UNESP - Universidade Estadual Paulista
“Júlio de Mesquita Filho”
Faculdade de Odontologia de Araraquara

VICTOR TRASSI FERNANDES SILVA DE SOUZA

EFEITO DE ADESIVOS MODIFICADOS POR NANOPARTÍCULAS DE TIO_2 DECORADAS OU NÃO COM PRATA EM DIFERENTES PROPRIEDADES

Araraquara

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Odontológicas, área de concentração em Dentística Restauradora, da Faculdade de Odontologia de Araraquara da Universidade Estadual Paulista – UNESP, como parte dos requisitos para obtenção do título de Mestre em Ciências Odontológicas.
Orientador: Profa. Dra. Alessandra Nara de Souza Rastelli

Araraquara

2017

Souza, Victor Trassi Fernandes Silva de
Efeito de adesivos modificados por nanopartículas de TiO_2 decoradas ou não com prata em diferentes propriedades / Victor Trassi Fernandes Silva de Souza.-- Araraquara: [s.n.], 2017
77 f.; 30 cm.

Dissertação (Mestrado em Dentística Restauradora) – Universidade Estadual Paulista, Faculdade de Odontologia
Orientadora: Profa. Dra. Alessandra Nara de Souza Rastelli

1. Streptococcus mutans 2. Prata 3. Nanopartículas 4. Titânio 5. Cura luminosa de adesivos dentários I. Título

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Dissertação para Obtenção do Grau de Mestre

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Araraquara, 21 de setembro de 2017

DADOS CURRICULARES

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DEDICATÓRIA

Dedico este trabalho, em primeiro lugar, ao nosso todo poderoso Senhor Deus, que sempre iluminou minha vida e permitiu que estivesse onde estou hoje.

Aos meus pais, **Wagner** e **Simone**, por serem minha fortaleza e sempre me proverem os melhores conselhos e apoio possíveis. Aos meus irmãos **Bruno** e **Lídia** por tornarem essa família completa e perfeita.

À minha namorada, **Isadora**, por seu infinito amor, carinho, compreensão e companheirismo nessa reta final. Você me tornou uma pessoa melhor e foi decisiva para eu conseguir completar essa pesquisa. Te amo mais do que é possível imaginar!

“A sabedoria é um paradoxo. O homem que mais sabe é aquele que mais reconhece a vastidão de sua ignorância.”

Friedrich Nietzsche

AGRADECIMENTOS

À Universidade Estadual Paulista Júlio de Mesquita Filho – UNESP, na presença de seu Magnífico Reitor, Prof. Dr. Sandro Roberto Valentini e Vice-Reitor Prof. Dr. Sérgio Roberto Nobre.

À Faculdade de Odontologia de Araraquara – UNESP, representada pela Profa. Dra. Elaine Maria Sgavioli Massucato (Diretora) e Prof. Dr. Edson Alves de Campos (Vice-Diretor).

Ao Programa de Pós-Graduação em Ciências Odontológicas, da Faculdade de Odontologia de Araraquara, coordenado pela Profª. Drª. Fernanda Lourenção Briguenti.

Aos docentes da disciplina de Dentística Restauradora: **Profa. Dra. Alessandra Nara de Souza Rastelli**, **Profa. Dra. Andrea Abi Rached Dantas**, **Prof. Dr. Edson Alves de Campos**, **Prof. Dr. José Roberto Cury Saad**, **Prof. Dr. Marcelo Ferrarezi Andrade**, **Prof. Dr. Osmir Batista de Oliveira Junior** e **Prof. Dr. Sizenando de Toledo Porto Neto** pela recepção na faculdade, ajuda e ensinamentos.

À **Profa. Dra. Alessandra Nara de Souza Rastelli**, pela oportunidade ímpar que me proporcionou de estar nessa ilustre Faculdade e desfrutar de tudo que ela me ofereceu. Agradeço efusivamente pelos conselhos, pelo apoio, pelos ensinamentos, pela compreensão e por me conduzir nos meus primeiros passos dentro da vida acadêmica. Por tudo isso serei eternamente grato.

Aos colegas de pós-graduação e graduação que tive o prazer de conhecer nesta jornada, especialmente ao **Hércules Bezerra Dias** por ter me auxiliado constantemente na realização deste trabalho.

À **Profa. Dra. Taís Maria Bauab**, da Faculdade de Ciências Farmacêuticas – UNESP, por ter disponibilizado as dependências de seu laboratório para parte importante deste trabalho e pela grande ajuda fornecida.

Aos colegas e funcionários do laboratório de Ciências Biológicas da Faculdade de Ciências Farmacêuticas – UNESP, por terem me auxiliado incontáveis vezes durante o tempo em que utilizei as dependências da Faculdade.

Ao **Prof. Dr. Luís Geraldo Vaz** pela disponibilização do Laboratório de Ensaios Mecânicos da Faculdade de Odontologia de Araraquara -UNESP.

Aos técnicos João Luiz e Naira do Laboratório Multiusuário de Análises Químicas do Instituto de Química – UNESP, pela ajuda durante a utilização do laboratório.

Aos funcionários Cristiano e José Alexandre da Seção de Pós-Graduação da Faculdade de Odontologia de Araraquara – UNESP.

Às funcionárias Creusa e Alessandra do Departamento de Odontologia Restauradora da Faculdade de Odontologia de Araraquara – UNESP.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), pelo aporte financeiro fornecido durante o curso de Mestrado.

E a todos que de alguma forma, mesmo sem saber, me ajudaram a concluir esta etapa.

Souza VTFS. Efeito de adesivos modificados por nanopartículas de TiO_2 decoradas ou não com prata em diferentes propriedades. [Dissertação de Mestrado]. Araraquara: Faculdade de Odontologia da UNESP; 2017.

RESUMO

O presente estudo teve como objetivo a avaliação do efeito antibacteriano de sistemas adesivos modificados com nanopartículas (NPs) de dióxido de titânio (TiO_2) decoradas ou não por prata (Ag) em diferentes concentrações, assim como o grau de conversão e a resistência de união à dentina sendo avaliada imediatamente e após três meses em saliva artificial. Os sistemas adesivos Adper^{TM} Scotch Bond Multi Purpose e Adper^{TM} Single Bond (3M Espe), e Clearfil SE Bond (Kuraray) foram modificados com concentrações 0 (controle); 1; 2 e 5% de NPs de TiO_2 (com e sem Ag). Foram confeccionados espécimes cilíndricos em resina composta (Filtek^{TM} Z250XT, 3M Espe) na cor A_2 com matrizes metálicas (4x2mm), nos quais foram aplicados os sistemas adesivos modificados ou não, de acordo com as instruções dos fabricantes, para o teste de atividade antibacteriana por contato direto. Os espécimes foram colocados em placa de 24 poços e incubados por 18 horas a 37ºC em atmosfera de microaerofilia juntamente com 100µL de suspensão bacteriana padronizada contendo *Streptococcus mutans* e 900µL de BHI caldo + sacarose (1%). Para a contagem de unidades formadoras de colônias (UFC/mL), foram realizadas diluições seriadas com as soluções resultantes e 50µL dessas diluições foram plaqueadas em placas de petri contendo BHI ágar, sendo incubadas a 37ºC em 10% de CO_2 por 48 horas. O grau de conversão foi avaliado por espectroscopia infravermelho por transformada de Fourier (FT-IR) para todos os adesivos modificados ou não em todas as concentrações, sob resolução de 4cm^{-1}, 64 escaneamentos de 400-4000cm^{-1}. Para o teste de microcisalhamento, os espécimes foram confeccionados incluindo fragmentos de dentes bovinos (13mm comprimento x 7mm largura x 2mm espessura) em tubos de PVC (1,2cm x 1,2cm) com resina acrílico autopolimerizável. Nestes espécimes, corpos-de-prova foram confeccionados utilizando-se a resina composta Filtek^{TM} Z250XT (3M Espe) utilizando-se matrizes de teflon em substrato bovino, sobre os quais os sistemas adesivos foram aplicados, sendo feitas as análises de resistência adesiva imediata e após armazenamento em água destilada a 37ºC (±1ºC) por 3 meses. O padrão de fratura dos espécimes foi analisado por microscopia óptica (lupa estereoscópica, 40x de

