UNIVERSIDADE ESTADUAL DE CAMPINAS

FACULDADE DE ODONTOLOGIA DE PIRACICABA

PEDRO HENRIQUE FREITAS

INIBIÇÃO DE METALOPROTEINASE E ATIVIDADE ANTIBIOFILME PELA INCORPORAÇÃO DE DOXICICLINA EM ADESIVO DENTINÁRIO

MATRIX METALLOPROTEINASE INHIBITION AND ANTIBIOFILM ACTIVITY BY INCORPORATION OF DOXYCYCLINE INTO DENTAL ADHESIVE

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Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisites exigidos para a obtenção do título de Doutor em Materiais Dentários.

Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Dental Materials.

Orientador: Prof. Dr. Simonides Consani

ESTE EXEMPLAR CORRESPONDE À VERSÃO FINAL DA TESE DEFENDIDA PELO ALUNO PEDRO HENRIQUE FREITAS E ORIENTADA PELO PROF. DR. SIMONIDES CONSANI.

PIRACICABA
2017

Agência(s) de fomento e nº(s) de processo(s): CAPES, 1777/2014

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca da Faculdade de Odontologia de Piracicaba
Marilene Girello - CRB 8/6159

F884i

Freitas, Pedro Henrique, 1987-
Inibição de metaloproteinases e atividade antibiofilme pela incorporação de doxiciclina em adesivo dentinário / Pedro Henrique Freitas. – Piracicaba, SP : [s.n.], 2017.

Orientador: Simonides Consani.
Tese (doutorado) – Universidade Estadual de Campinas, Faculdade de Odontologia de Piracicaba.

1. Adesivos dentinários. 2. Doxiciclina. 3. Metaloproteases. I. Consani, Simonides,1939-. II. Universidade Estadual de Campinas. Faculdade de Odontologia de Piracicaba. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: Matrix metalloproteinase inhibition and antibiofilm activity by incorporation of doxycycline into dental adhesive
Palavras-chave em inglês:
Dentin-bonding agents
Doxycycline
Metalloproteases
Área de concentração: Materiais Dentários
Titulação: Doutor em Materiais Dentários
Banca examinadora:
Simonides Consani [Orientador]
Polliana Mendes Cándia Scaffa
Lawrence Gonzaga Lopes
Rafael Nobrega Stipp
Livia Câmara de Carvalho Galvão
Data de defesa: 17-02-2017
Programa de Pós-Graduação: Materiais Dentários

UNICAMP

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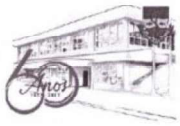

A Comissão Julgadora dos trabalhos de Defesa de Tese de Doutorado, em sessão pública realizada em 17 de Fevereiro de 2017, considerou o candidato PEDRO HENRIQUE FREITAS aprovado.

PROF. DR. SIMONIDES CONSANI

PROFª. DRª. POLLIANA MENDES CÁNDIA SCAFFA

PROF. DR. LAWRENCE GONZAGA LOPES

PROF. DR. RAFAEL NOBREGA STIPP

PROFª. DRª. LIVIA CÂMARA DE CARVALHO GALVÃO

A Ata da defesa com as respectivas assinaturas dos membros encontra-se no processo de vida acadêmica do aluno.

RESUMO

O objetivo neste estudo *in vitro* foi analisar o efeito da incorporação de diferentes concentrações de doxiciclina nas propriedades físico-químicas do adesivo, resistência da união à dentina, nanodureza e modulo de elasticidade da interface adesiva, atividade antibiofilme e atividade inibitória de metaloproteinases. Doxiciclina foi incorporada à um adesivo comercial de técnica úmida (Single Bond 2) nas concentrações de 0,05-, 0,1-, 0,5- e 1%. O adesivo não modificado foi utilizado como controle. Os efeitos das adições nas propriedades físico-mecânicas foram analisadas pelo pH, grau de conversão, , resistência flexural, modulo de flexão, sorção e solubilidade. Resistência de união, nanoinfiltração, nanodureza e módulo reduzido da interface dentina-adesivo foram avaliados após 24h e 1 ano de armazenagem em água. Biofilme de *S. mutans* foi crescido por 3 dias em discos de resina composta cobertos com adesivos, sendo a viabilidade bacteriana e o peso seco quantificados. Imagens do biofilme formado foram obtidas por MEV. O efeito inibitório da atividade das MMPs foi analisado por meio de zimografia *in situ* com microscopia confocal de fluorescência por varredura à laser. Adição de doxiciclina reduziu o pH dos adesivos modificados a partir da concentração de 0,5% alterou negativamente o grau de conversão a partir da concentração de 0,5%. Nenhuma alteração foi observada nos valores de resistência flexural, modulo de flexão, sorção e solubilidade. A incorporação de doxiciclina não afetou os valores de resistência da união imediata. Entretanto, o grupo controle apresentou diminuição significativa na resistência de união após um ano de armazenamento enquanto que os grupos com adição de doxiciclina apresentaram-se estáveis. O mesmo efeito pode ser observado nas imagens de nanoinfiltração, quando o grupo controle apresentou grande acumulo de nitrato de prata no período de um ano. Para os valores de nanodureza imediata apenas a camada híbrida foi estatísticamente superior na concentração de 0,1% comparada com o grupo controle. Após o período de um ano de armazenagem somente os valores de nanodureza da camada adesiva apresentaram diferença estatística, sendo que o controle apresentou valores superiores ao grupo 1%, os quais não foram significamente diferentes das concentrações intermediárias. Módulo de elasticidade do adesivo, camada híbrida e dentina não apresentaram diferença, qualquer que fosse a concentração. As concentrações de 0,5% e 1% reduziram a

viabilidade bacteriana e o peso seco. As imagens de MEV mostraram diminuição da densidade do biofilme formado de acordo com o aumento da concentração, assim como menor cobertura de bacterias ocorreu a partir da concentração de 0,1%. As imagens por confocal mostram aumento proporcional da inibição das MMPs de acordo com o aumento da concentração de doxiciclina. Concluindo, que a incorporação de doxiciclina mostrou se eficiente na inibição de MMP com capacidade de reduzir a viabilidade bacteriana e a biomassa de biofilme para as maiores concentrações.

Palavras-chave: Adesivos dentinários, doxiciclina, metaloproteinases.

ABSTRACT

The aim of this *in vitro* study was to analyze the effect of doxycycline incorporation in different concentrations into dental adhesives, on physical-chemical properties, microtensile bond strength, interfacial nanohardness and elastic modulus, antibiofilm activity and MMP inhibition. Doxycycline was incorporated into a wet-bonding commercial adhesive (Single bond 2) in concentrations of 0.05, 0.1, 0.5 and 1%. An adhesive with no addition was used as a control group. The effect of the additions on physical-chemical properties was analyzed by pH determination, degree of conversion, flexural strength, flexural modulus, water sorption and solubility. Microtensile bond strength, nanoleakage, nanohardeness and elastic modulus were analyzed after 24 h and 1 year of water storage. Biofilms of S. mutans were grown on adhesive-coated resin disks for 3 days to perform the bacterial viability and to determine the amount of biomass. Qualitatively SEM images were obtained from the biofilm. The inhibitory effect on MMP was analyzed by *in situ* zimography under confocal microscopy. The addition of doxycycline reduced the pH from 0.5% concentration and above, and also reduced the degree of conversion. No effect was observed on flexural strength, flexural modulus, water sorption and solubility between groups. The incorporation of doxycycline did not affect the microtensile bond strength values between the groups tested. However, the control group showed a decreased in bond strength after 1 year of water storage, and the doxycycline-containing groups showed stable bond strength after aged, instead. The same effect was observed in nanoleakage images, as the control group showed a densest silver nitrate accumulation after one year of storage. Immediate nanohardness values presented an increased with the addition of 0.1% of doxycycline compared to the control group for the hybrid layer. After one year of storage, the adhesive layer with 1% doxycycline decreased compared to the control group, and both groups were not different from other concentrations. No difference was observed for elastic modulus for all concentrations and storage times tested. The 0.5 and 1% concentrations of doxycycline decreased the bacterial viability and the biomass. SEM images shows a less dense biofilm following the increase of doxycycline, and the

reduction in adhered bacteria seems to appear from 0.1% concentration. Confocal images show a proportional increase of MMP inhibition according to the increase of doxycycline. In conclusion, the doxycycline-modified adhesives showed efficacy to inhibit MMP and reduced the bacterial viability and biomass for the higher concentrations.

