differences among groups, when light-cured for 20 s.

3. Results

3.1. Characterization of the resin-based material

For the Degree of Conversion, the data met the premises of normality (p=0.564) and homoscedasticity (p=0.361). There was a statistically significant difference for the factors "treatment – adhesives" (p<0.001), "exposure time of light-curing" ($p < 0.001$) and for the interaction "treatment and exposure time" (p<0.001). For all groups, light curing for 20 s had higher DC% than 10 s, and for this reason, 20 s was used in the following steps of the study. When comparing groups light cured for 20 s, the group Clearfil SE Protect Bond (CFPB) had higher DC% than all the other groups (p<0.001). However, these groups (Scotchbond Multi-Purpose – SBMP; Experimental adhesive without β-TCP; with 10 wt.% β-TCP and with 15 wt.% β-TCP) did not differ from each other (p>0.050). The means and standard deviations (SD) of the Degree of Conversion (in DC%) for the different times tested are presented in Fig. 1.

For Flexural Strength (FS, expressed in MPa), the data met the premises of normality (p=0.292) and homoscedasticity (p=0.069). The group Adper™ Scotchbond™ Multi-Purpose Adhesive (SBMP) presented higher flexural strength than the experimental adhesive with 15 wt.% β-TCP (Exp.15 wt.% β-TCP), p = 0.009. For Knoop Hardness, the data assumed the premises of normality (p=0.741) and homoscedasticity (p=0.112). Both commercial adhesives, Adper™ Scotchbond™ Multi-Purpose Adhesive (SBMP) and Clearfil™ SE Protect Bond (CFPB), presented higher KHN than all experimental adhesives groups (Exp. without β-TCP, Exp.10 wt.% β-TCP and, Exp.15 wt.% β-TCP). The means and standard deviations of flexural strength (in MPa) and hardness (in KHN) for all the experimental groups are shown in Table 2.

For cytotoxicity, there was a statistically significant difference among the treatment groups ($p < 0.01$). The experimental adhesive with 15 wt.% β-TCP (Exp.15 wt.% β-TCP) presented a lower percentage of cell viability than the control ($p < 0.01$), CFPB (p = 0.007), Exp.10 wt.% β-TCP (p = 0.045) and Exp. (p = 0.01). Only SBMP presented a lower percentage of cell viability than the control (p = 0.034). The medians and the interquartile range of cell viability (in %) for all the experimental groups are shown in Table 3.

Table 2
Means and standard deviations of FS (in MPa) and Knoop Hardness (in KHN).

Groups	FS	Hardness
Exp.	81.94 (6.91) ^{AB}	21.84 (0.71) ^{A}
Exp.10 wt.% β-TCP	74.84 (9.09) ^{AB}	22.76 (1.69) ^{A}
Exp.15 wt.% β-TCP	68.00 (18.70) ^{B}	22.10 (0.73) ^{A}
SBMP	95.94 (13.39) ^{A}	26.09 (1.70) ^{B}
CFPB	89.86 (4.76) ^{AB}	27.32 (1.27) ^{B}

Table 3
Medians and the interquartile range of cell viability (in %) for all the experimental groups.

Groups	Median	Interquartile Range [25 %–75 %]
Exp.	97.24^{B}	[91.69 – 104.66]
Exp.10 wt.% β-TCP	98.05^{AB}	[89.89 – 104.36]
Exp.15 wt.% β-TCP	75.70^{C}	[61.38 – 86.70]
SBMP	86.71^{BC}	[68.71 – 93.50]
CFPB	98.91^{AB}	[93.92 – 106.72]
NT	100^{A}	[100 – 100]

Different letters indicate statistically significant differences among groups ($p < 0.05$).