aumento). Os pressupostos de normalidade e homocedasticidade foram atendidos e os dados obtidos foram analisados por Análise de Variância ANOVA (3 fatores) e teste de Tukey (5%). As médias e desvio padrão do grau de conversão foram 25,58 ± 2,25 (SB), 32,48 ±7,3 (SBM) e 9,63 ± 7,19(CB); TiO_2 (1%): 29,57±1,4 (SB) 32,72±8,48 (SBM) 31,66±4,78 (CB); TiO_2 (2%): 30,14±8,23 (SB) 35,08±7,57 (SBM) 27,19±8,74 (CB); TiO_2 (5%): 27,19±12,11 (SB) 35,77±7,91 (SBM) 39,63±6,95 (CB); $AgTiO_2$-1% 27,35±10,36 (SB) 38,03±7,30 (SBM) 37,47± 7,37(CB); $AgTiO_2$-2% 27,72±10,05 (SB) 33,57±10,19 (SBM) 34,13±8,06 (CB); $AgTiO_2$-5% 28,21±9,03 (SB) 38,7±8,15 (SBM) 31,33±8,5 (CB) ($p<0,05$). As médias log10 e desvio padrão para o teste antibacteriano por contato direto foram: 6.5 ± 0,30 (SB), 6.63 ± 0,19(SBM) e 6.62 ± 0,14 (CB); TiO_2 (1%): 5,50 ± 0,40(SB) 5,62 ± 0,29 (SBM) 5,61 ± 0,19(CB); TiO_2 (2%): 5,44 ± 0,36(SB) 5,30 ± 0,50(SBM) 5,55 ± 0,08 (CB); TiO_2 (5%): 5,46 ± 0,49(SB) 5,47 ± 0,39(SBM) 5,57 ± 0,1 (CB); $AgTiO_2$-1% 5,84 ± 0,16 (SB) 5,56 ± 0,22 (SBM) 5,54 ± 0,07(CB); $AgTiO_2$-2% 5,68 ± 0,45 (SB) 5,41 ± 0,10 (SBM) 5,56 ± 0,12(CB); $AgTiO_2$-5% 5,47 ± 0,14 (SB) 5,50 ± 0,14(SBM) 5,52 ± 0,11(CB) ($p<0,05$). As médias dos valores resistência adesiva em MPa e desvio padrão para o teste de microcisalhamento (imediato) foram: 19,3 ± 2,7 (SB), 22,4 ± 3 (SBM) e 16,6 ± 2,9 (CB); TiO_2 (1%): 18,7 ± 2,4 (SB) 22,8 ± 4,8 (SBM) 15,2 ± 2,7 (CB); TiO_2 (2%): 17,9 ± 2,7 (SB) 21,5 ± 2,8 (SBM) 17,9 ± 3,4 (CB); TiO_2 (5%): 18,2 ± 3,01(SB) 21,9 ± 3,9(SBM) 17,5 ± 3,99 (CB); $AgTiO_2$-1% 19,5 ± 3,2 (SB) 23,5 ± 3,8 (SBM) 16,5 ± 3,1 (CB); $AgTiO_2$-2% 18,1 ± 3,1 (SB) 24,2 ± 3,9 (SBM) 14,8 ± 3,2 (CB); $AgTiO_2$-5% 17,2 ±4,5 (SB) 23,3 ± 4 (SBM) 14,1 ± 5 (CB) ($p<0,05$). As médias dos valores resistência adesiva em MPa e desvio padrão para o teste de microcisalhamento (3 meses) foram: 18,3 ± 1,83 (SB), 18,58 ± 2,31 (SBM) e 18,58 ± 2,23 (CB); TiO_2 (1%): 17,92 ± 1,98 (SB) 18,25 ± 2,05 (SBM) 17,5 ± 1,8 (CB); TiO_2 (2%): 17,83 ± 1,85 (SB) 18,17 ± 2,,04 (SBM) 17 ± 2,59 (CB); TiO_2 (5%): 17,75 ± 1,96 (SB) 18,17 ± 2,17 (SBM) 18,75 ± 2,01 (CB); $AgTiO_2$-1% 17,75 ± 1,87 (SB) 17,33 ± 1,88 (SBM) 17,42 ± 1,44 (CB); $AgTiO_2$-2% 17,50 ± 2,28 (SB) 17,25 ± 1,96 (SBM) 17,33 ± 2,31 (CB); $AgTiO_2$-5% 18,25 ± 1,91 (SB) 18,3 ± 2,54 (SBM) 19,17 ± 2,21 (CB) ($p<0,05$). Os resultados mostraram que todos os sistemas adesivos exibiram atividade antibacteriana independente do tipo de nanopartículas utilizada ($p<0,05$). Porém, nenhuma diferença foi constatada quando comparadas as diferentes concentrações e os diferentes tipos de adesivo ($p<0,05$). A adição de nanopartículas não afetou o grau de conversão dos sistemas adesivos, não sendo também constatada diferença nos valores de resistência de união, considerando todas as variáveis e os

dois períodos de tempo utilizados. Pode-se concluir que a adição de nanopartículas com propriedades antibacterianas promoveram atividade antibacteriana, sobre *Streptococcus mutans*, aos adesivos odontológicos, sem prejudicar o grau de polimerização e a adesão, podendo ser uma técnica viável para tornar adesivos odontológicos bioativos e diminuir a infiltração de bactérias em restaurações adesivas.

Palavras-chave: Streptoccocus mutans. Prata. Nanopartículas. Titânio. Cura luminosa de adesivos dentários.

Souza VTFS. Effect of adhesives modified with TiO_2 nanoparticles decorated or not with silver on different properties [Dissertação de Mestrado]. Araraquara: Faculdade de Odontologia da UNESP; 2017.

ABSTRACT

The present study evaluated the antibacterial effect, the degree of conversion and the micro-shear bond strength (immediate and after three months of water storage) of modified dental adhesive systems with TiO_2 or Ag/TiO_2 nanoparticles at different concentrations. The adhesive systems Adper^{TM} Single Bond (SB), Adper^{TM}Scotch^{TM} Bond Multi Purpose (3M Espe) (SBM) and Clearfil SE Bond (Kuraray) (CB) were modified with 1.0, 2.0 and 5.0 wt% of NPs. For the direct-contact test, sterilized specimens (n=36) were made using a metallic matrix (4x2mm) with the composite resin Filtek^{TM}Z250 XT (3M Espe), on which the dental adhesive systems were applied according to the manufacturer's instructions. Both materials were photo-activated using a LED light-curing unit (LED Radii Plus, SDI). The specimens were placed in a 24-well plate with 100μL of *Streptoccocus mutans* standardized suspension on their surfaces, 900μL of BHI broth and incubated for 18 hours at 37ºC under an atmosphere containing 5% CO_2. A six-fold serial dilution was performed with the resultant solutions. Fifty microliters (50μL) from each dilution was retrieved and spread on brain-heart infusion agar plates and incubated at 37°C under an atmosphere containing 5% of CO_2 for 48h and the colony forming units (CFU's) were registered. For the evaluation of the DC, the specimens were made by the modification of the dental adhesive systems with all concentrations of nanoparticles and stored for 24hs at 37ºC in an incubator. The FTIR analysis was conducted using an attenuated total reflectance unit (ATR) at a 4cm^{-1} resolution and 64 scans. For the micro-shear bond strength test, One hundred and twenty six standardized dentin specimens were made from bovine incisors and divided into twenty-one groups. The control and modified adhesive systems were applied on the surfaces of the specimens according to the manufacturer's instructions and composite resin cylinders (Filtek^{TM} Z250XT) were inserted into the bonded surfaces using a Teflon matrix. The test was performed using a universal testing machine, at a cross-head speed of 0,5mm/min until the specimen rupture. The failure modes of the specimens was determined using a stereomicroscope at 40x and was divided into adhesive, cohesive and mixed. The data was assessed by three-way ANOVA and Tukey's

Test ($p \leq 0.05$). The mean values and standard deviation for the control groups of the DC test were: 25,58 ± 2,25 (SB), 32,48 ±7,3 (SBM) e 9,63 ± 7,19(CB); TiO_2 (1%): 29,57±1,4 (SB) 32,72±8,48 (SBM) 31,66±4,78 (CB); TiO_2 (2%): 30,14±8,23 (SB) 35,08±7,57 (SBM) 27,19±8,74 (CB); TiO_2 (5%): 27,19±12,11 (SB) 35,77±7,91 (SBM) 39,63±6,95 (CB); $AgTiO_2$-1% 27,35±10,36 (SB) 38,03±7,30 (SBM) 37,47± 7,37(CB); $AgTiO_2$-2% 27,72±10,05 (SB) 33,57±10,19 (SBM) 34,13±8,06 (CB); $AgTiO_2$-5% 28,21±9,03 (SB) 38,7±8,15 (SBM) 31,33±8,5 (CB) ($p<0,05$). The mean log10 values and standard deviation for the antibacterial test were: 6.5 ± 0,30 (SB), 6.63 ± 0,19(SBM) e 6.62 ± 0,14 (CB); TiO_2 (1%): 5,50 ± 0,40(SB) 5,62 ± 0,29 (SBM) 5,61 ± 0,19(CB); TiO_2 (2%): 5,44 ± 0,36(SB) 5,30 ± 0,50(SBM) 5,55 ± 0,08 (CB); TiO_2 (5%): 5,46 ± 0,49(SB) 5,47 ± 0,39(SBM) 5,57 ± 0,1 (CB); $AgTiO_2$-1% 5,84 ± 0,16 (SB) 5,56 ± 0,22 (SBM) 5,54 ± 0,07(CB); $AgTiO_2$-2% 5,68 ± 0,45 (SB) 5,41 ± 0,10 (SBM) 5,56 ± 0,12(CB); $AgTiO_2$-5% 5,47 ± 0,14 (SB) 5,50 ± 0,14(SBM) 5,52 ± 0,11(CB) ($p<0,05$). The mean bond strength values (MPa) and standard deviation for the microshear test (immediate) were: 19,3 ± 2,7 (SB), 22,4 ± 3 (SBM) e 16,6 ± 2,9 (CB); TiO_2 (1%): 18,7 ± 2,4 (SB) 22,8 ± 4,8 (SBM) 15,2 ± 2,7 (CB); TiO_2 (2%): 17,9 ± 2,7 (SB) 21,5 ± 2,8 (SBM) 17,9 ± 3,4 (CB); TiO_2 (5%): 18,2 ± 3,01(SB) 21,9 ± 3,9(SBM) 17,5 ± 3,99 (CB); $AgTiO_2$-1% 19,5 ± 3,2 (SB) 23,5 ± 3,8 (SBM) 16,5 ± 3,1 (CB); $AgTiO_2$-2% 18,1 ± 3,1 (SB) 24,2 ± 3,9 (SBM) 14,8 ± 3,2 (CB); $AgTiO_2$-5% 17,2 ±4,5 (SB) 23,3 ± 4 (SBM) 14,1 ± 5 (CB) ($p<0,05$). The mean bond strength values (MPa) and standard deviation for the microshear test (3 months) were: 18,3 ± 1,83 (SB), 18,58 ± 2,31 (SBM) e 18,58 ± 2,23 (CB); TiO_2 (1%): 17,92 ± 1,98 (SB) 18,25 ± 2,05 (SBM) 17,5 ± 1,8 (CB); TiO_2 (2%): 17,83 ± 1,85 (SB) 18,17 ± 2,,04 (SBM) 17 ± 2,59 (CB); TiO_2 (5%): 17,75 ± 1,96 (SB) 18,17 ± 2,17 (SBM) 18,75 ± 2,01 (CB); $AgTiO_2$-1% 17,75 ± 1,87 (SB) 17,33 ± 1,88 (SBM) 17,42 ± 1,44 (CB); $AgTiO_2$-2% 17,50 ± 2,28 (SB) 17,25 ± 1,96 (SBM) 17,33 ± 2,31 (CB); $AgTiO_2$-5% 18,25 ± 1,91 (SB) 18,3 ± 2,54 (SBM) 19,17 ± 2,21 (CB) ($p<0,05$).The results showed that all adhesive systems exhibited effective antibacterial activity, regardless of the NPs used (TiO_2 or TiO_2/Ag). However, no statistical difference was observed between the different concentrations and also when the three types of adhesive systems were compared. The addition of NPs did not exert any influence on the DC and on the bond strength values. No significant difference was found between the control and experimental groups, considering both types of nanoparticles, all concentrations used and both periods of time. It can be concluded that the addition of antibacterial nanoparticles to dental adhesive systems can provide antibacterial activity to

these adhesive systems, without compromising their mechanical and adhesion properties, which may reveal a viable technique to reduce the amount of bacteria in adhesive restorations.