Keywords: dentin adhesive, doxycycline, metalloproteinases.

SUMÁRIO

INTRODUÇÃO

O avanço da Odontologia adesiva tem proporcionado o desenvolvimento de técnicas restauradoras conservadoras, além de proporcionar união dente-restauração (Cunha *et al*., 2008; Alonso *et al*. 2007). Contudo, a combinação de sistemas adesivos com resinas compostas não tem sido considerado um paradigma definitivo da Odontologia restauradora, considerando as falhas verificadas em alguns estudos clínicos (Hashimoto *et al*., 2000; Hebling *et al*., 2005; Carrilho *et al*., 2007a; Palaniappan *et al*., 2009; Ricci *et al*., 2010).

O insucesso das restaurações adesivas pode estar associado, em parte, à degradação da camada híbrida e deterioração das fibras colágenas (De Munck *et al*., 2003; Carrilho *et al*., 2007a; Hashimoto *et al*., 2010; Fukuoka *et al*., 2011), causando redução da resistência da união (RU) da interface adesiva com o decorrer do tempo (Carrilho *et al*., 2007a; Breschi *et al*., 2010; Hashimoto et al., 2010). Estudos prévios sugerem que o processo de degradação da interface adesiva está relacionado com a presença de enzimas proteolíticas (Carrilho *et al*., 2007a; Breschi et al., 2010), mais especificamente às metaloproteinases (MMPs) provenientes da matriz dentinária, as quais também estão envolvidas com a progressão da lesão cariosa (Vidal *et al*., 2014). Assim, a aplicação de substâncias inibidoras dessas enzimas pode reduzir a deterioração das fibras colágenas (Pashley *et al*., 2004; Hebling *et al*., 2005; Carrilho *et al*., 2007b), aumentando a longevidade da união adesiva.

Estudos recentes têm demonstrado que inibir essas proteases pode ser uma importante estratégica para manutenção da interface adesiva (Almahdy *et al*., 2012; Tjäderhane *et al*., 2013; Scheffel *et al*., 2014a; Scheffel *et al*., 2014b). A doxiciclina é uma tetraciclina capaz de inibir a atividade das MMPs independentemente da ação antimicrobiana (Golub *et al*., 1998), sendo considerada o inibidor mais potente e não seletivo de MMPs (Golub *et al*., 1991). A doxiciclina é o único inibidor de MMPs aprovado para uso clínico pelo FDA (US *Food and Drug Administration*) para o tratamento de doenças periodontais em dose "subantimicrobiana", ou seja, em doses que produzem concentrações plasmáticas menores do que as requeridas para efeito antimicrobiano (Novak *et al*., 2002; Lee *et al*., 2004). Além disso, o uso da doxiciclina tem demonstrado efeitos benéficos no

tratamento de outras doenças em que as MMPs desempenham ações patológicas (Axisa *et al*., 2002; Prall *et al*., 2002; Villarreal *et al*., 2003; Liu *et al*., 2003; Onoda *et al*., 2004; Tessone *et al*., 2005; Guimarães *et al*., 2010).

Assim, o estudo da doxiciclina como inibidora de MMPs na tentativa de preservar a união adesiva parece ser um propósito válido. Especialmente, no uso de adesivos de condicionamento total (Tezvergil-Mutluay *et al*., 2013; Zhang *et al*., 2009; Tersariol *et al*., 2010), onde as enzimas são expostas e ativadas pelo condicionamento ácido (Hebling et al., 2005; Brackett *et al*., 2009), podendo degradar lentamente as fibrilas de colágeno da camada híbrida, resultando em significativa diminuição da união (Koshiro *et al*., 2004; Carrilho *et al*., 2007).

2 ARTIGO: Matrix metalloproteinase inhibition and antibiofilm activity by incorporation of doxycycline into dental adhesive*

*Submitted to Biomaterials Journal

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ABSTRACT

The aim of this *in vitro* study was to analyze the effect of doxycycline (DOX) incorporation in different concentrations into dental adhesives, on physical-chemical properties, microtensile bond strength (μTBS), nanoleakage (NL), nanohardness (NH) and reduced modulus of elasticity (Er), antibiofilm activity (AA)

and MMP inhibition. DOX was incorporated into a wet-bonding commercial adhesive (Single bond 2) in concentrations of 0.05, 0.1, 0.5 and 1 %. An adhesive with no addition was used as a control group. The effect of the additions on physical-chemical properties was analyzed. Microtensile bond strength, nanoleakage, nanohardeness and reduced modulus of elasticity were analyzed after 24 h and 1 year of water storage. Biofilms of *Streptococcus mutans* were grown and bacterial viability and to determine the amount of biomass. Qualitatively scanning electron microscope (SEM) images were obtained from the biofilm. The inhibitory effect on MMP was analyzed by *in situ* zimography under confocal microscopy. The addition of DOX reduced the pH from 0.5% concentration and above, and also reduced the degree of conversion. No effect was observed on physical properties. The incorporation of DOX did not affect the μTBS values between the groups tested. The control group showed a decreased in bond strength after 1 year of water storage, and the DOX-containing groups showed stable bond strength after aged, instead. The same was observed in NL images, as the control group showed a densest silver nitrate accumulation after one year of storage. Immediate NH values presented an increased with the addition of 0.1 % of DOX compared to the control group and 1 % concentration for the hybrid layer. After one year of storage, the adhesive nanohardness with 1 % DOX decreased compared to the control group, and both groups were not different from other concentrations. No difference was observed for ER for all concentrations and storage times tested. The 0.5 and 1 % concentrations of DOX decreased the bacterial viability and the biomass and showed the reduction in adhered bacteria by SEM. Confocal images show a proportional increase of MMP inhibition according to the increase of DOX. In conclusion, the DOX-modified adhesives showed efficacy to inhibit MMP and reduced the bacterial viability and biomass for the higher concentrations. Furthermore, DOX incorporation did not influence the physical properties of the adhesives.

Keywords: dentin adhesive, doxycycline, metalloproteinases.

INTRODUCTION

Resin composite restorations have become the treatment of choice in restorative dentistry due to the higher aesthetics and conservative preparations compared to amalgam restorations (Sabbagh *et al.*, 2016). However, the longevity of the dentin-adhesive interface remains uncertain as a result of the physical and chemical factors challenging the adhesive interface. Over time, the resin-dentin bond may weak due to interfacial degradation caused by the association of both factors leading to restoration failure (Breschi *et al.*, 2008).

It is well known that collagen fibrils exposed by acid etching are incompletely infiltrated by adhesive monomers. This poorly resin-infiltrated dentin matrix leaves unprotect collagen fibrils susceptible to enzymatic degradation host-derived matrix metalloproteinases (MMPs). MMPs are a group Zn2+ and Ca2+ dependent proteases synthesized during dentinogenesis, and remaining inactive in mineralized dentin (Visse and Nagase, 2003). These enzymes are released and activated when dentin is demineralized by etching acids or acidic monomers, resulting in long-term progressive loss of collagen from the hybrid layers (Mazzoni *et al.*, 2007, Mazzoni *et al.*, 2006, Sulkala *et al.*, 2007).

