UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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LABORATÓRIO DE MATERIAIS DENTÁRIOS

MICHELE STÜRMER

AVALIAÇÃO DAS PROPRIEDADES FÍSICO-QUÍMICAS E BIOLÓGICAS DE UM ADESIVO EXPERIMENTAL CONTENDO NANOTUBOS DE TITÂNIA E TRIAZINA

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Trabalho de conclusão de curso de graduação apresentado à Faculdade de Odontologia da Universidade Federal do Rio Grande do Sul como requisito obrigatório para a obtenção do título de Cirurgião-Dentista

Orientador: Prof. Dr. Fabrício Mezzomo Collares

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RESUMO

O objetivo do presente estudo foi formular adesivos experimentais e avaliar a influência da incorporação de nanotubos de titânia puros (nTi) ou com triazina (nTi-TAT) nas suas propriedades físicas, químicas e biológicas. nTi foram sintetizados pelo método hidrotérmico e parte do produto foi misturado a 1,3,5-trimetilhexahidro-1,3,5-triazina (TAT). O adesivo foi formulado com 66,6% de Bis-GMA e 33,3% de HEMA, em peso. Foram adicionados, como sistema fotoiniciador: 1% de Canforoquinona e 1% de EDAB, em mol, e 0,1% de BHT, em peso. nTi ou nTi-TAT foram incorporados em 2,5 e 5% e um grupo sem carga foi utilizado como controle. Os grupos resultantes foram: $G_{nTi2.5\%}$, $G_{nTi5\%}$, $G_{nTi\text{-}TAT2.5\%}$, $G_{nTi\text{-}TAT5\%}$ e G_{CTRL}. nTi foram avaliados por microscopia eletrônica de transmissão (MET). Avaliou-se a atividade antimicrobiana (n=3), citotoxicidade (n=3), cinética de polimerização e grau de conversão (GC) (n=3), resistência coesiva (UTS) (n=10), dureza Knoop e amolecimento em solvente (ΔKHN) (n=3) e resistência de união à microtração imediata e longitudinal (μ-TBS) (n=20). O padrão de fratura foi analisado. A distribuição dos dados foi analisada usando o teste de Shapiro Wilk. Para análise estatística utilizou-se ANOVA de duas vias e teste post hoc de Tukey para o ensaio antimicrobiano e μ-TBS, e ANOVA de uma via para os demais ensaios. Utilizou-se um nível de significância de 5%. Análise por MET confirmou a morfologia e tamanho dos nTi. A cinética de polimerização variou entre os grupos e todos atingiram valores de GC acima de 50%, sem diferença entre eles ($p>0.05$). $G_{nTi5\%}$, $G_{nTi\text{-}TAT2,5\%}$ e $G_{nTi\text{-}TAT5\%}$ apresentaram maiores valores de dureza inicial ($p<0.05$). $G_{nTi2,5\%}$, $G_{nTi\text{-}TAT2,5\%}$ e $G_{nTi\text{-}TAT5\%}$ tiveram menor ΔKHN ($p<0.05$). $G_{nTi\text{-}TAT2,5\%}$ e $G_{nTi\text{-}TAT5\%}$ apresentaram melhores resultados de UTS, além de atividade antimicrobiana contra formação de biofilme na superfície das amostras polimerizadas superior ao G_{CTRL} e grupos contendo nTi ($p<0.05$), sem diferença entre 24 e 48h. Todos os grupos apresentaram porcentagens elevadas de viabilidade celular, $G_{nTi5\%}$ e $G_{nTi\text{-}TAT2,5\%}$ alcançaram os maiores valores ($p<0.05$). Adição de nTi e nTi-TAT, em ambas as concentrações, não alterou a μ-TBS imediata em comparação ao G_{CTRL} ($p>0.05$). Todos os grupos tiveram redução da μ-TBS imediata e, após um ano, $G_{nTi\text{-}TAT2,5\%}$ alcançou melhores resultados. Conclui-se que nTi-TAT incrementou as propriedades das resinas adesivas experimentais e é uma alternativa promissora para o desenvolvimento de materiais dentários com melhores propriedades terapêuticas.

Palavras-chave: Adesivos dentinários. Antibacterianos. Cárie Dentária. Dentina.

ABSTRACT

The aim of the present study was to formulate experimental adhesives and to evaluate the influence of the incorporation of pure titanium dioxide nanotubes (nTi) or with triazine (nTi-TAT) in their physical, chemical and biological properties. nTi were synthesized by a hydrothermal method and part of the product was mixed with 1,3,5-triacryloylhexahydro-1,3,5-triazine (TAT). The adhesive resin was formulated with 66.6 wt.% of Bis-GMA and 33.3 wt.% of HEMA. Chloroquinone and EDAB at 1% in mol, and BHT at 0.1 wt.% were added as the photoinitiator system. nTi or nTi-TAT were incorporated in 2.5wt.% and 5 wt.% and a group without filler addition was used as control. The resulting groups were: $G_{nTi2.5\%}$, $G_{nTi5\%}$, $G_{nTi-TAT2.5\%}$, $G_{nTi-TAT5\%}$ e $G_{CTRL.}$ nTi were evaluated by transmission electron microscopy (MET). Was evaluated the antibacterial activity (n=3), cytotoxicity (n=3), polymerization kinetics and degree of conversion (DC) (n=3), ultimate tensile strength (UTS) (n=10), Knoop hardness and softening in solvent (ΔKHN) (n=3) and immediate and longitudinal microtensile bond strength (μ-TBS) (n=20). The fracture pattern was analyzed. Data distribution was performed using the Shapiro Wilk test. Statistical analysis was performed using two-way ANOVA and Tukey's post hoc test for the antimicrobial assay and μ-TBS and one-way ANOVA for the remaining assays. All tests were performed at a level of significance of 0.05. Size and morphology of nTi were confirmed by TEM analysis. The polymerization kinetics varied between groups and all groups reached values of DC above 50%, with no difference among them ($p>0.05$). $G_{nTi5\%}$, $G_{nTi-TAT2,5\%}$ and $G_{nTi-TAT5\%}$ had higher values of initial microhardness ($p<0.05$). $G_{nTi2,5\%}$, $G_{nTi-TAT2,5\%}$ and $G_{nTi-TAT5\%}$ had lower ΔKHN ($p<0.05$). $G_{nTi-TAT2,5\%}$ and $G_{nTi-TAT5\%}$ presented better results of UTS, besides presenting antimicrobial activity against formation of biofilm on the surface of polymerized samples superior to G_{CTRL} and groups containing nTi ($p<0,05$), with no difference between 24 and 48 h. All groups presented high percentages of cell viability, $G_{nTi5\%}$ and $G_{nTi-TAT2,5\%}$ reached the highest values ($p<0.05$). Addition of nTi and nTi-TAT, at both concentrations, did not change the immediate μ-TBS compared to G_{CTRL} ($p>0.05$). All groups had immediate-to-longitudinal μ-TBS reduction and, after one year, $G_{nTi-TAT2,5\%}$ achieved better results. It is concluded that nTi-TAT increased the physical, chemical and biological properties of experimental adhesive resins and is a promising alternative for the development of dental materials with better therapeutic properties.