3.2. Dentin permeability

At baseline (T_0), there were no significant differences between the groups regarding dentin permeability (p = 1). The data of % Lp_1 and % Lp_2 satisfied the premise of normal distribution (p = 0.542) and homoscedasticity (p = 0.639). According to the Two Way Repeated Measures ANOVA (One Factor Repetition), there was a significative difference for the factors: experimental adhesive ($p < 0.001$), time ($p < 0.001$), and for the interaction adhesive x time (p = 0.002). Only the groups Exp.10 wt.% β-TCP and Exp.15 wt.% β-TCP did not present a significative difference among the times pos-treatment (T_1) and pos-cycle (T_2), p = 0.117 and p = 0.558 respectively. After treatment application, the Negative Control group presented higher permeability than all experimental groups, except Exp.15 wt.% β-TCP (p = 0.052). After the erosion/abrasion cycle, the Negative Control group presented higher permeability than all experimental groups; Group Exp. (Experimental without the nanoparticles) presented higher permeability than the group Exp.10 wt.% β-TCP ($p < 0.001$). Group CFPB (commercial adhesive with fluoride) presented higher permeability than group Exp.10 wt.% β-TCP (p = 0.045), as shown in Fig. 2.

4. Discussion

The present study synthesized an experimental resin-based dental material containing β-TCP nanoparticles. These nanoparticles used here were previously synthesized by our research group, characterized by X-ray diffraction (XRD), transmission electron microscopy (TEM) and their anti-erosive effect in solution was investigated [16]. The fillers optimal concentrations to be incorporated into the resin matrix were defined based on a previous study [33], which has tested the role of β-Tricalcium Phosphate concentration levels on the physical properties of a dental adhesive. The authors found that 10 wt.% β-TCP promoted higher micro-tensile bond strength (μTBS) than 5 wt.% β-TCP. Based in that previous results, it

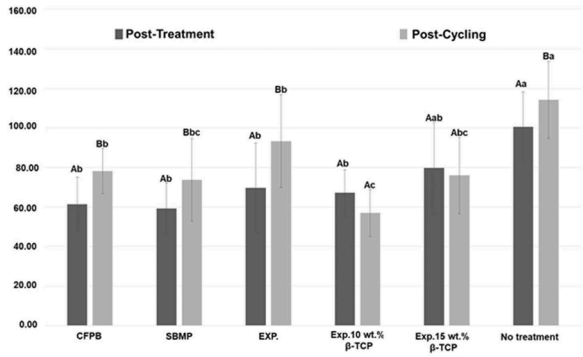

Fig. 2. Means and standard deviations of dentin permeability post-treatment and post-cycling, based on %Lp values. Different capital letters indicate differences between experimental time intervals for each group ($p \leq 0.05$). Different lowercase letters denote differences among groups within time intervals ($p \leq 0.05$).

becomes interesting to investigate the effect of an already known concentration (10 wt.%) and a higher concentration (15 wt.%) on the mechanical and biological properties of an experimental dental adhesive, as well as in its ability to reduce dentin permeability.

The first physical property evaluated was the degree of conversion, as an insufficient cure can impact the mechanical-chemical performance of resin-based materials [24]. Since an experimental material has an unknown %DC, the test was performed to observe if the polymeric scaffold/chain formation could be affected by the nanoparticles incorporation. In addition, the test allowed to establish the appropriate exposure time of light-curing to promote higher final double conversion. Two different light-curing times were tested: 10 s, as recommended by the manufacters of the commercial adhesives; and 20 s, which corresponds to a protocol previously investigated by other authors [23]. For all groups tested, 20 s presented higher %DC than 10 s. It was expected that a prolonged light-curing time could promote a higher level of polymerization, as previously demonstraded by authors that investigated Clearfil Se Bond %DC after curing for 20, 40 and 60 s [34]. In the present study, groups Exp. and Exp.10 wt.% β-TCP light-cured for only 10 s, presented a %DC lower than 60%. It is known that unfilled Bis-GMA-based resins have a degree of cure of up to 72 % [35] and for this reason, 20 s was defined as the most appropriate light-curing time for all the groups.

When light-cured for 20 s, the filled groups (Exp.10 wt.% β-TCP and Exp.15 wt.% β-TCP) did not differ from the unfilled group (Exp.) and the commercial adhesive SBMP. This data showed that the β-TCP nanoparticles incorporation into a basic adhesive formulation did not affect the %DC, despite the concentration of the filler, which is in agreement with the literature [36]. It can be suggested that there was no mismatch between the Refractive Index of the resin-based material matrix and the β-TCP nanoparticle fillers [37]. However, group CFPB presented a higher %DC than all groups. CFPB has silanized colloidal silica and a surface treated with sodium fluoride. Silica particles can reduce the polymerization time [38], as these fillers can increase the viscosity of the adhesive and, as a consequence, the rigidity of the polymeric chain increases. In addition, the presence of specific types of fillers can absorb surface-reacting monomers, acting as a site for chain growth [38,39] and providing a higher %DC.