In order to decrease the activity of these proteases and maintain the durability of bonded restorations, the use of MMP inhibitors has been demonstrated to be an alternative therapy. Doxycycline (DOX) is an antibiotic of tetracycline class that has antimicrobial activity and the most potent and non-selective inhibition effect on MMPs. The approach involving a sub-antimicrobial dose of DOX (SDD) has been applied to the treatment of many diseases where MMPs has a pathological role (Vandenbroucke and Libert, 2014). The SDD is the first-ever FDA approved MMP-inhibitor drug for any disease. In dentistry, DOX has been largely used against generalized aggressive periodontitis (Golub *et al.*, 2016, Kim *et al.*, 2016, Mahmoud and Samy, 2016). However, only recently the use of DOX as an MMP inhibitor for demineralized dentin and adjuvant on the biodegradation process has been studied (Feitosa *et al.*, 2014, Silva Sousa *et al.*, 2016, Stanislawczuk *et al.*, 2011, Toledano *et al.*, 2012).

The MMP inhibitors can be applied on demineralized dentin as a pretreatment before resin infiltration through a primer containing the therapeutic

agent or by the use of an adhesive containing the MMP inhibitor in it formulation. Besides the disadvantaged of increasing the time of restorative procedure by primer application, the use of a primer containing DOX do not have a significant effect on bond strength (Stanislawczuk *et al*, 2011), despite being effective on the inhibition of MMPs. When DOX was incorporated in nanotubes into dental adhesives it promoted MMP inhibition without jeopardizing physicochemical and bonding properties of the adhesive (Feitosa *et al*, 2014). However, few details regarding the loading efficiency of encapsulation process and release capacity of DOX from theses nanotubes is not available. Thus, initial studies should focus on the ability of doxycycline to be incorporated into the adhesive and determining optimum concentration for the inhibition of metalloproteinases, thereby preventing proteolytic degradation of collagen and stabilizing the bond.

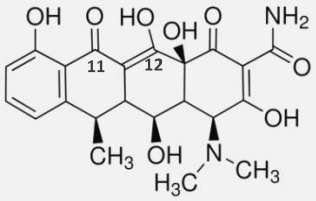
Therefore, the purpose of this *in vitro* study was to evaluate the effectiveness of DOX-containing adhesives (0.05-, 0.1-, 0.5- and 1 %) with the same adhesive without the additions by microtensile bond strength, nanoleakage, nanohardness and reduced modulus of the resin-dentin interface immediately and after 12 months of storage. The adhesives were physicochemical characterized by pH determination, degree of conversion, flexural strength, flexural modulus, water sorption and solubility analyses. The antibiofilm activity of these adhesives against *S. mutans* biofilm and the MMP inhibition was also evaluated.

MATERIALS AND METHODS

This study was performed under approval of the institutional ethics committee (#025/2014). The DOX powder was incorporated into a commercial two-step etch-and-rinse bonding system (Table 1) at concentrations of 0.05-, 0.1-, 0.5- and 1 % (w/v). The DOX concentrations were chosen according to their solubility characteristics, which limited the maximum incorporation to 1 % (w/v) into the adhesive resin. To avoid solvent volatilization and spontaneous polymerization from the ambient light, the modified adhesives were kept closed throughout the homogenization procedure and were manipulated under yellow light in a dark room with temperature and humidity controlled (23°C ± 2°C and 50 % ± 10 %). The adhesive and DOX powder were placed into the centrifugal laboratory mixing and

spun at 3000 rpm for 5 min to ensure adequate solubility of the powder within the adhesive. Due to the acidic nature of the DOX after 24 h of manipulation the pH of the adhesives was measured by a sensing electrode for hydrogen ion concentration. The same polywave light-curing unit was used in high intensity mode with radiant exposure of 15 J/cm^2 (Bluephase, Ivoclar Vivadent, Schaan, Liechtenstein) for all experiments. The unmodified adhesive was used as control.

Table 1 – MMP inhibitor, molecular structure, MMP effectiveness, adhesive, composition and application method

MMP inhibitor	Molecular structure	MMP effectiveness on dentin
Doxycycline hiclate (DOX) Sigma Aldrich; St Louis, MO, USA. Lot. #BCBP0625V		MMP-2, -8 and -9
Adhesive	**Composition**	**Application method**
Adper Single Bond 2 pH (4.7) 3M ESPE, St. Paul, MN, USA. Lot. #B14425	Etchant: 35% phosphoric acid, water, silica Adhesive: HEMA, water, ethanol, amines, Bis-GMA, methacrylate-functional, polycarboxylic acid, dimethacrylates. silanated colloidal	Acid etching for 15 s; rinse with water for 30 s; remove excess moisture with absorbent paper; apply two consecutive coats of the adhesive with a fully saturated brush tip; gentle air stream for 5 s; light-cure for 10 s

HEMA 2-hydroxyethyl methacrylate, Bis-GMA bisphenol a diglycidyl ether dimethacrylate

Degree of conversion

The degree of conversion (DC) of the unmodified and modified adhesives was analyzed using near-infrared (NIR) spectroscopy (Nicolet 6700, Thermo Fisher Scientific, Loughborough, UK). A preliminary reading for each uncured material was recorded under the absorption peak of the methacrylate functional group centered at 6165 cm^{-1} (Stansbury and Dickens, 2001). Light curing was performed for 10 s, the peak area of the sample before and after 10 min of the light-activation was recorded with 32 scans per spectrum at 4 cm^{-1} resolution, and the DC calculated as: DC = (1 - final peak area/initial peak area) × 100%. The analyses were performed in triplicate.

Flexural strength and flexural modulus

Flexural strength (FS) and flexural modulus (E) were performed using bars specimens (n = 10) obtained from a stainless-steel standard mold (25 mm length x 2 mm width x 2 mm height), according to the ISO 4049. The bars were polymerized between glass slides for 1 min each side and stored dry for one week in dark containers at room temperature (Bacchi *et al.*, 2015). After storage, the specimens were tested in three-point bending method carried out with an universal testing machine (Instron model 4411, Instron Corp., Canton, MA, USA) at a crosshead speed of 0.5 mm/min. The flexural strength (FS) was then calculated as: $FS(\sigma) = 3Fl/2bh^2$, where F stands for load at fracture (N), l is the span length, and b and h are the width and thickness of the specimens in mm, respectively. The flexural modulus (E) was determined from the slope of the initial linear part of stress–strain curve, calculated as: $E = Fl^3/4bh^3d$, where F is the load at some point on the linear region of the stress–strain curve; d is the slack compensated deflection at load F; and l, b, and h are as defined above.

Water sorption and solubility

Water sorption (WS) and solubility (SL) were determined according to the ISO 4049 except for the specimen dimensions (Fabre *et al.*, 2007). The adhesive was directly dispensed in four installments (Gaglianone *et al.*, 2012) (5 µL each) to the stainless-steel standard mold (5 mm diameter x 1 mm depth), and gently air blown for 10 s, after each addition, for solvent evaporation. The specimens (n = 10) were light-activated for 10 s each side and stored in desiccators at an oven at 37°C, containing silica gel, for 24 h. The specimens were repeatedly weighed daily until a constant mass (m_1) was obtained, i.e., until the mass variation was less than 0.1 mg per 24 h cycle. Thickness and diameter of each specimen was measured using a digital electronic caliper at four points. The specimens were then individually immersed in sealed glass vials containing 10 mL of deionized water and replaced at the oven for 7-days. After the storage time, the specimens were washed in running water, carefully wiped with a soft absorbent paper, weighted (m_2) and returned to desiccator until obtained the constant mass (m_3) in the same cycle described for m_1. WS and SL

over 7-days of water storage were calculated as: WS = (m_2 – m_3)/V; SL = (m_1 – m_3)/V.