Keywords: Streptoccocus mutans. Silver. Nanoparticles. Titanium. Luminous cure of dental adhesives.

LISTA DE ABREVIATURAS E SIGLAS

TiO_2 – Dióxido de titânio

Ag/TiO_2 – Partículas de dióxido de titânio decoradas com prata

SB – Single Bond

SBM – ScotchBond Multipurpose

CB – Clearfil SE Bond

GC – Grau de conversão

NPs – Nanopartículas

UFC – Unidades Formadoras de Colônias

PBS – Phosphate Buffered Saline (Solução Salina Tamponada Fosfatada)

SUMÁRIO

1 INTRODUÇÃO

O uso dos sistemas adesivos e de materiais restauradores na Odontologia sedimentou-se ao longo dos anos como uma ferramenta indispensável para o restabelecimento da estética e da função dentária nos pacientes em ambiente clínico, e também como um campo de pesquisa extremamente vasto para os cientistas no ambiente laboratorial. Sua utilização estabelece-se hoje como um dos mais importantes pilares do tratamento odontológicos nas mais diversas esferas e por todo o mundo.

Uma das mais importantes indicações dos sistemas adesivos odontológicos incluem a restauração de lesões cariosas. Nesse sentido, sabe-se que a cárie dental é uma doença crônica, infecto contagiosa, já estudada e conhecida há muito tempo, e seu estabelecimento está ligado a tipos específicos de patógenos (Leites et al.[21], 2006). Um deles, o *Streptococcus mutans*, vem sendo estudado extensivamente há décadas, sendo uma das principais bactérias relacionadas ao processo carioso. São cocos gram positivos, acidogênicos, acidúricos, microaerófilos e anaeróbios facultativos (Hamada et al.[16], 1980). Possuem potencial altamente cariogênico devido à sua alta capacidade de colonizar a superfície dentária (Florio et al.[13], 2002), produção de polissacarídeos extracelulares do tipo glicana (Cowman et al.[6], 1979), capacidade de produção de ácidos (Menaker[24], 1984), capacidade de sobrevivência em meio ácido, desenvolvendo suas atividades metabólicas (Hamada et al.[16], 1980), estando a presença desse microorganismo relacionada à alta prevalência de cárie (Leites et al.[21], 2006). Sendo assim, essa bactéria se constitui como elemento chave no panorama microbiológico da cárie dental, o que justifica os estudos sobre sua resposta frente a substâncias antibacterianas.

Observando o panorama da saúde pública mundial, constata-se que a cárie dental permanece ainda hoje como um grande desafio, sendo a infecção bacteriana mais comum em humanos e uma ameaça à saúde oral e sistêmica, demonstrando-se ainda um fator de grande impacto econômico e social para a humanidade (Kopperud et al.[20], 2012; Cheng et al.[5], 2012; Featherstone[10], 2004). Nesse cenário, compósitos resinosos, em combinação com os sistemas adesivos, desempenham papel importante na Odontologia Restauradora, já que demonstram boa performance restauradora e ótima estética (Mjor et al.[25], 2000). Entretanto, alguns desafios impõem problemas a essa opção restauradora . As lesões cariosas secundárias se constituem como um deles (Frost[14], 2002; Mjor et al.[25], 2000), juntamente com a dificuldade na remoção completa do tecido cariado e permanência de contaminação bacteriana nos preparos cavitários (Sakaguchi[31], 2005; Chen et al.[4], 2012; Almeidas Neves et al.[1], 2011). As cáries secundárias são, de fato, a maior razão pela qual as restaurações adesivas falham (Frost[14], 2002; Ferracane[12], 2013). Isso devido ao acúmulo de resíduos alimentares e formação de biofilme às suas margens (Svanberg et al.[32], 1990; Drummond et al.[8], 2008), bem como à característica demonstrada pelos compósitos, que tendem a acumular mais biofilme do que outros materiais restauradores (Sakaguchi[31], 2005; Beyth et al.[2], 2007). Portanto, passou-se a pensar na hipótese de que atividade antibacteriana que acontecesse adicionalmente após a confecção de preparos e restaurações poderia trazer uma nova luz para essa questão (Sahoo et al.[29], 2007). Com isso, e levando em consideração os fatores citados, nota-se que o investimento em estudos sobre materiais restauradores e adesivos inovadores que contenham propriedades antibacterianas e bioativas é imprescindível, com o objetivo de diminuir ou controlar a ameaça bacteriana secundária e aumentar a longevidade das restaurações adesivas.

Vários tipos de agentes antibacterianos vêm sendo utilizados com esta finalidade, sendo adicionados tanto em resinas compostas quanto nos sistemas adesivos (Cheng et al.[5], 2012). Entretanto, suas propriedades físicas e mecânicas podem ser afetadas (Saito et al.[30], 2007). Entre os principais agentes usados, podemos citá-los de acordo com a forma com que são usados: liberados pelos sistemas adesivos ao longo do tempo (clorexidina e cloreto de benzalcônio), agentes que se copolimerizam junto à matriz resinosa (brometo de metacriloiloxidodecilpiridínio – MDPB) e preenchedores inorgânicos (dióxido de titânio e óxido de zinco). No que tange aos agentes solúveis, Hiraishi et al.[17] (2010) demonstraram que a adição de clorexidina ao *primer ED 2.0* (Kuraray) permite efeito antibacteriano significativo para todas as concentrações (1 e 2%), tendo o Grupo com 2% de concentração demonstrado resistência à microtração consideravelmente diminuída. Saito et al.[30] (2007) também demonstraram que, adicionando-se BAC (cloreto de benzalcônio) a um adesivo ortodôntico (Superbond C&B, Sun Medical Co), em concentrações variando de 0.25 a 2.5%, obteve-se atividade antibacteriana significativa em comparação ao Grupo controle. Esta atividade aumentou de acordo com a concentração de BAC.

Para investigar melhor o desempenho dos agentes que se unem à matriz, Zhang et al.[38] (2013) incorporaram nanopartículas de MDPB (brometo de metacriloiloxidodecilpiridínio) e Ag em sistema adesivo universal de 3 passos (Scotch Bond Multipurpose, 3M Espe) para avaliar seu efeito antibacteriano, levando em conta a viabilidade e atividade metabólica do biofilme, bem como a resistência de união à dentina. Os resultados mostraram que a combinação do adesivo com as nanopartículas não trouxe perda à resistência de união e reduziu substancialmente a atividade metabólica do biofilme, sendo a combinação de MDPB e Ag a mais eficaz. Em 2014, Wang et al.[34] (2014) obtiveram resultados semelhantes, também em sistemas adesivos, desta vez somente com a incorporação de MDPB e sua ação sobre *S. mutans*.

Com o objetivo de estudar as nanopartículas inorgânicas, Tavassoli et al.[33] (2013) avaliaram a ação de nanopartículas de ZnO em resinas compostas do tipo *flow* (Heliomolar Flow, Ivoclar Vivadent AG) na inibição de *S. mutans,* realizando testes antibacterianos e investigando propriedades mecânicas e profundidade de polimerização. Os resultados demonstraram que o comportamento mecânico não era comprometido, enquanto que a profundidade de polimerização foi menor e a resistência adesiva aumentou com a presença de nanopartículas de ZnO. Além disso, com o aumento do conteúdo de nanopartículas, o crescimento bacteriano reduziu significativamente; no entanto, com o passar do tempo, a ação antibacteriana diminuiu. Kasrei et al.[19] (2014) também estudaram as propriedades antibacterianas do ZnO juntamente com a Ag utilizando-se resina composta do tipo *flow* (Opallis, FGM, Joinville, SC, Brasil), em *S. mutans* e *Lactobacillus*. Os compósitos contendo nanopartículas exibiram maior atividade antibacteriana em relação ao Grupo controle; o efeito do ZnO foi maior em *S. mutans* e não houve diferença significativa em *Lactobacillus.*

Na função de preenchedores inorgânicos temos, além do ZnO, o TiO_2, definido como uma substância fotocatalítica que se estabelece em dois importantes polimorfos: a fase rutilo estável e a anatase metaestável. Estes compostos polimorfos exibem características discrepantes e, invariavelmente, diferentes performances fotocatalíticas (Maness et al.[23], 1999). O surgimento de espécies reativas de oxigênio (radicais livres) causa a morte de bactérias e vírus, sendo essa formação proveniente da liberação de energia resultante de sua foto indução (Cui et al.[7], 2012; Fu et al.[15], 2005). O mecanismo de ação bactericida do TiO_2 se deve ao fato destes radicais agirem em fosfolipídios, lipoproteínas e ácidos nucleicos na membrana e parede celular, levando à destruição das mesmas e à perda de estruturas imprescindíveis à vida da célula bacteriana (Cai et al[3] 2014; Rai et al.[28], 2009). Por sua vez, a prata deve seus efeitos à ligação que possui com os grupos tiol presentes em enzimas respiratórias nas bactérias, inibindo, desse modo, o processo de respiração celular (Feng et al.[11], 2000).

Na primeira pesquisa a estudar o ação antibacteriana do TiO_2 com irradiação UV (1.2 e 7.5 mW/cm²) em sistemas adesivos, realizada por Welch et al.[37] (2010), observou-se diminuição no número das colônias bacterianas, dependendo da intensidade de irradiação e também do tempo ao qual as mesmas eram submetidas, não estabelecendo interferências nas propriedades mecânicas até proporção de 30% de nanopartículas na composição. Em 2014, Cai et al.[3] (2014) concluíram que um sistema adesivo (de formulação própria dos autores do estudo) contendo TiO_2, fazendo uso de 8,4 J/cm² de irradiação UV-A, permitiu diminuição quantitativa significativa das culturas bacterianas, da ordem de 90%, aproximadamente, sem comprometer as propriedades mecânicas.