According to the material's application, different types of proprerties and performance are required. In the present study, the experimental resin-based material was synthesyzed as a new approach to deliver a syntethic mineral system for tooth remineralization. However, dental adhesives are light-cured materials commonly used as part of a restorative system, with the main goal of establishing adhesion between dental composites and tooth structure, by sealing the dentin interface. However, they could also be a suitable material for sealing exposed dentinal tubules in cases of DH, where there is not much tissue loss that can be replaced by a restoration. Nonetheless, based on the main applicability of a dental adhesive, the present study investigated the physical and mechanical properties to certifiy that no differences would be found in comparison to commercial adhesives. Hence, after defining the exposure time of light-curing, the adhesives' flexural strength and hardness tests were performed.

According to the results, the increase in nanoparticle fillers concentration affected the mechanical properties, which is in agreement with a previous study [40]. Group SBMP presented higher flexural strength (FS) than the group Exp.15 wt.% β-TCP; and the first null hypothesis was rejected. It can be suggested that when higher concentrations were used, the nanoparticles formed agglomerates, which act as defected areas susceptible to stress accumulation; as a consequence, lower FS were observed. Additionally, the use of fillers with nanometric dimensions increases the amount of entrapped air in the resin matrix [41], contributing to a decrease in the FS.

Both commercial adhesives (CFPB and SBMP) had higher Knoop hardness number (KHN) than all experimental resin-based materials (filled or not), rejecting the first null hypothesis. As group CFPB had higher %DC than all groups, higher hardness values were expected, since a positive correlation between amount of cure and hardness has been stablished in the literature [42]. In addition, it was demonstrated that the incorporation of Ca/P fillers into dental adhesives can reduce the surface hardness of the polymerized monomer chain [43], due to the inorganic nature of the fillers, which may not have a chemical reaction with the resin matrix. However, in the present study, unfilled groups did not differ from filled groups. In this scenario, it was not possible to confirm that fillers decresead hardness. According to the literature, the addition of reinforciment fillers, such as glass particles, can benefit the hardness [44]. So, future studies are needed to investigate if glass particles incorporation can increase the hardness of adhesives with β-TCP nanoparticles.

According to the results of the present study, cell viability was also affected by the increase in filler concentration, and the second null hypothesis was also rejected. Ideally, all experimental groups should have a similar (or lower) cytotoxicity effect compared to the commercial adhesives, as all of them have the potential in reducing cell viability [45]. However, the group Exp.15 wt.% β-TCP promoted lower dental pulp stem cell proliferation than all the other groups, except SBMP. Although the incorporation of β-tricalcium phosphate has not been toxic when tested at lower dilutions [17], our results suggest that there is a concentration limit to be used in dental application, and this should be explored in the future using a range of materials with β-TCP nanoparticles, such as solutions, adhesives and gels, and different dilutions as well.

After performing the physical-mechanical and biological characterization, the ability of the experimental resin-based material to reduce dentin permeability was investigated. Dental adhesives have the advantage of being in contact with tooth structure for a prolonged period, compared to other types of desensitizers, such as toothpaste and gels. This is possible since dental adhesives are light-cured materials that form a mechanical barrier on the tooth surface. The mechanical forces, as abrasion, gradually remove this resin layer, and this effect can be increased with the impact of erosive acids [46]. However, in the present study, it was expected that the active principle incorporated into the adhesive systems could slowly release minerals ions, and due to their nanometric dimensions - smaller than the diameter of dentinal tubules under hypersensitive conditions - it was also expected that these nanoparticles could penetrate the dentinal tubules. In this way, the Ca/P system used would be able to perform a physical obliteration of the dentinal tubules, reducing dentin permeability. The formation of mineralization nodules was also expected, since calcium-based materials can induce dental pulp cells to differentiate in odontoblastic-like cells [47] and, β-TCP has a highlighted potential in the formation of new bone tissue [48].