Microtensile Bond Strength

Fifty caries-free human third molar teeth (n=7) were cleaned, stored in 0.2 % thymol solution under refrigeration, and used no longer than 3 months after extraction. The roots and crowns were removed using a slow-speed water-cooled diamond saw, exposing a flat surface of dentin (Isomet, Buehler; Lake Bluff, IL, USA). The exposed dentin surfaces were mechanically abraded using wet 600-grit SiC paper for 60 s to create a standardized smear layer. Restored teeth were randomly divided into the studied groups (n = 10). All adhesive systems were applied following the instructions for use (Table 1). The resin composite (Filtek Z350, shade A2; 3M ESPE) was placed in 2-mm-thick increments, and each composite layer was light-activated for 20 s. The entire restored teeth were stored in deionized water at 37°C for 24 h. After storage time, the bonded teeth from each group were sliced into beams (1.0 ± 0.01 mm^2) (Sano *et al.*, 1994) with a slow-speed water-cooled diamond saw attached to a section machine (Isomet 1000; Buheler). The beams from each tooth were divided by the storage time (24h and 1 year) and tested after these times. The beams were attached to tensile dispositive with cyanoacrylate gel (Super Bonder gel; Loccite, Rocky Hill, CT, USA) and tested for failure under tension in an universal testing machine (EZ-test, Shimadzu, Kyoto, Japan) at 1.0 mm/min. The exact cross-sectional area was measured, and the data were expressed in MPa. The microtensile bond strength (μTBS) of the beams from the same bonded tooth were averaged and used for the statistical analysis as the statistical unit.

Nanoleakage

One central bonded-beam from each tooth was selected after storage time and submitted to the nanoleakage (NL) survey using 50 wt % ammoniacal silver nitrate solution [Ag(NH3)2NO_3(aq)] (Tay *et al.*, 2002). The bonded-beams were immersed in the tracer solution for 24 h and then immersed in photo-developing solution for 8 h under a fluorescent light. After that, the specimens were rinsed

thoroughly in distilled water, embedded in epoxy resin and polished using wet #600, #1200, #2000 SiC papers and 3-, 1-, and 0.25-μm diamond pastes (Buehler) with polishing cloths. They were ultrasonically cleaned after each polishing step for 5 min. The specimens were coated with carbon and observed under SEM by means of backscattered electron mode.

Nanohardness and Reduced Modulus of Elasticity

Fifteen caries-free human third molar teeth (n=3) were prepared and restored as described before for μTBS. Three bonded teeth from each group were longitudinally sectioned in the mesio-distal direction through the bonded interface using a slow-speed water-cooled diamond saw (Isomet-1000; Buehler) to obtain 1.0-mm-thick bonded slices. Two slices from the center of the teeth were selected from each tooth for the nanoindentation test (n = 3). The resin-bonded dentin slices were individually embedded in an epoxy resin (Buehler) and manually polished using wet SiC papers (Norton) with decreasing abrasiveness (600, 1000, 1200, 1500 and 2000). The samples were also polished using discs with diamond suspensions of 9, 6, 3, 1 and 0.5 μm (Buehler). The samples were kept immersed in Hank's solution for aging (Habelitz *et al.*, 2002). For the nanohardness (NH) and reduced modulus of elasticity (Er) measurements, the Hysitron Custom Triboindenter (Hysitron: Minneapolis, MN, USA) computer-controlled nano-indenter with a cell Berkovich point was used. The hydrate samples were individually placed on a computer-controlled X-Y table, and an accurate calibration of the distance between the microscope and nanoindenter of the probe was performed on the standard fused quartz sample before starting the test. In all samples, five equally-spaced nanoindentations were programmed for the dentin, hybrid layer and adhesive layer (Figure 1). The nanoindentations were performed with a load of 1000 μN, and a standard trapezoidal load function of 5–2–5 s (Freitas *et al.*, 2016). The nanohardness and Young's modulus of each area were computed according to the Oliver and Pharr's method (Oliver and Pharr, 1992).

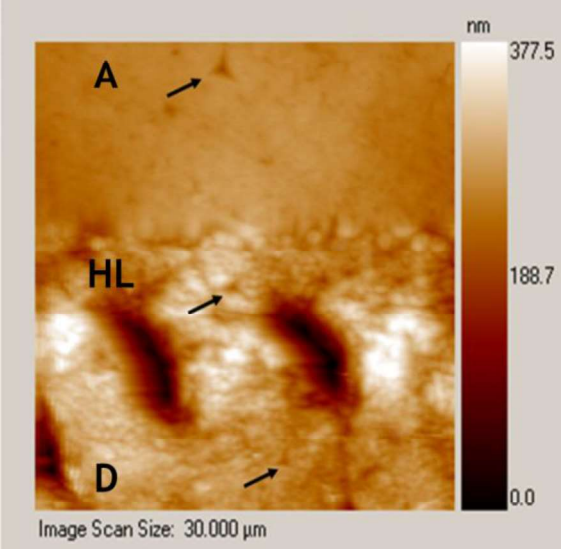


Figure 1. Topographic AFM image of the nanoindentations (arrows) on dentin (D), hybrid layer (HL) and adhesive layer (A).

Streptococcus mutans biofilm

The ability of *S. mutans* (UA159) to form biofilm on saliva-adhesive-coated disks was performed (Koo *et al.*, 2003). Thirty resin composite discs (n=6) were prepared in order to apply the adhesives and test against *S. mutans* biofilm. The resin composite was placed into a stainless-steel standard mold (2 mm thickness and 8 mm diameter) covered with a polyester strip and a glass microscope slide, pressured, to remove excess. The resin composite was light-activated for 20 s. The resin composite discs were sterilized in UV light for 1 min each side. The disks were divided in accordance with the adhesive group, and 20µL of the adhesive was applied on the entire surface of the composite resin discs and light-activated for 10 s. Human whole saliva was collected from one donor, clarified by centrifugation (10000 g, 4°C, 10 min), sterilized and diluted (1:1) in adsorption buffer (AB - 50mM KCl, 1mM KPO_4, 1mM $CaCl_2$, 0.1mM $MgCl_2$, pH 6.5), and supplemented with the protease inhibitor phenylmethylsulfonyl-fluoride (PMSF) at a final concentration of 1mmol/L. The disks were placed in a vertical position, in 24-well plates, and inoculated with approximately $2x10^6$ CFU/mL in buffered ultrafiltered (10 kDa cutoff membrane; Prep/Scale; Millipore, MA) tryptone yeast extract (pH 7.0), with the addition of 1% (w/v) sucrose, at 37°C, 5% CO_2. The biofilms were grown undisturbed during 24 h, and then the culture medium was replaced daily during 3 days of each experiment. At

the end of the experimental period, the biofilm was collected and processed to calculate the viable cells (colony forming units CFU/mL), and the dry-weight (biomass).