Diversas propriedades são determinantes para o bom desempenho clínico e laboratorial dos sistemas adesivos odontológicos atuais e variáveis como os valores de resistência de união e o grau de conversão são essenciais para que as restaurações adesivas mantenham-se funcionais por períodos longos de tempo (Faria-e-Silva et al.[9], 2010; Lodovici et al.[22], 2009; Watts et al.[36], 2003). O grau de conversão pode ser definido como a porcentagem da matriz resinosa total presente no adesivo que se polimeriza durante a exposição à luz (Kanehira et al.[18], 2006). Já está estabelecido que a alteração nos valores dessa propriedade podem levar a problemas na performance desses materiais, levando a valores menores de resistência de união, maior porosidade e permeabilidade da camada adesiva, maior eluição dos monômeros, e consequentemente, piores propriedades mecânicas (Kanehira et al.[18], 2006; Navarra et al.[26], 2012; Wang et al.[35], 2010). Já foi observado que a porcentagem de conversão para os sistemas adesivos varia entre 60 a 80% e que os resultados do grau de conversão variam após a modificação com compostos inorgânicos metálicos, tanto melhorando a porcentagem quanto levando a uma perda na qualidade da polimerização (Paul et al.[27], 1999).

Constata-se, entretanto, que uma avaliação mais extensa, minuciosa e criteriosa do TiO_2, sem a utilização da luz, em diferentes tipos de sistemas adesivos de diferentes protocolos de adesão e composição química, e em sua associação com a prata e outras nanopartículas, carece de investigação mais profunda, clínica e laboratorial. Levando em conta o explicitado acima, podemos observar que, ao passo em que algumas modalidades de nanopartículas já foram estudadas quanto a seu potencial antimicrobiano nas resinas compostas e sistemas adesivas, muitas lacunas no que diz respeito a esse campo de estudo ainda não foram preenchidas e o mesmo ainda oferece diversas perspectivas para maior investigação, principalmente com relação à definição de um sistema adesivo modificado que proponha ação antibacteriana prolongada, aplicável clinicamente e que não comprometa as suas propriedades físicas e mecânicas, o que poderia colocar em risco a eficácia da adesão, restando aos pesquisadores a continuidade dos estudos.

2 PROPOSIÇÃO

Objetivo Geral

1. Avaliar o efeito de nanopartículas de TiO_2 (dióxido de titânio) ou TiO_2/Ag (dióxido de titânio decorado por prata) como partículas de carga antibacterianas em diferentes tipos de sistemas adesivos odontológicos, visando avaliar a atividade antibacteriana em diferentes concentrações, além de sua possível interferência na resistência adesiva, analisando ainda o padrão de falha

Objetivos Específicos

1. Análise da atividade antibacteriana dos sistemas adesivos modificados com nanopartículas de TiO_2 ou TiO_2/Ag em diferentes concentrações (1, 2 e 5%) por meio do teste antibacteriano por contato direto.
2. Avaliação do grau de conversão de todos os sistemas adesivos modificados por ambos os tipos de nanopartículas e em todas a concentrações.
3. Avaliação da resistência adesiva obtida imediatamente após a confecção dos corpos- de-prova (imediato) e após 3 meses de armazenamento, por meio do teste de microcisalhamento.
4. Avaliação do tipo de falha na interface sistema adesivo/resina composta/dente bovino por meio de microscopia estereoscópica nos espécimes testados imediatamente após a confecção dos corpos-de-prova e após 3 meses de armazenamento.

3 PUBLICAÇÃO 1

Antimicrobial Activity and Degree of Conversion of Adhesive Systems Modified by Silver Decorated Titanium Dioxide Nanoparticles *

Running title: Antimicrobial Activity and Degree of Conversion of Different Adhesive Systems

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*a ser submetido para o periódico Brazilian Dental Journal

Abstract

The high prevalence of secondary caries and the unsatisfactory service expectancy of composite resin restorations remain as a serious and complex issue to dentists and researchers. Dental adhesive systems with antibacterial properties may reduce recurrent or secondary caries. Thus, the purpose of this study was to evaluate the antibacterial effect and the degree of conversion (DC) of dental adhesive systems containing titanium dioxide (TiO_2) or titanium dioxide decorated with silver (TiO_2/Ag) nanoparticles (NPs) using the direct-contact test and Fourier transform infrared analysis (FTIR) spectroscopy. The adhesive systems Adper^{TM} Single Bond (SB), Adper^{TM}Scotch^{TM} Bond Multi Purpose (3M Espe) (SBM) and Clearfil SE Bond (Kuraray) (CB) were modified with 1.0, 2.0 and 5.0 wt% of NPs. For the direct-contact test, the specimens (n=108) were made using a metallic matrix (4x2mm) with the composite resin Filtek^{TM}Z250 XT (3M Espe), on which the dental adhesive systems were applied according to the manufacturer's instructions. Both materials were photo-activated using a LED light-curing unit (LED Radii Plus, SDI). After that, the specimens were autoclaved at 121°C during 15 minutes before the direct contact test. The specimens were placed in a 24-well plate with 100μL of *Streptoccocus mutans* standardized suspension on their surfaces, 900μL of BHI broth and incubated for 18 hours at 37ºC under an atmosphere containing 5% CO_2. A six-fold serial dilution was performed with the resultant solutions. Fifty microliters (50μL) from each dilution was retrieved and spread on brain-heart infusion agar plates and incubated at 37°C under an atmosphere containing 5% of CO_2 for 48h and the colony forming units (CFU's) were registered. For the evaluation of the DC, the specimens (n=112 – 56

curated – 56 not curated) were made by the modification of the dental adhesive systems with all concentrations of nanoparticles and stored for 24hs at 37ºC in an incubator. The FTIR analysis was conducted using an attenuated total reflectance unit (ATR) at a 4cm^{-1} resolution and 64 scans. The data was assessed by three-way ANOVA and Tukey's Test ($p \leq 0.05$). The mean log10 values and standard deviation for the control and experimental Groups for the antibacterial tests were, respectively: 6.5 ± 0,30 (SB), 6.63 ± 0,19(SBM) e 6.62 ± 0,14 (CB); TiO_2 (1%): 5,50 ± 0,40(SB) 5,62 ± 0,29 (SBM) 5,61 ± 0,19(CB); TiO_2 (2%): 5,44 ± 0,36(SB) 5,30 ± 0,50(SBM) 5,55 ± 0,08 (CB); TiO_2 (5%): 5,46 ± 0,49(SB) 5,47 ± 0,39(SBM) 5,57 ± 0,1 (CB); $AgTiO_2$-1% 5,84 ± 0,16 (SB) 5,56 ± 0,22 (SBM) 5,54 ± 0,07(CB); $AgTiO_2$-2% 5,68 ± 0,45 (SB) 5,41 ± 0,10 (SBM) 5,56 ± 0,12(CB); $AgTiO_2$-5% 5,47 ± 0,14 (SB) 5,50 ± 0,14(SBM) 5,52 ± 0,11(CB) ($p<0,05$).The mean values and standard deviation for the control and experimental Groups for DC were respectively: 25,58 ± 2,25 (SB), 32,48 ±7,3 (SBM) e 9,63 ± 7,19(CB); TiO_2 (1%): 29,57±1,4 (SB) 32,72±8,48 (SBM) 31,66±4,78 (CB); TiO_2 (2%): 30,14±8,23 (SB) 35,08±7,57 (SBM) 27,19±8,74 (CB); TiO_2 (5%): 27,19±12,11 (SB) 35,77±7,91 (SBM) 39,63±6,95 (CB); $AgTiO_2$-1% 27,35±10,36 (SB) 38,03±7,30 (SBM) 37,47± 7,37(CB); $AgTiO_2$-2% 27,72±10,05 (SB) 33,57±10,19 (SBM) 34,13±8,06 (CB); $AgTiO_2$-5% 28,21±9,03 (SB) 38,7±8,15 (SBM) 31,33±8,5 (CB) ($p<0,05$). The results showed that all modified adhesive systems exhibited effective antibacterial activity, regardless of the NPs used (TiO_2 or TiO_2/Ag) ($p<0,05$). However, no statistical significant difference was observed between the different concentrations and also when the three types of adhesive systems were compared. ($p>0.05$). The addition of NPs did not exert any influence on the DC of the different adhesive systems ($p>0.05$). These results suggest that modified dental adhesives with antibacterial nanoparticles inhibit bacterial activity without compromise their polymerization.

Keywords: Adhesive Systems; Titanium Dioxide; Silver; Nanoparticles; *Streptococcus mutans*; Degree of Conversion

1.Introduction

Direct composite resin restorations have increased considerably in popularity and predictability and also have changed paradigms in modern dentistry. Now, these restorative procedures have been cited as an adequate and widely method used to treat carious lesions. However, the placement of effective composite resin restorations and the control of the development of secondary caries remain as a big challenge (1).

The cavity preparation without residual carious dentin and bacteria is difficult and consists the main reason for the establishment of secondary caries. The marginal micro-leakage and biofilm build-up also contribute to this condition (2). In this way, to prevent both cariogenic bacterial colonization and growth of remaining bacteria in the cavity preparation, an antimicrobial effect of composite resins and adhesive systems is a desired property.

The modification of dental products, such as composite resins, luting agents and adhesive systems to provide antimicrobial effect is achieved by adding antimicrobial agents, which can include monomers, chlorhexidine, glutaraldehyde and inorganic fillers (3).

Inorganic fillers, such as silver (AgN) and titanium dioxide (TiO_2) have proven antibacterial properties. Silver nanoparticles, in comparison to silver ions, which are more easily sequestered, are more likely to be not intercepted by substances such as phosphate and proteins, showing a more effective delivery mechanism and are used in a wide range of materials (4). The killing mechanism of these nanoparticles consist of cell wall rupture, protein denaturation and interference in the respiration process (5). Titanium dioxide is a metallic oxide, a photocatalytic substance and exhibits strong antibacterial activity under the presence of UV-light (6). Nevertheless, the bactericidal effect of TiO_2 nanoparticles without the use of light is also significant. Reactive oxygen species derived from TiO_2 disturb elements present in the cell membrane, such as phospholipids, lipoproteins and nucleic acids, which causes oxidative stress and ultimately induces cell death (7). The investigation of these nanoparticles in dental adhesive systems has demonstrated positive results, significant antibacterial activity and unaltered mechanical properties (8). Also, the incorporation of other types of antimicrobial components, such as antimicrobial monomers, into dental adhesive systems, orthodontic adhesives, resin cements and restorative composites have shown promising results (9). The control of secondary caries using modified dental materials is desirable, but their physical and mechanical properties should not be changed, otherwise they can show a bad performance, which could compromise the lifetime of the restoration.