Keywords: Dentin adhesives. Antibacterials. Dental caries. Dentin.

SUMÁRIO

1 INTRODUÇÃO

Os avanços na Odontologia adesiva vêm possibilitando a adoção de estratégias cada vez mais conservadoras. Resinas compostas e adesivos são amplamente utilizados na prática clínica restauradora devido às suas vantagens de preparo conservador, estética, preenchimento direto, possibilidade de reparo (DEMARCO *et al.*, 2012; FERRACANE, 2011). No caso da dentina, a adesão desses materiais resinosos envolve a penetração dos monômeros através dos espaços interfibrilares da mesma previamente condicionada, formando um embricamento micromecânico entre o colágeno e o polímero, chamado hibridização (NAKABAYASHI; KOJIMA; MASUHARA, 1982). Entretanto, a interface de união é o ponto fraco nessas restaurações, pois está propensa à degradação por enzimas colagenolíticas, por produtos bacterianos e fluidos orais (HASHIMOTO *et al.*, 2000; TJÄDERHANE *et al.*, 2013). Esta degradação da camada híbrida e a contração de polimerização podem levar a perda da integridade marginal e microinfiltração bacteriana (FERRACANE, 2013; KERMANSHAHI *et al.*, 2010) facilitando a falha mecânica e o desenvolvimento de cárie recorrente, a principal causa de falha de restaurações à longo prazo (DEMARCO *et al*., 2017; FERRACANE, 2013; KERMANSHAHI *et al.*, 2010; OPDAM *et al.*, 2014).

Muitos esforços têm sido dedicados ao desenvolvimento de estratégias para promover uma forte e durável adesão ao substrato. A incorporação de partículas inorgânicas, por exemplo, tem sido estudada com o objetivo de incrementar as propriedades mecânicas e físico-químicas das resinas adesivas (LEITUNE *et al.*, 2013a; LEITUNE *et al.*, 2013b; REIS *et al.*, 2013). A adição de partículas inorgânicas diminui a quantidade relativa de matriz polimérica, diminuindo o estresse da contração de polimerização e a degradação hidrolítica (CONDON; FERRACANE, 2000). O desenvolvimento de materiais antibacterianos também sido amplamente estudado objetivando a redução da adesão bacteriana e formação de biofilme na interface dente/restauração (FARRUGIA; CAMILLERI, 2015; FUGOLIN; PFEIFER, 2017; GARCIA *et al.*, 2018a; SCHIROKY *et al.*, 2017).

Nanopartículas, como nanotubos de dióxido de titânio (nTi) tem sido utilizados como carga inorgânica (DAFAR *et al.*, 2016; KHALED *et al*, 2010; RAMOS-TONELLO *et al.*, 2017) e podendo ter atividade antibacteriana (KIM *et al.*, 2017). Além disso, devido à sua estrutura tubular, podem agir como carreadores de fármacos (KUMERIA *et al.*, 2015; ROGUSKA *et al*., 2015; WANG *et al.*, 2016). TiO_2 é também um material biativo, como demonstrado em estudos anteriores ao ser incorporado a polímeros imersos em solução do tipo fluido corporal simulado (SBF) (SAITO *et al.*, 2011; SHIMIZU *et al.*, 2016). Entretanto, já foi demonstrado que esses

nanotubos tendem a sofrer aglomeração (KHALED *et al.,* 2010). Enquanto partículas inorgânicas bem dispersas melhoram as propriedades mecânicas por restringir a matriz circundante contra deformação (BELLI *et al.,* 2014), aglomerados podem reduzir a resistência mecânica dos polímeros agindo como sítios de concentração de tensões (WAGNER *et al.,* 2013).

Com o intuito de conferir propriedades antimicrobianas aos sistemas adesivos, diversas substâncias foram utilizadas, como clorexidina, fluoretos e partículas de prata. Entretanto, esses agentes antibacterianos são considerados agentes de liberação e possuem limitações, como a impossibilidade do controle da cinética de liberação, levando, consequentemente, a diminuição da atividade antibacteriana ao longo do tempo (COCCO *et al.*, 2015). Além disso, a liberação do agente pode afetar negativamente as propriedades físicas do polímero, tornando o material suscetível a degradação e aumentando a citotoxicidade (KURATA *et al.*, 2011). Uma alternativa poderia ser o uso de sistema de carreamento de fármacos (GENARI *et al.*, 2017; PRIYADARSHINI *et al.,* 2017) ou monômeros antibacterianos capazes de copolimerizar com a matriz resinosa (SCHIROKY *et al.*, 2017; VIDAL *et al.*, 2018).

Compostos quaternários de amônio (CQA) são moléculas antimicrobianas muito utilizadas em materiais dentários experimentais (ANDRÉ; CHAN; GIANNINI, 2018; ANTONUCCI e*t al.,* 2012). Seu mecanismo de ação antibacteriano ainda não é bem elucidado, mas acredita-se que seja por uma interação eletrostática com os elementos carregados negativamente da membrana e parede bacteriana, levando à disrupção e perda dos componentes intracelulares (BEYTH *et al.*, 2006). A incorporação de CQA com grupos metacrilato em resinas adesivas proporciona atividade antibacteriana sem prejuízo às outras propriedades do polímero, devido à capacidade de copolimerização com os demais monômeros, ficando imobilizados na matriz polimérica. O brometo de metacriloiloxidodecilpiridínio (MDPB) foi o primeiro monômero antibacteriano incorporado em um sistema adesivo comercial (IMAZATO; RUSSELL; MCCABE, 1995; IMAZATO *et al.*, 1999). Mais tarde, o dimetil metacrilato de cloreto de amônia (DMAE-CB) também foi incorporado em outro adesivo comercial demonstrando propriedades antibacterianas (CHAI *et al.*, 2011). Ambos são monômeros compostos pela combinação de um grupo metacrilato, polimerizável, com um grupo amônia quaternária, responsável pelas propriedades antibacterianas. Ao contrário dos dimetacrilatos tipicamente utilizados em resinas e adesivos dentários, estes monômeros catiônicos geralmente têm apenas um grupamento metacrilato. A incorporação de altas concentrações de

monometacrilato poderia afetar significativamente as estruturas da rede polimérica e comprometer as propriedades do material (ANTONUCCI *et al.*, 2012). A utilização de monômeros dimetacrilato, por sua vez, poderia resultar em um polímero menos linear, com aumento do grau de conversão, e em um material menos lixiviável em comparação com outros monômeros de monometacrilato que contêm grupos de amônio quaternário, devido aos múltiplos grupos reativos (ANTONUCCI *et al.*, 2012).