In situ zymography

Fifteen bonded teeth were (n=3) cut into resin–dentin slabs and a self-quenched fluorescein-conjugated gelatin was used as the MMP substrate (E-12055, Molecular Probes, Eugene, OR, USA). The substrate was prepared from a 1 mL stock solution of DQ-gelatin (DQ-gelatin, E12055; Molecular Probes, Eugene, OR, USA). An anti-fading agent (Mounting Medium with Dapi H-1200, Vectashield, Vector Laboratories LTD, Cambridgeshire, UK) and fluorescein-conjugated gelatin were added in the dilution buffer (NaCl 150 mM, $CaCl_2$ 5 mM, Tris–HCl 50 mM, pH 8.0) in a proportion of 1:1:8 (Mazzoni *et al.*, 2013). Fifty microliter of the fluorescent-gelatin solution was spread on top of each sample and after incubated in a 100 % relative humidity dark chamber at 37°C. To observe the hydrolysis of quenched fluorescein-conjugated gelatin substrate, which resulted from gelatinolytic activity, each sample was examined under a confocal laser scanning microscope (Leica SP5 CLSM, Heidelberg, Germany), at 488 and 530 nm with excitation and emission wavelengths. The images were obtained at 24 h after incubation. Each resin–dentin interface was entirely characterized, and images representing the MMP-activity observed along the bonded interfaces were obtained.

Statistical Analysis

Values are expressed as means ± standard deviation. All data were analyzed for normality data and homogeneous variance. The pH data failed normality test and was analyzed by Kruskal-Wallis and Dunn's multiple comparisons test. Statistical analyses for DC, FS, FM, WS, WL, bacterial viability, and dry-weight were performed by One-way ANOVA followed by Tukey's multiple-comparison test. Repeated measures analyses of variance (rANOVA) followed by Tukey's multiple-comparison test were performed for μTBS, NH and Er. A value of $p < 0.05$ was

considered significant. Qualitatively analyses were performed for SEM and confocal images.

RESULTS

The data of physicochemical characterization from control group and the modified adhesives by DOX incorporation are described in Table 2. There were statistically differences ($p < 0.05$) between the values of pH and DC of tested adhesives. The highest concentrations (0.5 and 1 %) showed a significant decrease of pH and DC compared to the other groups. The values for FS, FM, WS and WL did not show statistical difference between the groups.

The μTBS values (Figure 2) did not statistically differ between the control and the modified adhesives at storage for 24 h. After 1 year of storage, statistically difference was observed only between the control and the experimental groups. In addition, the control group showed a statistically decrease for μTBS values after 1 year of storage period, result that was not observed for the experimental groups. The silver nitrate uptake was observed throughout the hybrid layer with the same pattern for all groups at 24 h. Slight changes in the nanoleakage pattern after aging were observed for the experimental groups (Figure 3, b.2, c.2, d.2 and e.2), whereas the control group showed intense silver penetration after 1 year (Fig 3, a.2).

Table 2 – Physicochemical characterization of control and modified adhesives by doxycycline incorporation

Adhesive	pH	DC	FS	FM	WS	SL
Control	4.67 ± 0.03 a	89.07 ± 0.33 a	16.50 ± 3.68 a	1.29 ± 0,24 a	237.36 ± 21.94 a	89.79 ±8.43 a
Dox 0.05%	4.60 ± 0.02 a	88,23 ± 0.71 a	15.08 ± 3.91 a	1.60 ± 0.37 a	240.47 ± 12.21 a	89.82 ± 8.22 a
Dox 0.1%	4.51 ± 0.03 ab	88.16 ± 0.99 a	15.34 ± 4.11 a	1.35 ± 0.39 a	246.57 ± 22.33 a	93.91 ± 7.22 a
Dox 0.5%	4.34 ± 0.04 b	82.46 ± 0.54 b	16.08 ± 5.53 a	1.28 ± 0.28 a	248.80 ± 18.53 a	98.06 ± 8.26 a
Dox 1%	4.25 ± 0.03 b	69.11 ± 0.37 c	15.86 ± 2.17 a	1.26 ± 0.16 a	261.88 ± 20.64 a	101.88 ± 11.68 a

pH - Potential of hydrogen; DC - Degree of conversion (%); FS - Flexural strength (Mpa); FM - Flexural modulus (GPa); WS - Water sorption (µg⁄mm3); SL - Solubility (µg⁄mm3). Identical letters in the columns indicates no statistically difference (p>0.05)

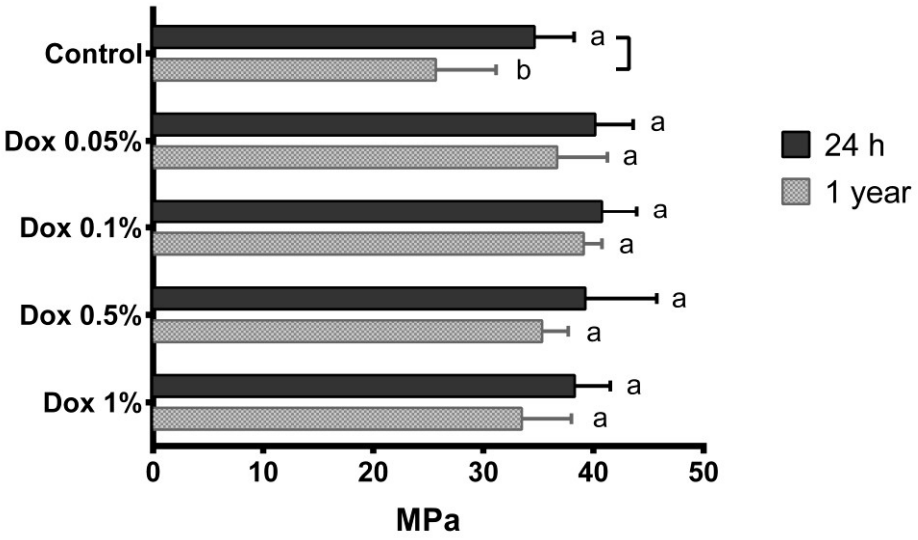


Figure 2. Microtensile bond strength (MPa) results for the different study groups at storage for 24 h and 1 year, bar compares the storage periods.