Degree of conversion (DC) in one of the most important properties evaluated to determine optimal and successful adhesion to dental substrate (10). Low percentages of DC can lead to a series of unwanted effects in the performance of dental adhesive systems, causing inefficient restorations (11). These effects include decreased shear bond strength values, suboptimal mechanical properties, high monomer elution, increased permeability and phase separation (12).Thus, maintaining adequate DC values in modified adhesive systems is essential to attain better adhesion and to prolong the longevity of restorations.

Although a large number of studies have investigated the use of antimicrobial nanoparticles in dental adhesives, the literature does not provide studies regarding the combination of two different types of nanoparticles and how TiO_2 behaves without the use of UV-light. Thus, the objective of this study was to evaluate the antibacterial effect of different adhesive systems modified with TiO_2 or TiO_2/Ag at different concentrations and their influence on degree of conversion. The null hypothesis was that there is no antibacterial activity in the modified adhesive systems and that there is no difference in degree of conversion.

2.Materials and Methods

2.1.Experimental Design

This study was a laboratory research in which the following factors were evaluated: antibacterial activity and degree of conversion of three different adhesive systems, two types of nanoparticles (TiO_2 or TiO_2/Ag) at three different concentrations (1, 2 and 5%).

The independent variables were the adhesive systems, type of nanoparticles and the concentrations tested. The dependent variables consisted of the antibacterial activity and the degree of conversion.

For both tests, the statistical analysis was performed assessing the normal distribution and homoscedasticity of the data, using the Kolmogorov-Smirnov and then three-way ANOVA and Post-Hoc Tukey's test were used. The Poisson regression was also utilized. The intraclass correlation coefficient was determined to calibrate the examiners when counting the plates. The analysis were performed using the following softwares: GraphPad (GraphPad Software Inc, La Jolla, CA, USA) and Assistat 7.5 (Federal University of Campina Grande, Campina Grande, PB, Brazil).

2.2. Adhesive Systems and Nanoparticles Used

Three commercially available adhesive systems were used in this study: Adper^{TM} Single Bond and Adper^{TM} Scotch^{TM} Bond Multi Purpose (3M Espe Dental Products, St.Paul, Minnesota, 55144, USA), and Clearfil SE Bond (Kuraray Noritake Dental Inc. 1621 Sakazu, Kurashiki, Okayama 710-0801, Japan).

The TiO_2 and TiO_2/Ag nanoparticles used have an average size of 56nm and were produced according to the specifications of the Pechini method,[23] by the Crystal Growth and Ceramics Materials Research Group (Physics Institute of São Carlos - IFSC , University of São Paulo - USP, São Carlos, São Paulo, Brazil).

2.3. Direct Contact Antibacterial Test

The methodology for this test is explained in appendix A. For the direct contact test circular specimens (n=108) were made with Filtek^{TM}Z250 XT (3M Espe Dental Products, St.Paul, Minnesota, 55144, USA, #1518700267HB004209993) composite resin using a metallic matrix (4mm length x 2mm width) and light-cured with a LED light-curing unit (LED Radii Plus, SDI Limited, Bayswater, Victoria, Australia) for 40 seconds. The TiO_2 and TiO_2/Ag nanoparticles were weighted using a precision balance (Ohaus Adventurer^{TM}, 10285042, Toledo do Brasil Indústria de Balanças Ltda, São Bernardo do Campo, SP, Brazil) to ensure that the correct concentration (1, 2 and 5% wt) was used. Then, the nanoparticles were incorporated into the adhesive systems, resulting in 3 control and 18 experimental Groups. The nanoparticles were added into 0.5mL of the adhesive systems, which were placed in Eppendorf Tubes® (3810X, Eppendorf AG, Hamburgo, Germany). The eppendorf tubes were then slightly shaken and placed in an ultrasonic bath (1440 Plus, Odontobras, Ribeirão Preto, SP, Brazil) during 1 minute. The adhesive systems modified or not were then applied (8µL) according to the manufacturer’s instructions on the surface of the composite resin specimens. The specimens were then sterilized using an autoclave at 121ºC for 15 minutes.

In order to achieve a standardized suspension of *Streptococcus mutans* (ATCC 25175) (FioCruz Foundation, Rio de Janeiro, RJ, 21045900, Brazil) for the direct contact test, 5 colonies were placed in a Falcon tube with 5mL of Bacto^{TM} BHI Broth (Brain Heart Infusion, Difco Laboratories, Becton Dickinson and Company, USA) supplemented with 1% sucrose and incubated for 18 hours at 37ºC in a 10% CO_2 incubator (3110 Forma Series II water jacketed CO_2 Incubator, Thermo Fisher Scientific, Waltham, MA, USA). After that, a standardized suspension of *S. mutans* at the absorbance of 0.08-0.1 was achieved using a spectrophotometer (Pharmacia, Pharmacia LKB, Ultrospec III) and the turbidity was adjusted to the 0.5 Mac Farland Scale. The composite resin specimens with the adhesive systems were place in a 24-well plate (Corning, USA) with 100µL of the standardized bacterial suspension and incubated for 1 hour at 37ºC under an atmosphere containing 10% of CO_2. Then, 900 µL of BHI broth + 1% sucrose was added into each well and the plate was incubated for 18 hours at 37ºC under an atmosphere containing 10% of CO_2. After that, each well was washed three times using 1000µL of phosphate buffered saline solution (PBS) and then, each specimen was transferred into a Falcon tube containing 5mL of PBS and centrifuged for 5 minutes. The final suspensions of each tube were submitted to a six-fold serial dilution. Finally, 50µL of each resultant dilution was retrieved and spread on petri dishes containing brain-heart infusion agar + 1% sucrose, which were placed in an incubator under an atmosphere containing 10% of CO_2 at 37ºC for 48 hours. After the incubation period, the colony forming units (CFU's) were visually counted in two separate occasions. A reproducibility test was executed before the beginning of the study. Sample plates were examined by the same examiner immediately after being taken out of the incubator, after one and 7 days, showing a good intraclass correlation coefficient of 0.81, which allowed the study to be performed.[24] The results showing the CFU's count above 300 or below 30 were discarded. The data was analyzed by three-way (Adhesive System x Type of Nanoparticle x Concentration) analysis of variance (ANOVA) and Tukey's Test at 5% confidence level ($p \leq 0.05$). The experiment was performed in triplicate at different occasions.

2.4.Degree of conversion

The degree of conversion test was performed for both nanoparticles at different concentrations for the different adhesive systems, including cured and uncured specimens. Eight specimens were made for each Group (n=64). The adhesive systems were modified as previously described and 10µL of the samples were placed in a glass slide and photo-activated according to the manufacturer's instructions. The light tip of the LED light-curing unit (LED Radii Plus, SDI Limited, Bayswater, Victoria, Australia) was placed at a distance of 2mm from the surface of the specimens. The specimens were then stored in an incubator for 24 hours at 37ºC. After the storage time, the FTIR analysis was performed using an attenuated total reflectance (ATR) unit (Vertex 70, 202, Bruker Optik GmbH, Ettlingen, Germany) under the following conditions: 64 scans and a $4cm^{-1}$ resolution. The degree of monomer conversion was determined by the comparison between the absorbance intensity of aliphatic carbon-to-carbon double bonds (peak at $1638cm^{-1}$) and the aromatic component (peak at $1608cm^{-1}$), before and after specimen photo-activation. The final degree of conversion percentage was calculated by subtracting the remaining double carbon bonds percentage from 100%, according to the formula:

$$DC\ (\%)\,\frac{1-\left(\frac{C=C_{aliphatic}}{C-C_{aromatic}}\right)cured}{\frac{C=C_{aliphatic}}{C-C_{aromatic}}\,uncured}$$

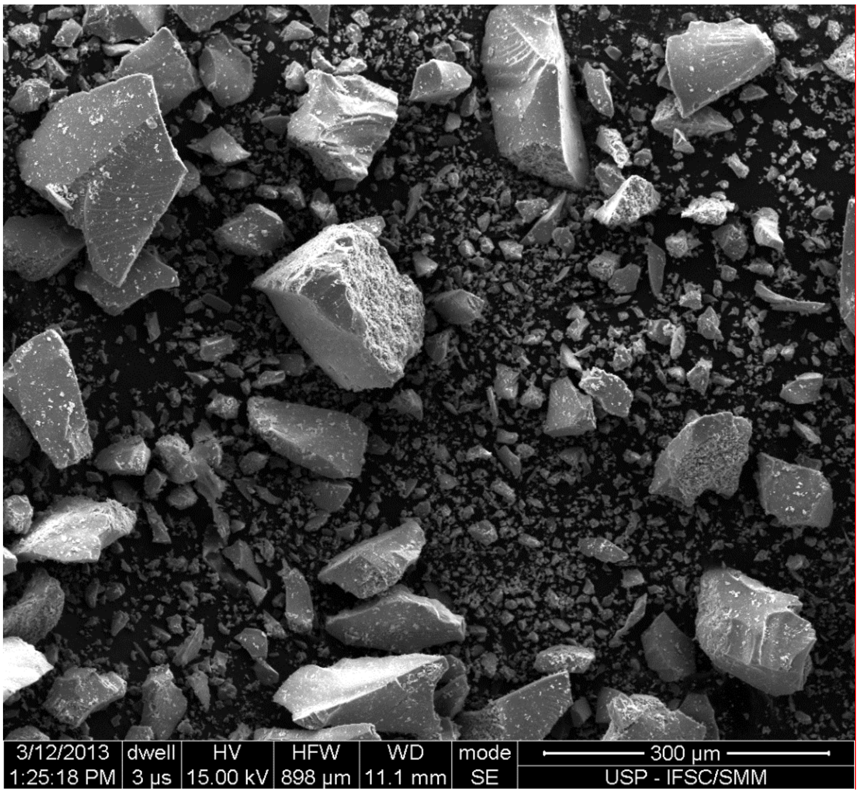

Figure 1. TiO_2/Ag nanoparticles under SEM observation (300um). Dias, H.B. (2014).