A triazina (TAT) é um CQA que apresenta propriedades biológicas como atividade antibacteriana, antiviral, antifúngica e antiprotozoária (EL-FAHAM *et al.*, 2016). Na área odontológica, já foi testada em adesivos, mostrando maior estabilidade frente a solvente (ALTMANN *et al*., 2015; SCHIROKY *et al.*, 2017) e atividade antibacteriana contra células planctônicas de *Streptococcus mutans* (SCHIROKY *et al.*, 2017); além de redução do crescimento por contato direto (ALTMANN *et al.*, 2017). A TAT é uma molécula tri-funcional – apresentando 3 ligações alifáticas duplas (C=C) na sua estrutura química – e sua copolimerização pode, possivelmente, aumentar a densidade de ligações cruzadas do polímero final, sendo esta uma vantagem em relação aos monômeros monometracrilatos, como MDPB e DMAE-CB. Além da obtenção de um grau de conversão satisfatório, uma resina adesiva com maior densidade de ligações cruzadas gera um polímero menos linear e mais resistente à hidrólise e à degradação ao longo do tempo, com melhores propriedades mecânicas (COLLARES *et al.*, 2011; SCHNEIDER *et al.*, 2008) e menor citotoxicidade devido a menor quantidade de monômeros livres em contato com tecidos. Além disso, as triazinas são usadas como herbicidas e, por serem básicas, são mais adsorvidas que a maioria dos demais herbicidas (SENESI; ORAZIO; MIANO, 1995). Dessa forma poderia ser adsorvida na superfície de cargas inorgânicas para polímeros odontológicos, como nanotubos de titânia, melhorando as propriedades do polímero final por se ligar ao restante da blenda, influenciando, por exemplo, na transferência de tensão entre a matriz orgânica e carga inorgânica.

2 OBJETIVOS

O objetivo do presente estudo foi formular adesivos experimentais e avaliar a influência da incorporação de nanotubos de titânia puros (nTi) ou com triazina (nTi-TAT) nas suas propriedades físicas, químicas e biológicas.

3 ARTIGO CIENTÍFICO

Este trabalho de conclusão de curso se apresenta na forma de artigo científico, escrito na língua inglesa e segue as normas referente ao periódico Dental Materials (ISSN: 0109-5641) para qual será submetido.

Titanium dioxide nanotubes with quaternary ammonium compound addition to improve physicochemical and biological properties of adhesive resins

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Highlights:

- Titanium dioxide nanotubes-incorporated adhesives showed no antibacterial activity
- Nanotubes with triazine compound provided antibacterial activity for the adhesive
- The μ-TBS was improved with the addition of nanotubes with triazine compound
- All adhesives showed no cytotoxicity against human pulp fibroblasts

Abstract

Objective: This study aimed to evaluate the influence of nTi or nTi dopped with TAT (nTi-TAT) into an experimental adhesive resin.

Methods: nTi were synthesized by a hydrothermal method and part of the product was mixed with TAT. An experimental adhesive resin was formulated with Bis-GMA, HEMA and a photoinitiator system and it was used as control group ($G_{CTRL.}$). nTi or nTi-TAT were incorporated at 2.5wt.% or 5wt.% ($G_{nTi2.5\%}$, $G_{nTi5\%}$, $G_{nTi\text{-}TAT2.5\%}$, $G_{nTi\text{-}TAT5\%}$). Nanotubes were evaluated by transmission electron microscopy (TEM). The adhesives were evaluated for antibacterial activity, cytotoxicity, polymerization kinetics and degree of conversion (DC), Knoop microhardness and softening in solvent (ΔKHN), ultimate tensile strength (UTS) and immediate and long-term microtensile bond strength (μ-TBS).

Results: Size and morphology of nTi were confirmed by TEM analysis. Polymerization kinetics varied between groups. All groups reached values of DC above 50% with no significant difference. $G_{nTi5\%}$, $G_{nTi\text{-}TAT2.5\%}$ and $G_{nTi\text{-}TAT5\%}$ had higher values of initial microhardness ($p<0.05$). $G_{nTi2,5\%}$, $G_{nTi\text{-}TAT2.5\%}$ and $G_{nTi\text{-}TAT5\%}$ had lower ΔKHN ($p<0.05$). $G_{nTi\text{-}TAT2.5\%}$ and $G_{nTi\text{-}TAT5\%}$ presented better results of UTS ($p<0.05$). nTi-TAT containing groups presented antimicrobial activity against biofilm formation compared to G_{CTRL} ($p<0.05$). All groups presented high percentages of cell viability with higher vaues for $G_{nTi5\%}$ and $G_{nTi\text{-}TAT2.5\%}$ ($p<0.05$). Addition of nTi or nTi-TAT, at 2.5wt.% or 5wt.%, did not change the immediate μ-TBS ($p>0.05$). After one year, all groups presented a lower μ-TBS. $G_{nTi\text{-}TAT2.5\%}$ presented higher μ-TBS among groups after 1 year. ($p<0.05$).

S*ignificance:* The addition of nTi-TAT into an experimental adhesive resin improved its physicochemical properties, besides inducing antibacterial activity against biofilm *S. mutans* without cytotoxic effect against pulp fibroblasts.

Keywords: Polymers; Nanotechnology; Chemomechanical properties; Anti-bacterial agents; Dentin bonding agents.

1. Introduction

Composite resin restorations replacement accounts for a large part of operative work in clinical practice [1]. Reliable bonding of dental materials to tooth substrates depends on the mechanical and chemical properties of the polymer and the hybrid layer (HL) [2, 3]. However, the HL represents the weakest link of the restoration and, in the oral environment, is prone to degradation by bacterial and oral fluids [4, 5]. Marginal gaps caused by polymer degradation and polymerization contraction [6-8], as well as excessive roughness of composite resin over time [9], may lead to the development of recurrent caries. Therefore, enhancing the quality and stability of the polymer and the HL formed, besides reducing the bacterial colonization are effective strategies for achieving long-term success of restorative treatments.

Inorganic fillers have been used to reinforce polymers, such as adhesive resins. Their addition decreases the relative amount of organic matrix, decreasing the stress of the polymerization contraction and the hydrolytic degradation [10] [11], thus, providing reliable marginal sealing. Nanoparticles are advantageous alternatives for adhesive resins, because they could also permeate through interfibrillar spaces of collagen fibrils [12]. Titanium dioxide nanotubes (nTi) have been used as inorganic filler [13-15], and due to their tubular structure, they can also act as drug carriers [16]. TiO_2 is also a bioactive material, such property can be observed by the ability to stimulate osteoconduction, apatite-forming ability and induction of cell proliferation [17].