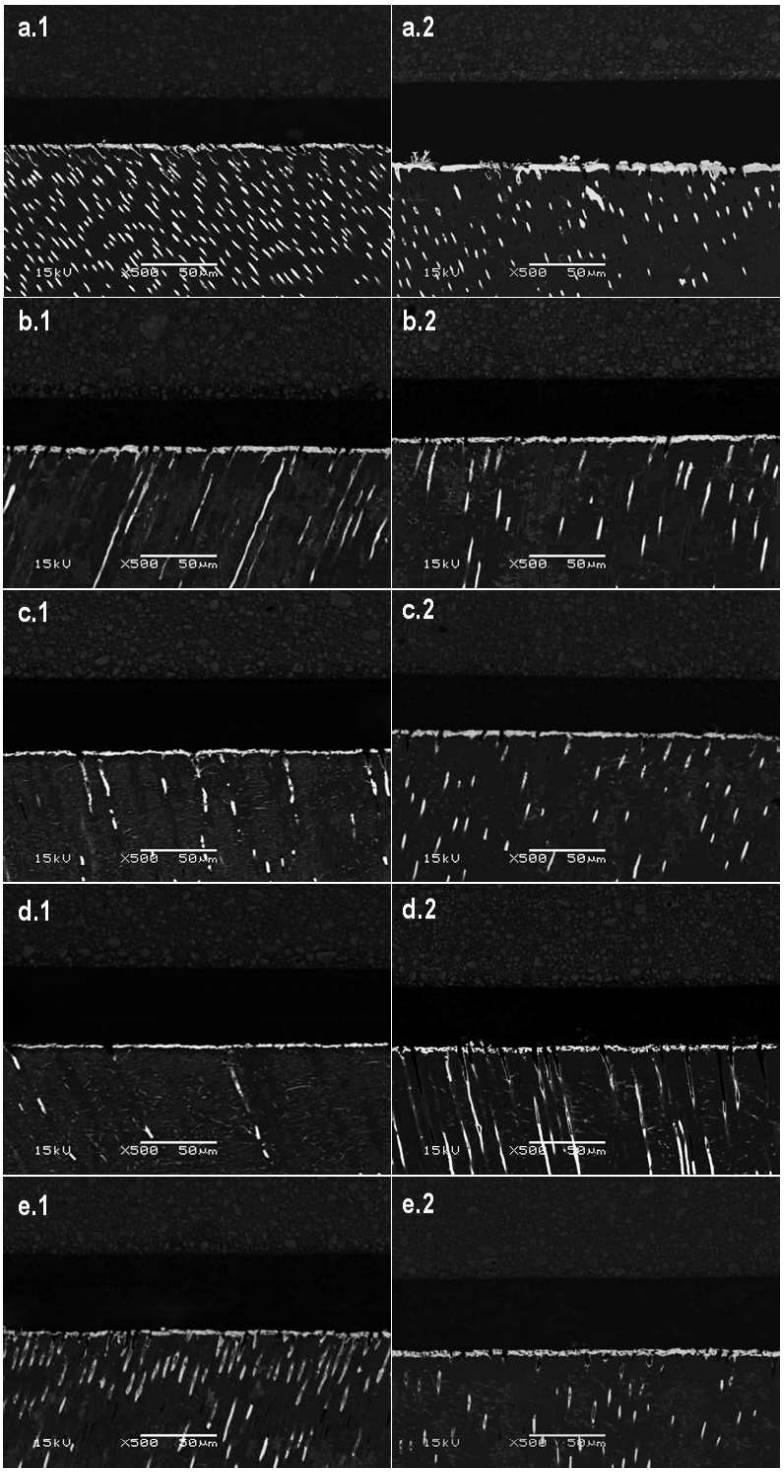


Figure 3. Back-scattering image of ammoniacal-silver-nitrate-stained resin-dentin interfaces. (a) Control; (b) Dox 0.05 %; (c) Dox 0.1 %; (d) Dox 0.5 %; (e) Dox 1 %; (.1) after 24 h of storage and (.2) after 1 year of storage.

Nanohardness (Table 3) and Young's modulus (Table 4) values for the adhesive layer, hybrid layer and dentin were significantly different for water storage period ($p < 0.05$). For nanohardness, only the hybrid layer at 24 h and the adhesive layer after 1 year showed statistical difference. The hybrid layer showed higher values for 0.1 % concentration compared to control and 1 %, and did not showed statistical difference between intermediate concentrations (0.05 and 0.5 %). After one year of water storage, the 1 % concentration showed significant decrease of the adhesive nanohardness among the control group, although this value was not different from the groups with lower concentration of DOX. No difference was observed for elastic modulus of the dentin, hybrid layer and adhesive for all concentrations.

Table 4 – Young's Modulus (GPa) of the dentin, hybrid layer and adhesive layer

	Adhesive		Hybrid Layer		Dentin	
Adhesive	**24 hours**	**1 year**	**24 hours**	**1 year**	**24 hours**	**1 year**
Control	4.60 ± 0.68 a	3.19 ± 0.60 a	6.72 ± 1.03 a	4.41 ± 0.98 a	0.65 ± 0.11 a	7.85 ± 2.05 a
Dox 0.05%	4.45 ± 0.98 a	3.02 ± 0.46 a	6.68 ± 0.57 a	4.92 ± 1.00 a	0.64 ± 0.08 a	8.25 ± 2.53 a
Dox 0.1%	4.44 ± 0.76 a	2.74 ± 0.47 a	6.93 ± 0.97 a	4.74 ± 0.53 a	0.67 ± 0.06 a	9.16 ± 2.25 a
Dox 0.5%	4.14 ± 0.63 a	2.87 ± 0.77 a	6.58 ± 1.14 a	4.60 ± 0.64 a	0.64 ± 0.08 a	9.21 ± 2.49 a
Dox 1%	4.44 ± 0.73 a	2.37 ± 0.86 a	6.30 ± 1.04 a	4.38 ± 1.28 a	0.69 ± 0.08 a	8.74 ± 1.90 a

Identical letters in the columns indicates no statistically difference (p>0.05)

Table 3 – Nanohardness (GPa) of dentin, hybrid layer and adhesive layer

	Adhesive		Hybrid Layer		Dentin	
Adhesive	**24 hours**	**1 year**	**24 hours**	**1 year**	**24 hours**	**1 year**
Control	0.35 ± 0.04 a	0.16 ± 0.03 a	0.38 ± 0.02 a	0.25 ± 0.08 a	0.65 ± 0.11 a	0.34 ± 0.10 a
Dox 0.05%	0.35 ± 0.06 a	0.15 ± 0.04 ab	0.39 ± 0.02 ab	0.28 ± 0.05 a	0.64 ± 0.08 a	0.35 ± 0.11 a
Dox 0.1%	0.36 ± 0.07 a	0.15 ± 0.02 ab	0.41 ± 0.02 b	0.29 ± 0.05 a	0.67 ± 0.06 a	0.37 ± 0.12 a
Dox 0.5%	0.34 ± 0.06 a	0.13 ± 0.04 ab	0.40 ± 0.02 ab	0.28 ± 0.12 a	0.64 ± 0.08 a	0.39 ± 0.14 a
Dox 1%	0.29 ± 0.06 a	0.12 ± 0.01 b	0.39 ± 0.02 ab	0.27 ± 0.07 a	0.69 ± 0.08 a	0.38 ± 0.11 a

Identical letters in the columns indicates no statistically difference (p>0.05)

Analyses from *S. mutans* biofilm are described in Figure 4. A decrease in bacterial viability and total amount of biomass (dry-weight) were observed for the 0.5 and 1 % concentrations. Representative images from *S. mutans* biofilm are shown in

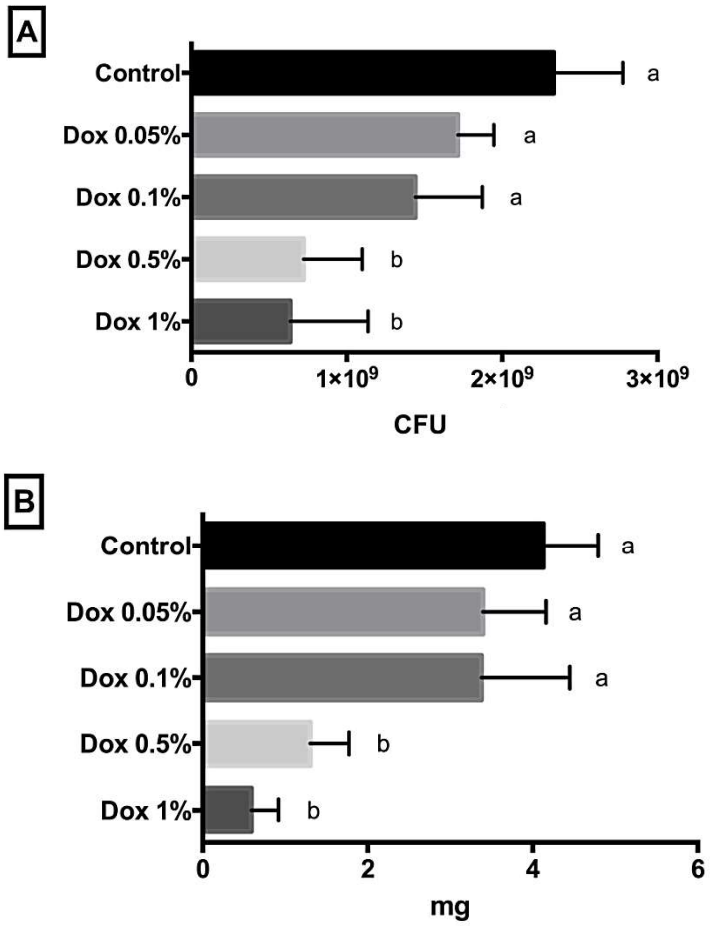


Figure 4. Effect of doxycycline incorporation into the adhesives on *S. mutans* biofilm grown (A) Bacterial viability. (B) Dry weight.