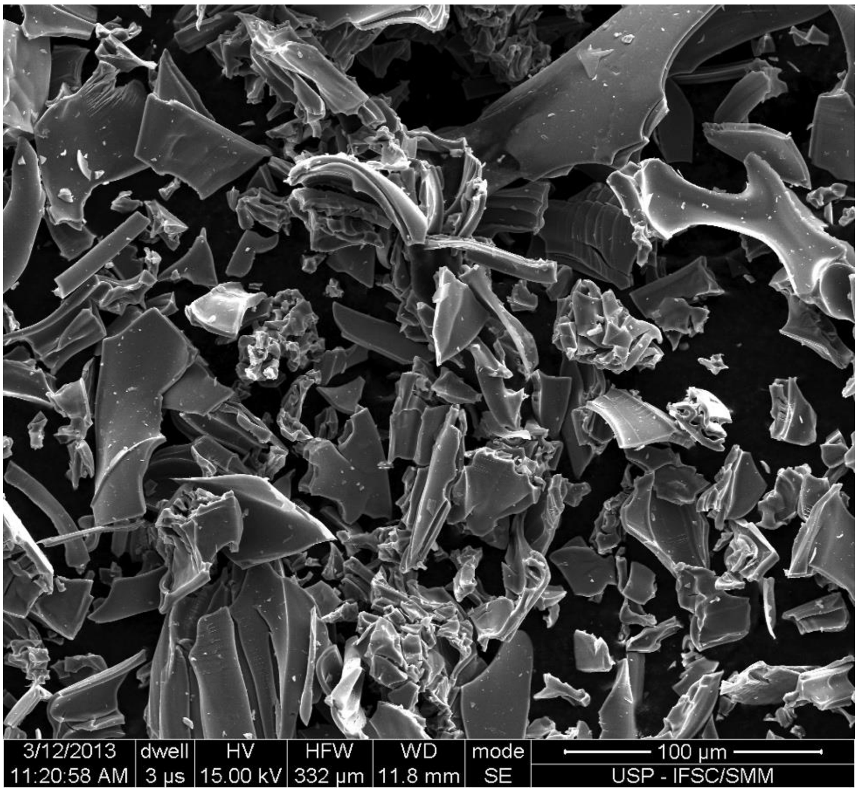

Figure 2. TiO_2 nanoparticles under SEM observation (100um). Dias, H.B. (2014).

4.Results

The results showed significant difference between the control and the experimental Groups for all adhesive systems modified with TiO_2 or TiO_2/Ag nanoparticles at all concentrations, which means that *S. mutans* was significantly reduced ($p<0.05$). However, the increase in concentration did not provide better antibacterial effect ($p \geq 0.05$). Also, considering the two nanoparticles used (TiO_2 and $AgTiO_2$) the antibacterial effect was not different ($p \geq 0.05$). Additionally, no difference was found among the three types of adhesive systems, which means that the effect showed by Single Bond was the same as the one showed by ScotchBond Multipurpose and Clearfil SE Bond ($p \geq 0.05$). The mean values for the CFU's count transformed into log10 and the standard deviations are shown in Table 2 and in Figures 6 and 7.

The mean values and standard deviation for DC are presented in Table 2 and in the Figures 3, 4 and 5. The addition of nanoparticles at any concentration did not statistically change the DC values for the different adhesive systems tested (p>0.05).

Table 1. Mean, standard deviation, quartil, minimum and maximum log10 values for the antibacterial direct contact test

Adhesive	Group	Concentration	N	Mean	S.D.	Minimum	1º Q.	2º Q.	3º Q.	Maximum
Clearfil	Control	0	67	6,63	0,14	6,38	6,53	6,60	6,73	6,89
	$AgTiO_2$	1	26	5,54	0,07	5,42	5,49	5,53	5,59	5,67
		2	23	5,56	0,12	5,39	5,50	5,53	5,60	5,97
		5	24	5,52	0,11	5,35	5,42	5,54	5,58	5,73
	TiO_2	1	29	5,61	0,19	5,42	5,53	5,58	5,64	6,52
		2	29	5,55	0,08	5,43	5,50	5,54	5,60	5,73
		5	30	5,57	0,10	5,38	5,51	5,55	5,60	5,79
Scotchbond	Control	0	66	6,63	0,19	6,09	6,51	6,60	6,75	7,07
	$AgTiO_2$	1	28	5,56	0,22	5,34	5,47	5,52	5,60	6,58
		2	22	5,41	0,10	5,29	5,34	5,39	5,48	5,62
		5	27	5,50	0,14	5,30	5,39	5,51	5,58	5,79
	TiO_2	1	33	5,62	0,29	4,64	5,60	5,66	5,72	6,17
		2	30	5,30	0,50	4,58	4,75	5,54	5,58	6,56
		5	29	5,47	0,39	4,60	5,42	5,49	5,54	6,55
SingleBond	Control	0	53	6,67	0,30	6,09	6,52	6,68	6,82	7,48
	$AgTiO_2$	1	26	5,84	0,16	5,57	5,69	5,83	5,98	6,08
		2	24	5,68	0,45	4,38	5,61	5,78	6,04	6,07
		5	21	5,47	0,52	3,58	5,51	5,59	5,72	5,89
	TiO_2	1	33	5,50	0,40	4,74	5,34	5,68	5,75	5,95
		2	29	5,44	0,36	4,41	5,30	5,53	5,67	6,05
		5	24	5,46	0,49	3,58	5,48	5,59	5,71	5,95

Table 2. Mean, standard deviation, quartil, minimum and maximum degree of conversion values

Adhesive	Group	Concentration	N	Mean	S.D.	Minimum	1º Q.	2º Q.	3º Q.	Maximum
Clearfil	Control	0	64	38,38	7,19	31,05	33,58	35,54	40,26	55,84
	$AgTiO_2$	1	64	37,47	7,37	33,11	34,98	36,04	37,58	92,54
		2	64	34,13	8,06	23,43	31,06	35,12	35,60	91,80
		5	64	31,33	8,50	20,20	29,59	30,76	32,84	90,69
	TiO_2	1	0	-	-	-	-	-	-	-
		2	64	37,78	8,74	30,09	33,71	34,91	40,45	92,65
		5	64	39,63	6,95	36,03	37,18	38,74	40,50	92,68
Scotchbond	Control	0	64	32,40	8,00	27,17	29,60	32,12	33,20	92,85
	$AgTiO_2$	1	64	38,03	7,30	31,12	36,64	37,12	38,65	92,62
		2	64	43,57	10,19	33,30	36,80	39,49	52,22	92,97
		5	64	38,70	8,15	22,93	36,65	39,60	40,72	93,60
	TiO_2	1	64	32,72	8,48	24,63	28,70	32,91	33,75	92,03
		2	64	35,08	7,57	31,27	32,70	33,49	35,44	92,75
		5	64	35,77	7,91	27,85	33,44	36,12	36,80	93,27
SingleBond	Control	0	64	25,58	12,25	2,84	21,03	24,00	31,20	56,58
	$AgTiO_2$	1	64	17,35	10,36	2,80	12,10	16,42	20,37	86,55
		2	64	27,72	10,05	10,62	21,44	27,12	32,65	85,94
		5	64	28,21	9,03	14,11	24,86	27,67	31,13	88,68
	TiO_2	1	64	29,57	10,41	14,97	23,37	31,27	33,82	89,37
		2	64	30,14	8,23	24,49	26,12	28,92	32,81	89,03
		5	63	27,19	12,11	5,16	28,49	30,31	32,46	85,15

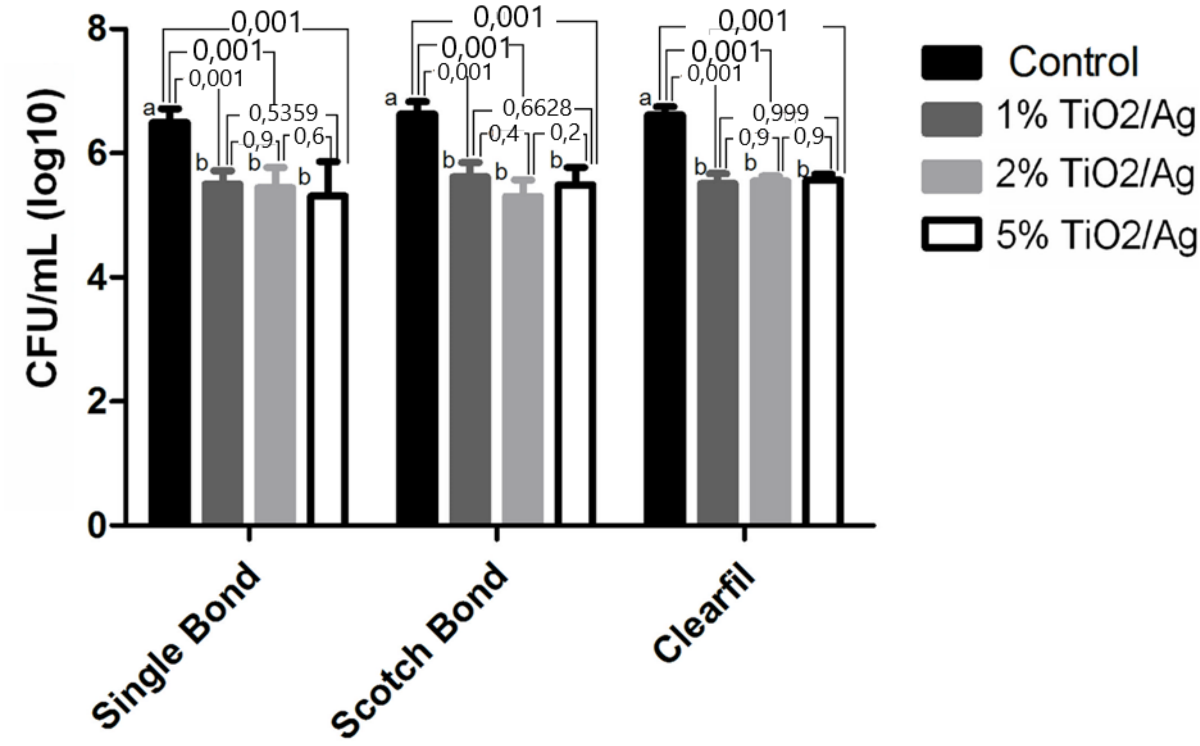

Figure 3. Mean CFU/ml (log10) values for the adhesive systems modified with TiO_2/Ag nanoparticles. Different letters means statistically significant difference.