Initially, an EDTA solution was used to open dentinal tubules. EDTA has a chelating action and a higher ability in opening dentinal tubules than sodium hypochlorite and chlorhexidine gel [49,50]. For these reasons, EDTA has been used in *in vitro* studies to increase dentin permeability [12,20,30]. At this time point (T_0), groups were compared and no diferences were observed, implying in similar permeability before treatment applications. After treatment applications (T_1), the group negative control (no treatment), showed a higher permeability than all groups, except when compared to the group Exp.15 wt.% β-TCP. It can be suggested that increasing the concentration of fillers in etch-and-rinse adhesive system resulted in higher viscosity and as consequence, less resin infiltration into demineralized dentin [51]. The fillers were not expected to be dissolved into the resin matrix, only dispersed. As previously discussed, under higher concentrations, particle aglomerates are formed, reducing their ability to penetrate the small interfibrillar spaces. All these suggestions are confirmed by a previous study [40], which performed TEM micrographs and found hydroxyapatite nanoparticles dispersed on resin matrix without a penetration in the hybrid layer. However, when erosive/abrasive challenges were performed, ions release might occured, allowing the material to provide a long-term effect.

After the erosion/abrasion cycle (T_2), only experimentally filled groups (Exp.10 wt.% β-TCP and Exp.15 wt.% β-TCP) did not have a statistically significative difference between the times T_1 (After Treatment Application) and T_2 (After Erosion/Abrasion), showing their ability in resisting the chemical/mechanical insults, which most DH treatments fail to do [12,52]. β-TCP nanoparticles might promote rapid and stable occlusion of dentin tubules, as it has been seen for Calcium-Doped Silica Mixed with Phosphate-Doped Silica Nanoparticles [53], and this justifies the results obtained for the filled groups in the present study. In addition, under a low pH environment, there is an increase in calcium and phosphate ions release, as demonstrated in a study using Tetracalcium phosphate (TTCP) particles incorporated into a resin matrix [44]. Since the lowest is the calcium and phosphate particles, the highest is the ions release [41], it is possible to assume that a high ionic release occurred for the adhesives filled with β-TCP nanoparticles after the erosive challenge. Under the presence of these ions and the presence of bioactive calcium phosphate nanoparticles [54], such as the β-TCP nanoparticles used here, hydroxyapatite can be precipitated and therefore, both experimentally filled groups maintained their post-treatment effect in the post-cycling moment. This assumption is confirmed by a previous study, in which a resin-based material containing β-TCP provided a higher supersaturated concentration of minerals, promoting hydroxyapatite precipitation [55].

The group Exp.10 wt.% β-TCP promoted a higher reduction in dentin permeability than the group CFPB, so the third null hypothesis was also rejected. Studies have shown that CFPB adhesive (a 2-step self-etching primer) has a positive impact in earlier stages of dentin wear [56] and in the formation of inhibition zone after artificial caries challenge, as it has fluoride in its composition [57]. In the present study, this group was used as a benchmark product for two reasons: the manufacturer recommends CFPB for the treatment of tooth sensitivity and because of the role of fluoride in reducing dentin permeability [58] and tooth wear [59]. Fluoride allows the formation of calcium fluoride-like deposits (CaF_2) on the tooth surface; however, this layer does not resist the continuous erosive attacks [59] and this can justify the difference between CFPB and Exp.10 wt.% β-TCP. The absence of difference between the other groups after erosive/abrasive cycling may be related to a similar degree of water permeation through the resin matrix caused by simulated pulp pressure [60].

Group SBMP (3-step etch-and-rinse adhesive) was also tested in the present study since it does not have fluoride in the composition, nor any other agent that could promote tooth remineralization. In this way, the effect of the material on dentin sealing by a mechanical mechanism could be investigated and compared with experimental groups, expected to provide biological and chemical effects on tooth remineralization. In addition, SBMP was considered a positive control in a previous study that investigated the effect of a dental adhesive contaning calcium/phosphate nanoparticles on tooth remineralization [61].