The *in situ* zymography (Figure 6) revealed an intense MMP activity at the hybrid layer and at the dentinal tubules for the control group (Figure 6, a1 and a3). The MMP activity was not observed at hybrid layer for modified adhesives containing DOX (Figure 6, b1, b3, c1, c3, d1, d3, e1 and e3). Despite an expressive reduction of MMP activity for the experimental groups, 0.05 % and 0.1 % concentration of DOX were not able to completely eliminate MMP activity in dentinal tubules. In addition, a proportional increase in inhibition of MMPs activity occurred as the concentration of DOX as increased with an apparently total inhibition observed from the concentration of 0.5 %.

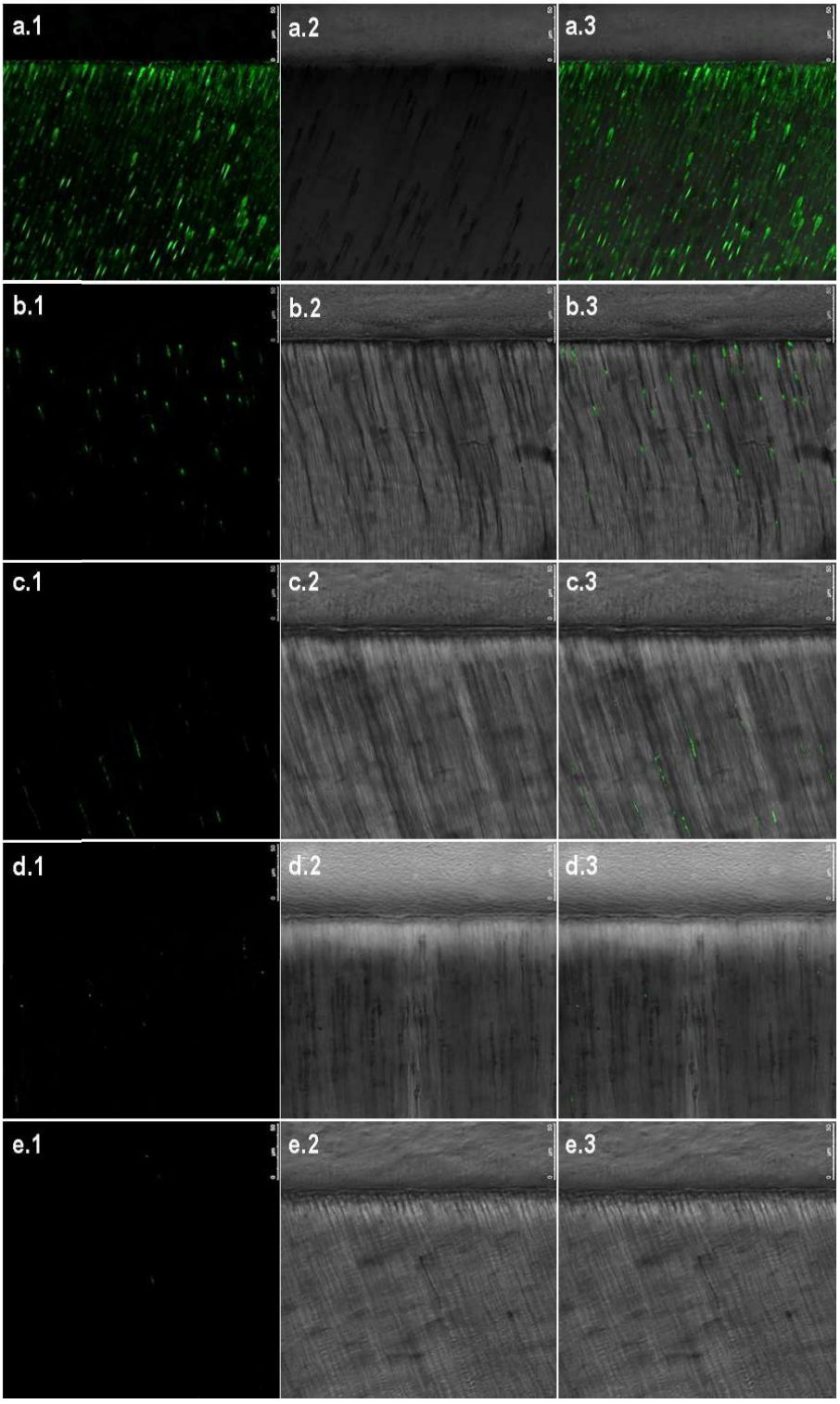


Figure 5. Resin-dentin bonded interface incubated for 24 h with quenched fluorescein-labeled gelatin (a) Control; (b) Dox 0.05 %; (c) Dox 0.1 %; (d) Dox 0.5 %; (e) Dox 1 %); (.1) Acquired image in green channel, showing fluorescence for MMP; (.2) Differential interference contrast, showing the optical density of the resin-dentin interface; (.3) Merged images 1 and 2. Bar = 50 μm.

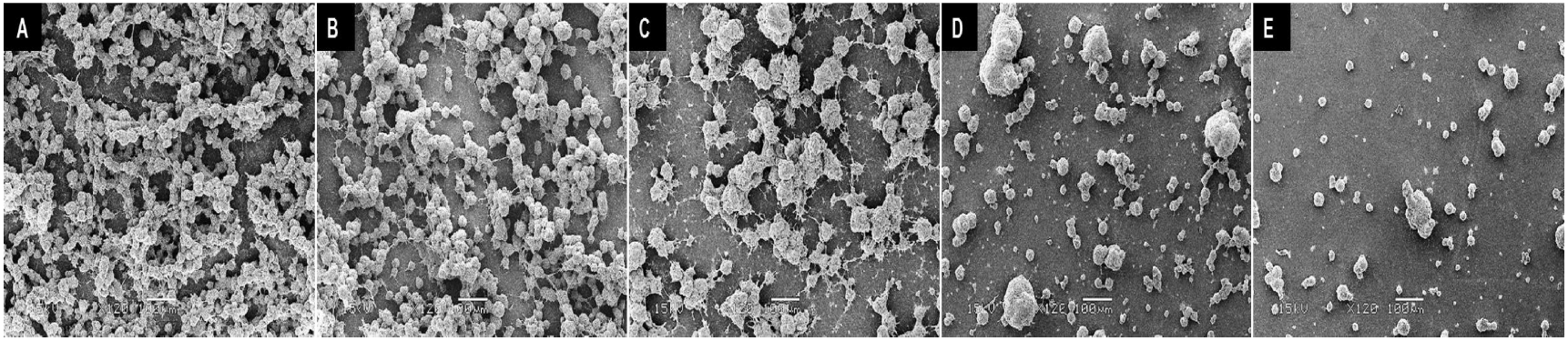


Figure 6. SEM images of biofilms of *S. mutans* in 120x magnifications. (A) Control SB; (B) Dox 0.05 %; (C) Dox 0.1 %; (D) Dox 0.5 %; (E) Dox 1 %.