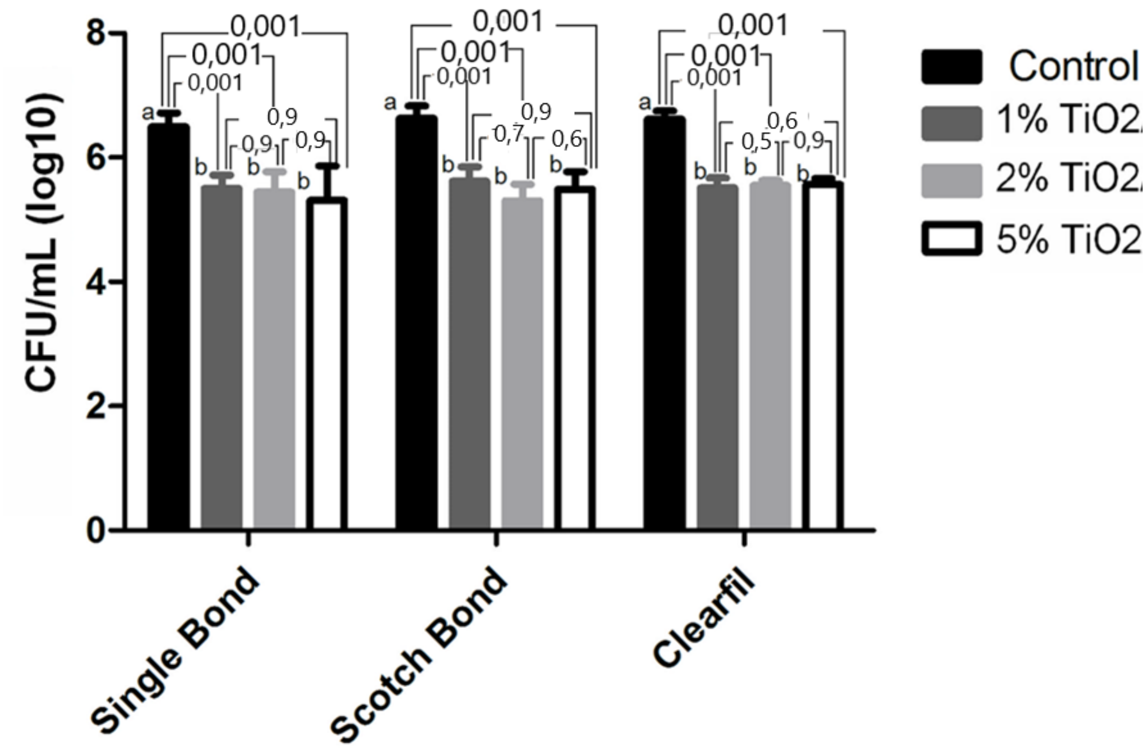

Figure 4. Mean CFU/ml (log10) values for the adhesive systems modified with TiO_2 nanoparticles. Different letters means statistically significant difference.

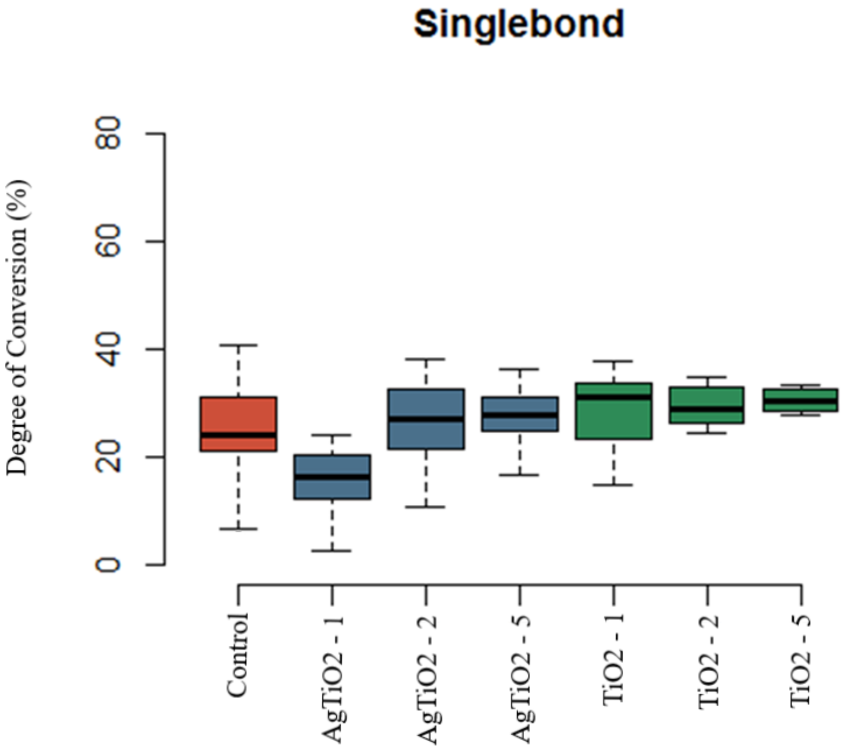

Figure 5. Boxplot for the degree of conversion of Single Bond.for the different Groups.

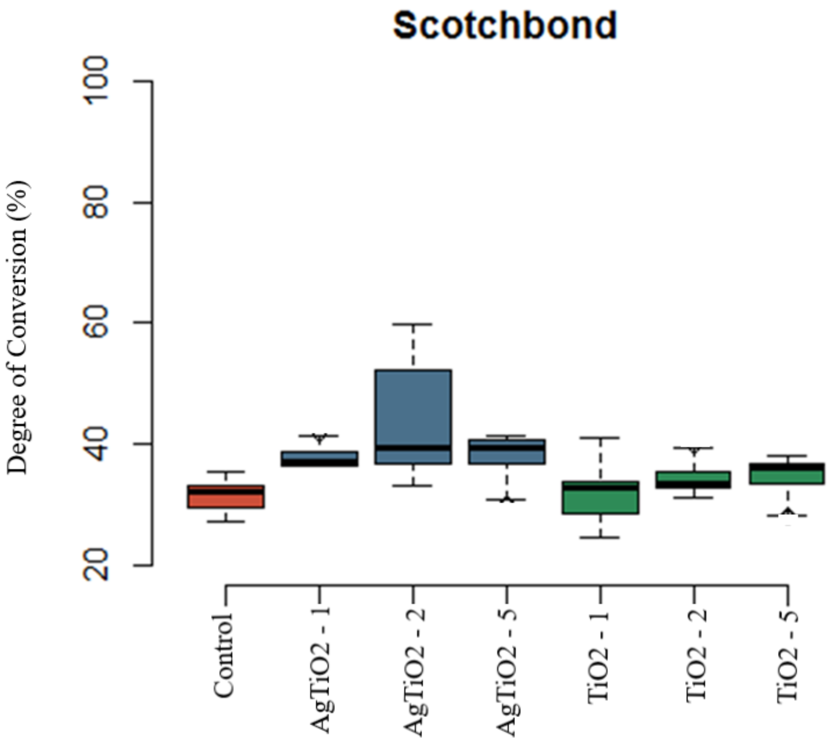

Figure 6. Boxplot for the degree of conversion of ScotchBond Multipurpose for the different Groups.

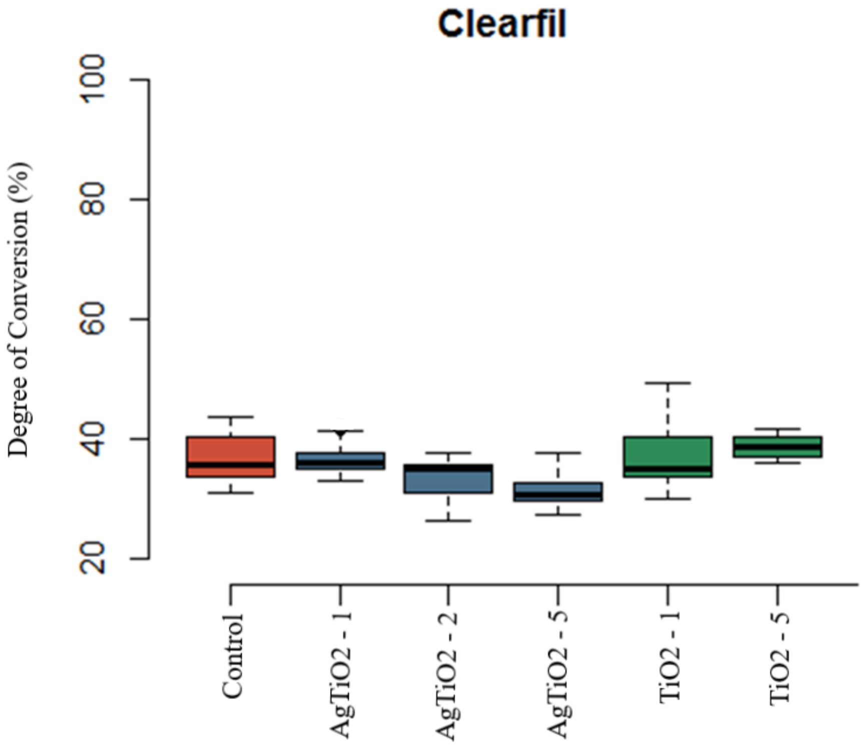

Figure 7. Boxplot for the degree of conversion of Clearfil SE Bond for the different Groups.

5. Discussion

Different approaches have been made to produce dental materials that can inhibit the bacterial growth.[8, 25] The current study evaluated the inhibition of bacterial growth of different types of adhesive systems (two-steps etch-and-rise, one-step etch-and-rinse and two-step self-etch), modified with TiO_2 or TiO_2/Ag nanoparticles by direct contact technique. Additionally, the degree of conversion was also evaluated.

The results for the current study showed that antibacterial effect was detected in the tested adhesive systems. The antibacterial potential of TiO_2 nanoparticles at low concentrations was statistically significant different from the control Groups ($p<0.05$), leading to a reduced level of the bacteria tested.