To create adhesive resins with antibacterial effect, quaternary ammonium compounds (QAC) have been studied [18, 19]. The incorporation of QAC with methacrylate groups into adhesive resins provides long-term antibacterial activity with no damage to other properties of the polymer due to copolymerization [20]. Thus, they are released, which could negatively influence the physical properties - turning the material susceptible to degradation - and increase the cytotoxicity. 1,3,5-triacryloylhexahydro-1,3,5-triazine (TAT) is a QAC with three aliphatic double bonds (C=C). When purely incorporated in adhesive resins, TAT was able to improve its physicochemical properties, such as the resistance against softening in solvent and bond strength [21-23]. TAT also demonstrated antibacterial effect by direct contact against biofilm formation and against planktonic cells of *Streptococcus mutans* [21, 23], bacteria related to the establishment and progression of dental caries [24]. Thus, the aim of this study was to evaluate the influence of nTi or nTi dopped with TAT (nTi-TAT) into an experimental adhesive resin.

2. Materials and methods

2.1 Preparation of TAT-dopped nTi and nTi analysis by transmission electron microscopy (TEM)

nTi were synthesized by a hydrothermal method using commercial TiO_2 P25 (1.0 g) and 10 M NaOH solution (100 mL) as reagents. A teflon-lined autoclave filled with the reagents was kept at 110 ºC under autogenous pressure and performed a cycle of 24 h. The resulting product was subjected to cooling, filtration, washing processes with deionized water. Then, the product was washed in pH 1.6 solution ajust by HCl, washed again with deionized water until pH 7, and dried at 100 ºC [25].

Part of the nTi synthesized were mixed with 1,3,5-triacryloylhexahydro-1,3,5-triazine (Sigma-Aldrich Chemical Co., St. Louis, Missouri, USA) (ratio 1:1 by weight) and ethanol PA by a magnetic stirrer until the solvent vaporization. The fillers remained at 37 °C until complete solvent vaporization.

The pure nTi were analyzed by TEM (JEM 1200 EXll, JEOL, Tokyo, Japan). Powder was dispersed in isopropyl alcohol PA and put under sonication for 15 min. A 10 µL drop was dispensed in a carbon-coated copper grid (TEM: Electron Microscopy Sciences, Hatfield, PA, USA), which was placed in a desiccator for 24 h. The analysis was performed under an acceleration voltage of 80 kV.

2.2 Preparation of experimental adhesive resins

Bisphenol A glycerolate dimethacrylate (Bis-GMA) at 66.6 wt.% and 2-hydroxyethyl methacrylate (HEMA) at 33.3 wt.% were mixed to formulate the adhesive resins. Camphorquinone (CQ) and ethyl 4-dimethylaminobenzoate (EDAB) at 1 mol% and butylated hydroxytoluene (BHT) at 0.01 wt.% were added as photoinitiator system. Monomers and photoinitiator were purchased from Sigma-Aldrich Chemical Co. (St. Louis, Missouri, USA). The previous formulated fillers were incorporated at two concentrations: 2.5 and 5 wt.%. An adhesive resin without inorganic filler was used as control group. The 5 resulting groups were: control group (G_{CTRL}), group with 2.5 wt.% of nTi ($G_{nTi2.5\%}$), and four test groups: group with 5 wt.% of nTi ($G_{nTi5\%}$), group with 2.5 wt.% of nTi with TAT ($G_{nTi\text{-}TAT2.5\%}$) and group with 5wt.% of nTi with TAT ($G_{nTi\text{-}TAT5\%}$).

Samples for all tests, except for ultimate tensile strength and microtensile bond strength, were prepared using a polyvinylsiloxane matrix with 1 mm thickness and 4 mm diameter. A light-emitting diode (Radii cal, SDI, Bayswater, Australia) at 1200 mW/cm^2 was used to perform the monomer photoactivation for all tests. All the samples were photoactivated for 30 s on each side, except for the polymerization kinetics test in which the samples were evaluated during 50 s of photoactivation on the top.

2.3 Antibacterial activity evaluation

The antibacterial activity was evaluated for biofilm formation and planktonic bacteria against *Streptococcus mutans* (NCTC 10449). For the antibacterial activity test by direct contact inhibition, six samples from each group were attached to the lid of a test plate that were submitted to hydrogen peroxide plasm sterilization [26, 27]. Three samples from each group were used for 24 h assay and the other three samples were used for 48 h assay. In each well, 900 μL of brain-heart infusion (BHI) broth (Sigma-Aldrich Chemical Co, St. Louis, Missouri, USA) with 1 wt.% of sucrose was added with more 100 μL of a suspension of an overnight broth culture of the *Streptococcus mutans* previously subcultured as described elsewhere [27]. The lid with the samples was placed on the sterile well-plate and the samples surfaces were exposed to the culture medium with the 10^6 CFU/mL of bacteria at 37 °C for 24 h. After 24 h, freshly BHI broth with sucrose containing 10^6 CFU/mL of bacteria was added in each well with 900 μL of BHI broth and sucrose for samples of 48 h assay. Then, the samples of 24 h assay were removed from the lid and vortexed in 1 mL of saline solution (0.9%) to be diluted until 10^{-6} dilution. Two 25-mL drops of each dilution were plated on BHI agar Petri dishes and incubated at 37 °C for 48 h. The number of colonies were visually counted and the colonies forming units per milliliter (CFU/mL) were obtained using the dilution where the colonies were counted in each dish. These procedures were repeated with the samples from the 48 h assay.

For antibacterial activity against planktonic bacteria, the experiment was performed using the same samples and broth as cited above. After the 24 h-contact between samples and BHI broth with *Streptococcus mutans*, 100 μL from each well was diluted in 900 μL of saline solution until 10^{-6} dilution. Two drops (25 μL each) from each dilution were plated on BHI agar and incubated for 48 h at 37 °C. The colonies were counted and transformed to CFU/mL as cited above. These procedures were repeated with the broths of samples from the 48 h assay.

2.4 Cytotoxicity

For the cytotoxicity assay, human fibroblasts were collected from a third molar extracted after approval of the Research Ethics Committee of this local institution (nº 1.739.340). Eluates were prepared using three samples from each group previously sterilized with hydrogen peroxide plasm. Each sample was immersed in 1 mL of Dulbecco's Modified Eagle's Medium (DMEM) for 24 h in eppendorf tubes. In this same day, 100 µL of broth containing the cells were seeded in triplicate at a concentration of $5x10^3$ in each well. After 24 h, 100 µL of eluate from each sample was placed in each well to treat the cells. One group of wells containing the cells did not enter in contact with eluates to be used as assay control. After 72 h at 37 °C, the cells were fixed with 50 µL of trichloroacetic acid solution at 50%, incubated at 4 °C for 1 h and washed 6 times with running water. The plates were dried at room temperature. Then, 50 µL of sulforhodamide B (SRB, Sigma-Aldrich, St. Louis, USA) at 0.4% was added in each well to stain the protein of viable cells. The plates were incubated for 30 min at room temperature, washed 4 times with solution of acetic acid at 1% and allowed to completely dry at room temperature. Trizma solution at 10 mM (100 µL) was added in each well and the plates were incubated for 1 h to allow complete solubilization of the dye. The plates were read at 560nm and cell viability was normalized against cell viability of untreated cells (wells from assay control). The results were expressed in percentage of viability.