DISCUSSION

The dentin etching leaves the collagen fibrils unprotected and vulnerable to degradation by endogenous matrix metalloproteinases (MMPs) capable of degrading all extracellular matrix components (Mazzoni *et al*, 2013). Human dentin contains MMPs-2, -8, and -9 which are capable of degrading type I collagen fiber (Mazzoni *et al*, 2013, Sulkala *et al*, 2007). Dentin collagenolytic and gelatinolytic activities can be suppressed by protease inhibitors, indicating that MMP inhibition could be beneficial in the preservation of collagen fibrils (Pashley *et al.*, 2004). DOX possess an unexpected ability to inhibit host-derived MMP activity and connective tissue destruction in various tissues, and by mechanisms unrelated to their antibiotic properties (Golub *et al*, 2016). Their use as a MMP inhibitor is approved by ADA for the use of host-modulation therapy with a sub-antimicrobial dose (SD) (Golub *et al*, 2016). This formulation, referred to as SD, given orally 20 mg, twice daily, yielded peak serum levels of 0.3 – 0.7 μg/mL (Golub *et al*, 2016). DOX serum levels of less than 1 μg/mL are considered too low to have an antibacterial effect (Golub *et al*, 2016). However, in the present study DOX was incorporated into the adhesive resin and applied topically on dentin after acid etching. DOX concentrations were chosen according to their solubility characteristics, which limited the maximum incorporation to 1 % (w/v) into the adhesive resin. The lower concentrations studies were 0.5, 0.1 and 0.05 %.

The incorporation of 0.5 and 1 % of DOX negatively influenced the DC values compared to other groups. Similarly, the pH showed statically difference for these groups. However, these alterations did not influence the values of flexural strength, modulus, sorption and solubility. Thus, we might consider that until a certain level the decrease of the DC does not seem to influence the mechanical properties. One reason for these findings is even when there is a difference in DC, the cross-linking density of polymerization reactions can maintain stable the mechanical properties (Dauvillier *et al.*, 2000, Oliveira *et al.*, 2016). Higher enzymatic activity at the adhesive interface of the control group does not seem to affect the dentin–resin bond strength at 24 hours. However, the control group showed a significant decrease of μTBS values compared to the modified adhesives after 1 year of storage. These

findings might be related to the effectiveness of DOX to avoid the collagen degradation. In addition, the pH of the modified adhesives did not influence on μTBS, showing that within a certain range differences of pH values may not be an imperative parameter to correlate with the bond strength (Gregoire and Millas, 2005). The same can be observed for the nanoleakage where the control group showed a higher silver nitrate uptake after 1 year of storage while slight changes in the nanoleakage after aging were observed for modified adhesives.

SD is also used in dentistry against generalized aggressive periodontitis by topical DOX application. The release from a commercial gel DOX formulation was reported to release during the first 2 hours of application a peak average of 1,500 to 2,000 μg/mL DOX found in the gingival crevicular fluid (Gamal and Kumper, 2012, Kim *et al.*, 2002, Stoller *et al.*, 1998). These peak averages are higher than 0.1% (1000 μg/mL) and lower than 0.5 % (5000 μg/mL), the concentrations of DOX incorporated into the adhesives. This information might help to explain the effect of DOX modified adhesives on biofilm of *S. mutans* grown. The groups containing 0.5 % and 1 % decreased the bacterial viability and reduced the biomass compared to control, 0.05 % and 0.1 % groups. This reduction was also observed in SEM images of the biofilms grown on adhesive-coated disks. DOX is effective against Gram-positive and Gram-negative bacteria, protozoa, and some anaerobes bacteria (Bokor-Bratic and Brkanic, 2000). DOX exerts the antibacterial action by inhibiting the microbial protein synthesis that requires access into the cell wall and lipid solubility. DOX binds the ribosome to prevent ribonucleic acid synthesis by avoiding addition of more amino acid to the polypeptide (Walker *et al.*, 2004).

Zymography *in situ* findings demonstrated the effectiveness of all groups with DOX incorporation. The control group revealed an intense green fluorescence at the hybrid layer and at the dentinal tubules, indicating strong activity of MMPs. The effect of MMPs inhibition was observed at hybrid layer for modified adhesives containing DOX. Proportional increase in inhibition of MMPs occurred as the concentration of DOX increased with almost complete inhibition in 0.5 % and 1 % groups. It has been already reported that DOX in 0.5 % was able to inhibit completely collagen degradation in demineralized dentine (Toledano *et al*, 2012). However, all experimental groups showed some expression of MMPs at dentinal tubules. MMPs are multidomain proteins with a catalytic domain containing a Ca^{2+} and Zn^{2+} ion

(Vandenbroucke and Libert, 2014). The site on the DOX molecule responsible for the anti-MMP activity, b-diketone moiety is located on carbon-11 and carbon-12 (Table 1) (Golub *et al.*, 1991). This site acts altering MMP to create distorted geometry around the catalytic domain, resulting in conformational changes in the enzyme protein (Smith *et al.*, 1999). Although the groups with the highest concentrations (0.5 % and 1 %) presented antimicrobial activity, these groups showed the greatest capacity of inhibition. It seems reasonable to consider that the therapeutic role of MMP inhibition of DOX works independently of the antimicrobial function. However, the increased risk for development of drug-resistance bacteria can be considered to the use of high concentrations.

Nanohardness and reduced elastic modulus showed significant decrease after 1 year of storage. Adhesive nanohardness showed a statistically decrease for the 1 % DOX modified adhesive, probably caused by the lower degree of conversion for this group. The nanohardness of the hybrid layer at 24 hours showed higher values for 0.1% concentration compared to control and 1 %, but did not showed statistical difference between intermediate concentrations (0.05 and 0.5 %). However, after aging no difference was observed for the nanohardness values of the hybrid layer. One possible explanation is that the tested area for the nanoindentation test is totally exposed to degradation, while for the μTBS test, the entire sample is tested and only the external area is exposed to degradation. Moreover, the values of hybrid layer nanohardness were numerically higher for the DOX modified adhesives, although no statistical difference was observed.

CONCLUSION

It can be conclude that: (1) the DOX incorporation into adhesives did not interfere with the dentin bond strength; (2) the different concentrations of DOX did not interfere with the physical properties of the modified adhesives; (3) the higher concentrations of DOX reduced the degree of conversion and pH of the modified adhesives; (4) all concentrations test had efficacy of MMP inhibition compared to control group; (5) higher concentrations showed a decrease in bacterial viability and biomass and (6) 0.1 % concentration showed better results regarding the reduction of

MMP activity without decreasing the bacterial viability and also better results for the hybrid layer nanohardness.

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3 CONCLUSÃO

Pode-se concluir que: (1) a incorporação de doxiciclina em adesivos não interferiu no mecanismo de união da dentina; (2) diferentes concentrações de doxiciclina não interferiram nas propriedades físicas dos adesivos modificados; (3) concentrações mais elevadas de doxiciclina reduziram o grau de conversão e o pH dos adesivos modificados; (4) todas as concentrações tiveram eficácia na inibição de MMP em comparação com o grupo de controle; (5) maiores concentrações mostraram uma diminuição na viabilidade bacteriana e biomassa e (6) concentração de 0,1% mostrou melhores resultados devido apresentar atividade de MMP sem diminuir a viabilidade bacteriana e também melhorar os resultados para a dureza da camada híbrida.

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ANEXOS

Anexo 1

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CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"Influência da adição de hiclato de doxiciclina na durabilidade da união, propriedade físico-mecânica e atividade antimicrobiana de adesivos dentinários experimentais "**, protocolo nº 025/2014, dos pesquisadores Pedro Henrique Freitas e Simonides Consani, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 07/05/2014.

The Ethics Committee in Research of the Piracicaba Dental School - University of Campinas, certify that the project **"Influence of addition of doxycycline hyclate in the durability of marriage, physical and mechanical properties and antimicrobial activity of experimental dentin adhesives"**, register number 025/2014, of Pedro Henrique Freitas and Simonides Consani, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee on May 07, 2014.

Prof. Dr. Felippe Bevilacqua Prado
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Profa. Dra. Lívia Maria Andaló Tenuta
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Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
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