In general, dental adhesives can be classified as etch-and-rinse (E&R) and self-etch (SE) adhesives. Based on the number of steps, the 3-step total-etch or 2-step self-etching adhesive systems are known to provide better dentin surface sealing, as a layer of hydrophobic monomers is formed [62]. However, a systematic review showed that the adhesion on eroded substrate is a challenge, and a surface pre-treatment is required [63]. Based on these studies, and considering that an experimental adhesive was tested in the present study, the conventional 3-step total-etch technique was adopted, since it is a well-established method in literature and with more predictable adhesion and restorative longevity results.

When the etch-and-rinse system is used, 5–8 μm of the intertubular dentin matrix can be demineralized [64]. However, even with this additional demineralization promoted by the acid etching, the experimental adhesive with 10 wt.% β-TCP, used in a three-step method, promoted higher reduction in dentin permeability than two-step adhesives, such as CFPB. It can be suggested that with this additional demineralization, the nanoparticles had the chance to penetrate more through the tubules, and as a consequence, induced the formation of mineralized tissue.

The synthesys of the experimental resin-based material was performed based on previous studies [22,25], without the addition of 10-methacryloyloxydecyl dihydrogen phosphate (10-MDP). However, 10-MDP can increase the adhesion to dentin substrates, as chemical interaction occurs with hydroxiapatite, allowing the formation of MDP-Ca salts. These salts improve adhesive stability, protect collagen fibers [65], and their effects on dentin permeability needs to be investigated.

Although different experimental models are available to simulate erosive tooth wear, the present study used 5-day cycling, in which alternating episodes of erosion and remineralization are performed associated with toothbrushing simulation. This *in vitro* cycling model has been used to investigate the prevention of ETW and the progression of dentin permeability [31]. The parameters adopted, such as acid concentration, exposure frequency and time, and also the amount of brushing cycles were previously defined in literature [66], to simulate a patient with high risk of ETW. In the present study, 15 s of brushing were used as it was demonstrated that individuals perform oral hygiene by brushing with toothpaste twice a day [67], and, according to this study, each brushing lasts about 120 s, and therefore, each face is brushed for 15 s, totaling 30 s per day. Based on this previous evidence, studies [52,68,69] have been using 15 s of brushing (2x/day) to test the effect of desensitizing agents on dentin permeability and/or erosive tooth wear. Also, the hydraulic conductance, adopted to assess dentin permeability reduction, has different parameters in literature, such as the pressure used. Some authors adopted 10 PSI [30], while other authors 5 PSI [20,21]. Based on the machine capacity available during this study, 5 PSI was used, allowing to compare the results with recent studies [20,21].

The present study used artificial saliva because this is the first time that the experimental material was synthesized and tested, so its behavior against erosion/abrasion was unknown. Nonetheless, the ability of the present material in reduce dentin permeability should also be tested in future studies, using more clinically relevant models with human saliva and using an *in situ* model. The present study also did not evaluate the adhesive thickness on dentin surface, neither their viscosity. However, the treatment application was performed by a single operator, previously trained, using a disposable applicator dispenser with the same size for all groups. It seems the experimental resin based dental material synthesized in the present study has a great potential for tooth remineralization, therefore its effect in preventing dental caries, enamel and dentin wear should be further explored.

5. Conclusions

The resin-based dental material synthesized with 10 and 15 wt.% β-TCP nanoparticles reduced dentin permeability, resisting to the erosive/abrasive challenges, when all the other treatments tested failed to do. The incorporation of 10 wt.% β-TCP nanoparticles into the resin-based dental material did not affect its mechanical properties and biocompatibility. However, when the concentration of fillers increased (15 wt.% β-TCP), lower mechanical properties were observed compared to the commercial adhesives, and dental pulp cells viability was affected.

CRediT authorship contribution statement

Leonardo Custódio Lima: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. **Flávia Rodrigues Oliveira Silva:** Conceptualization, Data curation, Writing – original draft, Writing – review & editing. **Ítallo Emídio Lira Viana:** Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Giovanna Corrêa Denucci:** Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Christen Leigh Mumaw:** Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing.

Chandler Walker: Conceptualization, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing. **Anderson T. Hara:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Taís Scaramucci:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Sabrina Feitosa Sochacki:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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