2.5 Polymerization kinetics and degree of conversion (DC)

The polymerization kinetics and the DC were evaluated via Fourier Transform Infrared Spectroscopy (FTIR, Bruker Optics, Ettingen, Germany) with a spectrometer equipped with an attenuated total reflectance device (ATR). First, a background spectrum was ran using only a polyvinyl siloxane matrix (ADSIL, Vigodent, Rio de Janeiro, Brazil) to subtract any unwanted residual peaks from samples spectrums. Second, three samples for each group of experimental adhesive resins were distributed in the polyvinyl siloxane matrix for monomer analysis. Then, subtracting the saved background spectrum, polymerization kinetics was carried out for 50 s, with photoinitiation performed at 1 mm between the tip of the of light-cured unit unit and the top of each sample. Data processing was carried out with Opus 6.5 software (Bruker Optics, Ettlingen, Germany), in the range of 4000-800 cm^{-1} with 2 scans per second at the velocity of 160 kHz and resolution of 4 cm^{-1}. DC was calculated from according to a previous study [28] at 30 s. The graphs of DC versus time and polymerization rate (Rp) versus time were plotted.

2.6 Softening in solvent

Three samples from each group were embedded in self-curing acrylic resin and polished (Model 3v, Arotec, Cotia, SP, BR). After being stored-dried at 37 °C for 24 h, Knoop hardness (KHN) measurements were performed using a digital microhardness tester (HMV 2, Shimadzu, Tokyo, Japan). Five indentations at 10 g / 5 s were made in each sample before (KHN1) and after (KHN2) immersion in a solution of ethanol and water (70:30) for 2h. The relative hardness reduction was calculated (ΔKHN%) for each specimen and for each group.

2.7 Ultimate tensile strength (UTS)

Ten samples from each group were prepared using a metallic matrix with an hourglass design (8 mm long, 2 mm wide, 1 mm thick and a cross-sectional area of 1 mm^2). After storage for 24 h, the constriction area of each sample was measured using a digital caliper and fixed in metallic jigs with cyanoacrylate resin. The samples were loaded under tension in a universal testing machine (EZ-SX Series, Shimadzu, Kyoto, Japan) at 1mm/min of crosshead speed. The values were acquired in newton (N) and expressed in megapascal (MPa).

2.8 Microtensile bond strength (μ-TBS)

The immediate μ-TBS was evaluated using one hundred bovine mandibular incisors (20 per group), who had their buccal surface removed and the dentin surface exposed. A 600-grit SiC paper was used under wet conditions for 30 s to create a smear layer on the dentine surface. The dentine surface was etched with 37% phosphoric acid for 15 s, washed for 30 s and dried. A commercial primer (Primer Scotch Bond MultiPurpose; 3M ESPE) was actively applied for 20s and then the solvent was evaporated. The experimental adhesive resins were applied and photoactivated for 20 s. A composite build-up (Filtek Z350 XT, 3M ESPE, St Paul, USA) was performed in two increments of 2 mm and each one was light cured for 40 s, as indicated by the manufacturer. The teeth were stored in distilled water at 37 °C for 24 h and then sectioned into four to six sticks (area of 0.5 mm^2). The sticks were fixed to a microtensile device and tested on a mechanical testing machine (EZ-SX Series, Shimadzu, Kyoto, Japan il) at a crosshead speed of 1mm/min until failure.

For the long-term analysis, we also used one hundred bovine mandibular incisors (20 per group) and the test was performed as cited above, but the sticks were stored in distilled water at 37°C for one year.

The fracture pattern was analyzed and the fracture classified as adhesive (A), cohesive in dentin (CD), cohesive in composite resin (CR) or mixed (M). The results were expressed in percentage.

2.9 Statistical Analysis

Data distribution was evaluated by Shapiro-Wilk test. Two-way ANOVA and Tukey post-hoc test were used in the comparison of groups' antibacterial activity and μ-TBS. One-way ANOVA and Tukey post-hoc test were used to compare groups for DC, KHN1, ΔKHN and UTS. Paired t-test was used to compare KHN1 and KHN2 in each group. All tests were performed at a significance level of 0.05.

3. Results

TEM imagens shows the distribution of pure nTi (Figure 1). Their nanotubular morphology and size were confirmed with microscopy analysis.

The values of antibacterial activity tests are shown as colony forming units per milliliter (CFU/mL) in Table 1. All groups with TAT had lower bacterial growth on the surface of polymerized samples compared to G_{CTRL}, $G_{nTi2.5\%}$ and $G_{nTi5\%}$ ($p<0.05$). The lowest CFU/mL values were observed for $G_{nTi\text{-}TAT5\%}$ (3.64 ± 0.08) followed by $G_{nTi\text{-}TAT2.5\%}$ (4.10 ± 0.08). The values were not different from 24 to 48 h. There was no variation among groups regarding planktonic bacteria ($p>0.05$). Table 1 also shows the results of cytotoxicity evaluation. The highest values, 97.84% ± 2.20% and 93.83% ± 3.97% for $G_{nTi5\%}$ and $G_{nTi\text{-}TAT2.5\%,}$ respectively, were significantly higher than $G_{CTRL,}$ which reached 82.06% ± 3.45 ($p<0.05$).

The polymerization behavior of the experimental adhesive resins is presented in Figure 2. G_{CTRL} began the polymerization process before groups with filler addition (Fig. 3A) and achieved the maximum Rp (Rp_{max}) earlier. Fig. 3B). In groups with filler addition, Rp_{max} was reached at different times (Fig. 2B). Values of DC of adhesive resins are listed in Table 2. DC ranged from 57.00% ± 4.58% to 62.60% ± 0.57% with no significant difference among groups.

Values from UTS are shown in Table 2. The values varied between 51.47 MPa ± 7.65 MPa and 85.37 MPa ± 8.49 MPa. $G_{nTi\text{-}TAT2.5\%}$ and $G_{nTi\text{-}TAT5\%}$ presented higher values: 85.37 MPa ± 8.49 and 82.76 MPa ± 5.22 MPa, respectively, with no statistical difference between these two groups ($p<0.05$). All Knoop hardness values and variation of hardness (ΔKHN) are shown in Table 2. The KHN1 was higher for $G_{nTi5\%}$ (21.19 ± 0.51), $G_{nTi\text{-}TAT2.5\%}$ (22.61 ± 1.02) and $G_{nTi\text{-}TAT5\%}$ (22.83 ± 0.87) ($p<0.05$). All the adhesive resins reduced KHN1 after immersion in solvent ($p<0.05$) and this reduction was lower for $G_{nTi\text{-}TAT2.5\%}$ (39.35% ± 3.98%) and $G_{nTi\text{-}TAT5\%}$ (38.96% ± 2.85%).

Table 3 shows the results of immediate and long-term μ-TBS evaluation and the percentage of each fracture pattern. No difference was found among groups in the immediate test ($p>0.05$). All groups showed a reduction in μ-TBS after 1 year of storage. Regarding to long-term evaluation, values ranged from 35.06 MPa ± 3.69 MPa to 43.31 MPa ± 5.21 MPa, and the highest value was observed for $G_{nTi\text{-}TAT2.5\%}$ ($p<0.05$). The adhesive mode failure was predominant in all groups.

4. Discussion

Adhesive systems have been innovated to present better physicochemical properties [11, 29] and antibacterial activity [30]. This study evaluated the effect of the addition of nTi as inorganic filler and as carrier of a quaternary ammonium compound, TAT, as antibacterial agent in experimental adhesive resins. It was possible to formulate adhesive resins with reliable physicochemical properties, better hydrolytic stability and bond-strength at long-term, with antibacterial activity and no adverse effects on biocompatibility.

The analysis of nTi carried out using TEM confirmed the nano-dimension, tubular morphology and the lumen of nTi. nTi used in this work were synthesized according to a previous study [25] where the nTi presented mean diameter of 60.5 ± 7.0 nm and lumen entrance diameter of 14 nm. Due to the tubular structure, nTi were used in this study as carriers of an antibacterial agent, TAT, which is capable of copolymerizing with the adhesive resin. Thus, if some TAT were free outside the nanotubes, it could increase the crosslinking density in the resin, as suggested by UTS, Knoop hardness and softening in solvent results, and would continue to provide antibacterial effect. In addition to act as carriers, nTi may also have antibacterial effect by photocatalytic effect depending on the relationship between anatase and rutile they present [31]. nTi groups did not demonstrate antibacterial activity in this study. Anatase appears to have a better performance [31], but there is no consensus in the literature, since the effect also depends on particle size [32]. According to a previous study used as a reference for the synthesis of nTi, the anatase: rutile ratio in nTi is 52:48 [25]. It is possible that nanotubes with higher anatase generated an antibacterial effect.

While nTi groups were not different from G_{CTRL}, $G_{nTi\text{-}TAT2.5\%}$ and $G_{nTi\text{-}TAT5\%}$ groups reduced the bacterial growth on the surface of the polymerized samples as occurred in previous study [23]. It is believed that the mechanism of QAC's antibacterial action consists of the electrostatic interaction, followed by the disruption of bacterial membrane integrity [33]. Results of antibacterial activity against planktonic bacteria suggests that TAT associated to nTi induced no variation of leached compounds among groups. Therefore, in addition to good physicochemical properties, the incorporation of nTi induced antibacterial activity and may be an alternative to improve the therapeutic activity of adhesive resins.

Due to the possibility of proximity to the pulp tissue in some restorative treatments, it is important that the formulated adhesive resins do not demonstrate cytotoxic effects against pulp

fibroblasts. nTi is presented biocompatibility in previous study [14]. According to the physicochemical and antibacterial assays carried in this study, TAT groups probably exhibit higher crosslinking density, reducing unreacted monomers in the matrix that could be leached over time. Thus, less free monomers could be exposed to the pulp tissue [23]. This study evaluated possible cytotoxicity of the adhesive resins by the colorimetric assay of Sulforhodamine B (SRB) in relation to fibroblast viability. Cell viability was greater or equal for groups with filler addition compared to $G_{CTRL.}$ Moreover, all the experimental adhesive resins presented cell viability higher than 70%, a value recommended by ISO 10993-5 [34]. Our results suggest that adhesive resins reinforced with nTi and nTi-TAT have suitable biological properties.

The polymerization behavior of the experimental adhesive resins were evaluated by FTIR-ATR. DC versus photoactivation time (Fig. 2A) showed a right displacement in the groups with filler addition, indicating a slight delay of the polymerization reaction. Higher viscosity of the resin due to filler addition may reduce the mobility of the chains and create rigid centers for chain initiation during polymerization [35, 36]. Figure A also showed that with about 20 s of photoactivation, G_{CTRL} had achieved almost the maximum DC, while $G_{nTi5\%}$ and $G_{nTi\text{-}TAT5\%}$ presented lower values. However, the conversion for these groups continued increasing, resulting in similar DC to all groups by the end of photoactivation. Filler addition could decrease the DC due to lower light transmission through the material and difference in refractive index between the fillers and the resin matrix [37, 38]. However, there was no difference observed among groups. All groups presented DC above 50%, value found for commercial adhesive resins [39]. These are promising results since polymers with high DC achieve higher mechanical properties and are less susceptible to degradation [40, 41]. In addition, less cytotoxic effects are expected due to the lower number of free monomers that could leach over time [42, 43]. Regarding Rp per time, meaningful differences were observed between groups, with higher R_{pmax} for G_{CTRL}, which achieved the Rp_{max} earlier than other groups. Groups with same concentration of filler had similar R_{pmax}. For 2.5wt.% of filler, the results approached G_{CTRL} values, while for 5wt.% there was a reduction of R_{pmax}.

The Knoop hardness and softening in solvent tests allowed a better understanding of the stability of the polymer's network. In this study, $G_{nTi5\%,\ GnTi\text{-}TAT2.5\%}$ and $G_{nTi\text{-}TAT5\%}$ had higher KHN1 values. The value of Knoop hardness found for $G_{nTi5\%}$ may be explained by the pressure on the inorganic filler [44], since this group presents the highest amount of inorganic filler.

Higher values obtained for $G_{nTi\text{-}TAT2.5\%}$ and $G_{nTi\text{-}TAT5\%}$ are due to the addition of nTi and TAT, since TAT it is trifunctional and could induce a higher density of crosslinks in the matrix. All groups reduced KHN1 after immersion in the solvent, which was expected, since adhesive resins are susceptible to softening when in contact with alcohol [26, 45]. Lower ΔKHN was observed for $G_{nTi2.5\%}$, $G_{nTi\text{-}TAT2.5\%}$ and $G_{nTi\text{-}TAT5\%}$ compared to G_{CTRL}. TAT may have improved the dispersion of the nanotubes in the nTi-TAT groups or induced higher density of crosslinks, leading to lower softening of these groups. In spite of having higher KHN1, $G_{nTi5\%,}$ may have formed a polymer with lower crosslinking density and agglomerates of nTi which led to the highest ΔKHN among all test groups, with no difference for G_{CTRL}. Regarding to UTS analysis, $G_{nTi\text{-}TAT2.5\%}$ and $G_{nTi\text{-}TAT5\%}$ also reached higher values. TAT is a cationic compound and due to its surfactant properties, may have improved the dispersion of stress within the adhesive resins by adsorbed around the nanotubes. In addition, TAT may have remained as a crosslinking agent, generating polymers with higher crosslinking density, increasing the UTS.

To assess the effectiveness of the adhesive system at hybrid layers, the μ-TBS assay was performed. No difference was found in the results of immediate μ-TBS among groups. Regarding to long-term evaluation, after one year of storage in distilled water, $G_{nTi\text{-}TAT2.5\%}$ presented better performance. The long-term evaluation of μ-TBS correlates with *in vivo* outcomes [46], regarding to the hydrolytic degradation of the polymer and the collagen matrix. This is directly related to the reminiscent presence of water during the adhesive procedure, hydrophilicity of the functional monomers and water sorption [47, 48]. In vivo, the adhesive interface is expose to other challenges such as degradation by saliva enzymes or chemicals released by biofilm bacteria [4, 5]. Therefore, the incorporation of nTi-TAT may could overcome the degradation by enzymes and bacterial products that were not evaluated by μ-TBS. More interesting results could be found, mainly in patients susceptible to the development of dental caries, due to the reduction of bacterial adhesion. Even though it is beyond the scope of the current study, other studies will be carried out to investigate these effects, since the observed results are promising.

5. Conclusion

New antibacterial adhesive resins were formulated with the addition of titanium dioxide nanotubes with triazine. In addition to improve physicochemical and biological properties of the polymer, this filler improved the u-TBS at long-term when incorporated at 2.5w.%.

Figures and tables

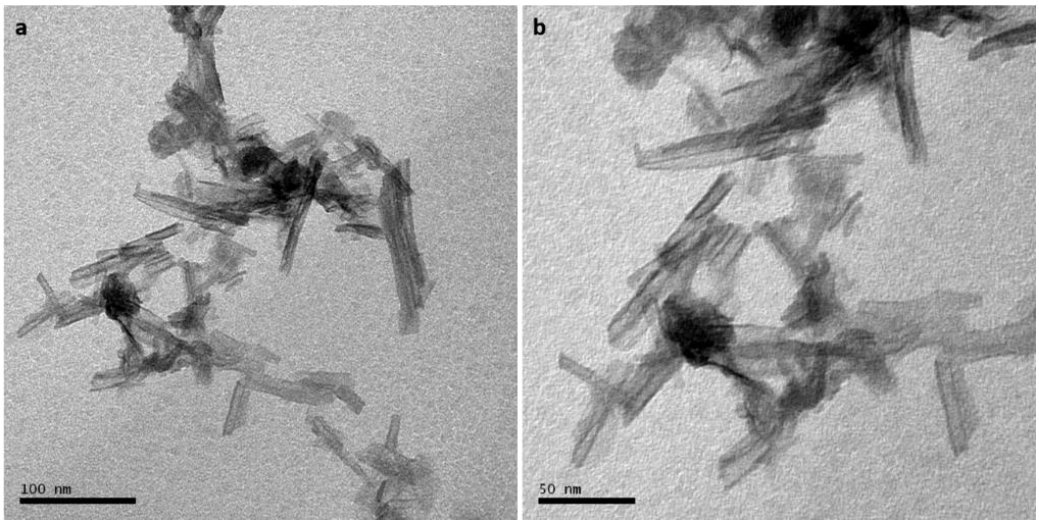

Figure 1. TEM images of nTi nanotubes in isopropyl alcohol at 500 x (a) and 2000 x (b).

Table 1: Mean and standard deviation values of CFU/mL with bacteria in biofilm and planktonic bacteria from the evaluation of antibacterial activity of the experimental adhesive resins after 24 and 48 h and values of the viable cells in percentage from cytotoxicity assay.

Group	Antibacterial activity after 24 h		Antibacterial activity after 48 h		Viable cells
	Biofilm	Planktonic bacteria	Biofilm	Planktonic bacteria	-
G_{CTRL}	5.32 (±0.04)^{Ca}	8.21 (±0.02)^{Aa}	5.35 (±0.06)^{Ca}	8.22 (±0.01)^{Aa}	82.06 (±3.45)^{C}
$G_{nTi2.5\%}$	5.34 (±0.05)^{Ca}	8.23 (±0.04)^{Aa}	5.36 (±0.02)^{Ca}	8.22 (±0.05)^{Aa}	91.35 (±3.22)^{ABC}
$G_{nTi5\%}$	5.32 (±0.01)^{Ca}	8.23 (±0.04)^{Aa}	5.38 (±0.07)^{Ca}	8.29 (±0.03)^{Aa}	97.84 (±2.20)^{A}
$G_{nTi\text{-}TAT2.5\%}$	4.10 (±0.08)^{Ba}	8.28 (±0.08)^{Aa}	4.16 (±0.01)^{Ba}	8.22 (±0.07)^{Aa}	93.83 (± 3.97)^{AB}
$G_{nTi\text{-}TAT5\%}$	3.64 (±0.08)^{Aa}	8.21 (±0.05)^{Aa}	3.61 (±0.05)^{Aa}	8.21 (±0.07)^{Aa}	86.27 (±6.47)^{BC}

Different capital letters indicate statistical difference in the same column ($p<0.05$).

Different lowercase letters indicate statistical difference in the same line for the same assay (for biofilm formation or planktonic growth) ($p<0.05$).

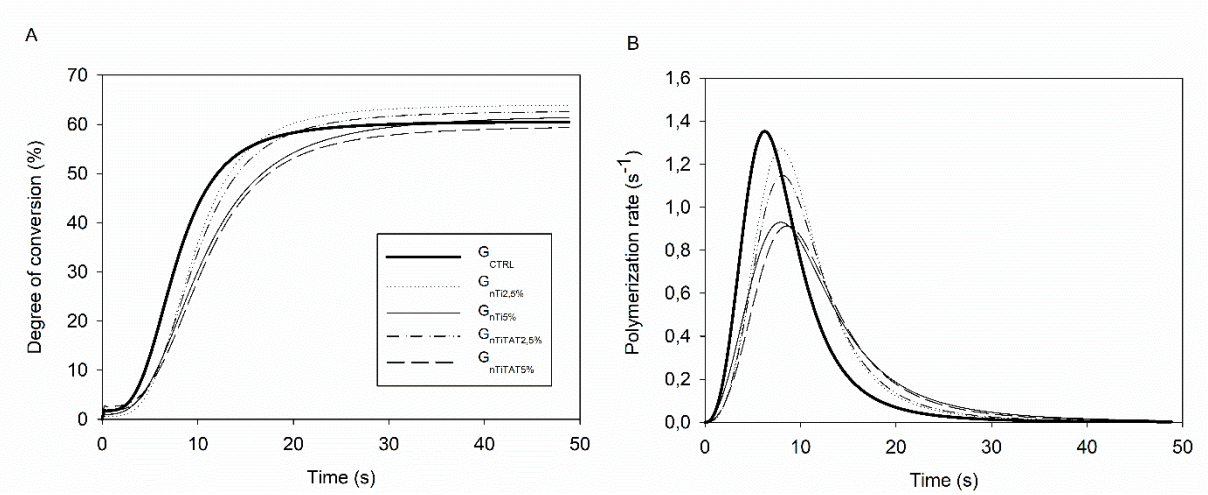

Figure 2. Graphs of polymerization behavior of experimental adhesive resins. (A) Degree of conversion (DC) versus photoactivation time. (b) Polymerization rate (Rp) versus photoactivation time.

Table 2: Mean and standard deviation values of degree of conversion (DC), ultimate tensile strength (UTS), initial Knoop hardness number (KHN1) and final Knoop hardness number (KHN2) and values of variation of Knoop hardness (ΔKHN%) of experimental adhesive resins.

	DC (%)	**KHN1**	**KHN2**	**ΔKHN**	**UTS (MPa)**
G_{CTRL}	59.90 (±1.63)^{A}	19.61(0.52)^{Ba}	8.80 (±0.73)^{b}	55.13 (±3.28)^{B}	51.47 (±7.65)^{B}
$G_{nTi2.5\%}$	62.60 (±0.57)^{A}	16.97 (±1.50)^{Ca}	9.44 (±0.78)^{b}	44.23 (±4.55)^{A}	58.91 (±13.86)^{B}
$G_{nTi5\%}$	58.66 (±1.57)^{A}	21.19 (±0.51)^{Aa}	9.88 (±0.44)^{b}	53.35 (±2.14)^{B}	64.80 (±8.22)^{B}
$G_{nTi\text{-}TAT2.5\%}$	61.20 (±2.15)^{A}	22.61 (±1.02)^{Aa}	13.68 (±0.38)^{b}	39.35 (±3.98)^{A}	85.37 (±8.49)^{A}
$G_{nTi\text{-}TAT5\%}$	57.00 (±4.58)^{A}	22.83 (±0.87)^{Aa}	13.92 (±0.32)^{b}	38.96 (±2.85)^{A}	82.76 (±5.22)^{A}

Different capital letters indicate statistical significant difference in the same column ($p<0.05$).

Different lowercase letters indicate statistical significant difference in the same line for KHN1 and KHN2 of the same material ($p<0.05$).

Table 3: Mean and standard deviation values of immediate and long-term μ-TBS in MPa and percentage of fracture pattern.

Group	Immediate μ-TBS	Fracture Pattern			Long-term μ-TBS	Fracture Pattern		
		CD	CR	A		CD	CR	A
G_{CTRL}	52.21 (±9.08)^{Aa}	95	5		35.06 (±3.69)^{Bb}	86.25	10	3.75
$G_{nTi2.5\%}$	50.69 (±9.51)^{Aa}	90	3.75	6.25	37.28 (±6.65)^{ABb}	92.40	3.80	3.80
$G_{nTi5\%}$	50.07 (±10.62)^{Aa}	96.25		3.75	40.83 (±4.52)^{ABb}	90	6.25	3.75
$G_{nTi\text{-}TAT2.5\%}$	49.74 (±8.63)^{Aa}	96.50	2.25	1.25	43.31 (±5.21)^{Ab}	93.5	3.75	2.75
$G_{nTi\text{-}TAT5\%}$	52.14 (±9.39)^{Aa}	91.25	3.75	5	40.88 (±4.50)^{ABb}	90	6.25	3.75

Different capital letters indicate statistical significant difference in the same column ($p<0.05$).

Different lowercase letters indicate statistical significant difference in the same line for the same assay (immediate and long-term μ-TBS)

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4 CONSIDERAÇÕES FINAIS

Diversos estudos têm sidos realizados objetivando o desenvolvimento de materiais odontológicos com melhores propriedades através da incorporação de novas cargas aos mesmos (GARCIA *et al.*, 2017; GARCIA *et al.*, 2018a; LEITUNE *et al.*, 2013a; LEITUNE *et al.*, 2013b). No presente trabalho foi possível desenvolver resinas adesivas experimentais à base de metacrilato com incorporação de nanotubos de titânia associados ou não com triazina.

Como resultado, foram obtidas resinas adesivas com melhor desempenho que o controle nas propriedades de dureza inicial, amolecimento em solvente e resistência coesiva, sem alteração no grau de conversão. Os materiais formulados com nTi-TAT apresentaram efeito antibacteriano em relação à formação de biofilme, a principal causa do desenvolvimento de cárie recorrente, sem acréscimo à citotoxicidade. Além disso, os resultados de resistência de união longitudinal sugerem que foi possível com a incorporação de 2,5% de nTi-TAT, uma menor degradação da interface após 1 ano de armazenamento em água destilada.

Dentro das limitações deste trabalho foi possível concluir que nTi em associação com a TAT, adicionados como carga a uma matriz resinosa a base de metacrilato, não causou danos e ainda incrementou suas propriedades. O desenvolvimento de novos sistemas adesivos com melhores propriedades mecânicas e físico-químicas, e com potencial bioativo, apresentam grande potencial para técnicas restauradoras mais conservadoras e tratamentos mais longevos. Dessa forma, considera-se nTi-TAT como promissoras cargas para desenvolvimento de materiais odontológicos com melhores propriedades terapêuticas. A avaliação *in vitro* das propriedades do material formulado é indispensável para maior compreensão da interação de seus componentes bem como do seu provável comportamento no ambiente oral. Entretanto, estudos clínicos são necessários para observação do comportamento e as propriedades do material *in vivo.*

Como perspectivas futuras destaca-se a avalição longitudinal da atividade antibacteriana da triazina, uma vez que ficando imobilizada na matriz polimérica, possivelmente produzirá efeito antibacteriano ao longo do tempo, não sendo este efeito capaz de ser avaliado apenas por um ensaio de 24 e 48h. Além disso, levando em consideração a biocompatibilidade dos nTi (KHALED *et al.*, 2010) além das propriedades bioativas do TiO_2 já observadas em estudos anteriores ao ser incorporado a polímeros imersos em SBF (SAITO *et al.*, 2011; SHIMIZU *et al.*, 2016), destaca-se como perspectiva futura a avaliação da bioatividade do material

formulado. A possível capacidade de deposição mineral é uma alternativa interessante para conferir aos materiais restauradores características capazes de aprimorar o processo de remineralização dentinária e as propriedades da dentina afetada por cárie.

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