The decoration of the TiO_2 nanoparticles with silver nanoparticles (AgN) also presented significant antibacterial potential, however similar to TiO_2, for all adhesive systems studied ($p<0.05$). It was also observed that the different concentrations (1, 2 and 5%) did not exert any influence ($p>0.05$). No difference was detected among the adhesive systems at any concentration, considering both types of nanoparticles. Moreover, DC remained unaltered at all concentrations, for both types of nanoparticles and for all modified adhesive systems. The null hypothesis was partially rejected, because the DC was not altered after the modification of the adhesive systems under the different concentrations of the nanoparticles.

The reduction of the amount of bacteria on the surface of the specimens reached one log10, for both TiO_2 and TiO_2/Ag nanoparticles are seen on Figures 1 and 2. A past study showed that the antibacterial properties of TiO_2 over *Escherichia coli* were observed under irradiation with UV-light, however in the dark the antibacterial properties were insignificant (14). Another study showed different antibacterial results, demonstrating that the nanoparticles showed significant antibacterial activity without the presence of light, although it was limited when compared to chlorhexidine or Ag nanoparticles (13). A possible explanation could be related to the differences of the microorganisms tested and the use of different methodologies to evaluate the antibacterial activities, which was found in the study from Maness and others (14) that investigated *E.coli*.

Regarding the modification of adhesive systems with antibacterial nanoparticles, the findings of the current study are in agreement with the results in the literature. Previous studies reported antibacterial effect of TiO_2 nanoparticles incorporated into Adper^{TM} Scotchbond^{TM} 1 XT up to 30% wt, with or without the presence of UV-light (15). Based on the photo-catalytic nature of TiO_2, the presence of UV-light causes the release of multiple reactive oxygen species, which significantly reduce the amount of bacteria, even sterilizing *in-vitro Staphylococcus epidermidis* when the irradiation time was a maximum of 120 minutes, although the light itself did not show any effect on the microorganism (15). Titanium dioxide (TiO_2) also has the antibacterial potential to kill other microorganisms, such as *Streptococcus mutans*, *Lactobacillus acidophilus*, *Actinomyces viscosus* and *Candida albicans* when incorporated into an orthodontic adhesive and as coating for orthodontic brackets and implants (16). It has been showed that the increase in nanoparticles concentration can cause enhancement of their antibacterial properties, however in the current study this fact was not observed. Maybe this fact can be explained by the low concentrations of nanoparticles used. Another factor that may exert influence is that when nanoparticles are incorporated into a adhesive system, they tend to become involved and encapsulated into the resin matrix, making the direct contact with bacteria more difficult, which may compromise the bactericidal effect

(15). We hypothesize that the poor dispersion and distribution of nanoparticles across the resin matrix may reduce bactericidal effect, although no reference was found in the specific subject.

Some studies have already successfully determined that silver nanoparticles in adhesive systems or composite resins show antibacterial effect (17). However, studies using TiO_2 decorated with silver nanoparticles in commercially available adhesive systems are still, to our knowledge, unpublished. This is the first study performed about this subject. Silver nanoparticles cause the death of bacteria through three processes: the attachment to the cell membrane surface, disturbing the optimal function, the penetration inside the cell and interacting with compounds like DNA and the release of silver ions, which give additional contribution (18). In adhesive systems, silver nanoparticles show potent antibacterial effect against microorganisms (19).Therefore, a potent bactericidal activity would be expected in small concentrations, as has been confirmed with epoxy resin containing TiO_2/Ag nanoparticles against *S. mutans* and an orthodontic adhesive with only 0.33% wt of AgN, which confirms the results obtained in the current study (19, 21). Chamber et al (19) showed that the incorporation of TiO_2/Ag into a bulk of an epoxy resin reduced the antibacterial activity, although it remained significant. Our findings are in accordance with these previous results. Silver nanoparticles can also be used in combination with composite resin-based materials. Indeed, this combination provides the desired antimicrobial activity (17).

The development of new and innovative restorative materials with biocidal action has to be carried out with caution. The original properties of these products could not be changed otherwise they are not clinically useful (20, 21). The degree of conversion is one of these properties and determines, along with other parameters, the adhesive’s final mechanical and physical properties (22).

The mean values and standard deviation or DC percentage obtained are shown in Table 3. These results are in agreement with previous researches (20, 22). However, Sun et al

(23) observed that adding a small amount (0,1%) of TiO_2 nanoparticles, the DC can improve significantly. In another study, the same author observed an improvement of 22% by adding 0,1% of nanoparticles, which suggests that this effect is limited only to extremely low concentrations and that higher concentrations of nanoparticles do not provide better DC values (24). On the other hand, analysis of DC of adhesive systems with silver nanoparticles showed that the values decreased up to 0,33% concentration of NAg, but the chemical and physical properties were not compromised, which is similar to the current findings (21). Faria e Silva et al (25) in 2010, showed that DC values for Scotch Bond, Single Bond and Clearfil SE Bond were around 60, 75 and 60%, respectively. Our results for the adhesive systems modified and not modified with nanoparticles at different concentrations are in according to this study. Furthermore, the fact that TiO_2 nanoparticles exhibit strong photoactivity has an impact on the photoiniation process.. The nanoparticles work as an additional coinitiator, which allows the reaction to occur normally and also the polymerization of the the resin matrix (23).

Dental adhesive systems are crucial for the placement of composite resin restorations and also they have a wide range of clinical uses. Thus, the clinical handling of such materials should be easy, less sensitive technique, which tends to make the procedures faster and more comfortable to the patients. The modification of adhesive systems with nanoparticles can increase the viscosity and can make the use extremely difficult.

Although the current study and the literature can help us to understand why TiO_2/Ag had an antibacterial potential, a reasonable assumption would be that the combination of two antibacterial nanoparticles could increase the bactericidal effect. However, this effect was not observed in this current research.

No statistically significant difference was found between the DC of the control and the experimental Groups, but there was a difference considering the antibacterial direct contact test for the two types of nanoparticles, which leads the null hypothesis to be partially rejected. Thus, for the DC test the null hypothesis was accepted, but for the antibacterial test it was rejected.

6.Conclusions

Based on the results obtained in the current study, it was possible to state that the antibacterial effect presented by TiO_2 and TiO_2/Ag nanoparticles on different types of adhesives systems at low concentrations was significant and this modification did not significantly change the monomer conversion percentage. These nanoparticles could also be an option to modify dental materials in order to reduce the negative impact of secondary caries show on the incidence of restoration replacement and failure. The possibilities for this field of study remain wide and the perspectives for the future are positive. Nevertheless, upcoming research should focus on the mechanical properties and clinical aspects of the incorporation of antibacterial agents in dental adhesives. In clinical situations, it is necessary to investigate how the adhesive systems will behave: if the antibacterial effect will be maintained and the performance will not be affected. Only when these questions would be answered, the implementation of a safe modification of the adhesive system will be achievable.

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4 PUBLICAÇÃO 2

The micro-shear bond strength of dental adhesive systems modified with antibacterial nanoparticles*

Running title: Shear bond-strength of modified dental adhesives

Clinical relevance: The modification of different adhesive systems with nanoparticles that provide antibacterial effect does not bring significant changes to bond strength.

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* a ser submetido para o periódico Operative Dentistry

Abstract

The aim of this study was to evaluate the micro-shear bond strength of three commercially available dental adhesive systems containing two different types of antibacterial nanoparticles (titanium dioxide or titanium dioxide decorated with silver) at three concentrations in two distinct periods of evaluation (immediately and after 3 months). *Methods:* The adhesive systems Adper^{TM} Single Bond, Adper^{TM} Scotch^{TM} Bond Multi Purpose and Clearfil SE Bond were modified with 1, 2 and 5 wt% of nanoparticles. Two hundred and fifty two (n=252) standardized dentin specimens were made using bovine incisors and divided into twenty-one Groups. The control and modified adhesive systems were applied on the surfaces of the specimens according to the manufacturer's instructions and composite resin cylinders (Filtek^{TM} Z250XT) were built-up on the bonded surfaces using a Teflon matrix. The test was performed using a universal testing machine, at a cross-head speed of 0,5mm/min until the specimen rupture. The mode of failure of the specimens was determined by the using a stereomicroscope at 40x and divided into adhesive, mixed and cohesive. The values were given in MPa and analyzed with three-way and post hoc Tukey's test at 95% significance level. The mean bond strength values (MPa) and standard deviation for the microshear test (immediate) were: 19,3 ± 2,7 (SB), 22,4 ± 3 (SBM) e 16,6 ± 2,9 (CB); TiO_2 (1%): 18,7 ± 2,4 (SB) 22,8 ± 4,8 (SBM) 15,2 ± 2,7 (CB); TiO_2 (2%): 17,9 ± 2,7 (SB) 21,5 ± 2,8 (SBM) 17,9 ± 3,4 (CB); TiO_2 (5%): 18,2 ± 3,01(SB) 21,9 ± 3,9(SBM) 17,5 ± 3,99 (CB); $AgTiO_2$-1% 19,5 ± 3,2 (SB) 23,5 ± 3,8 (SBM) 16,5 ± 3,1 (CB); $AgTiO_2$-2% 18,1 ± 3,1 (SB) 24,2 ± 3,9 (SBM) 14,8 ± 3,2 (CB); $AgTiO_2$-5% 17,2 ±4,5 (SB) 23,3 ± 4 (SBM) 14,1 ± 5 (CB) ($p<0,05$). The mean bond strength values (MPa) and standard deviation for the microshear test (3 months) were: 18,3 ± 1,83 (SB), 18,58 ± 2,31 (SBM) e 18,58 ± 2,23 (CB); TiO_2 (1%): 17,92 ± 1,98 (SB) 18,25 ± 2,05 (SBM) 17,5 ± 1,8 (CB); TiO_2 (2%): 17,83 ± 1,85

(SB) 18,17 ± 2,,04 (SBM) 17 ± 2,59 (CB); TiO_2 (5%): 17,75 ± 1,96 (SB) 18,17 ± 2,17 (SBM) 18,75 ± 2,01 (CB); $AgTiO_2$-1% 17,75 ± 1,87 (SB) 17,33 ± 1,88 (SBM) 17,42 ± 1,44 (CB); $AgTiO_2$-2% 17,50 ± 2,28 (SB) 17,25 ± 1,96 (SBM) 17,33 ± 2,31 (CB); $AgTiO_2$-5% 18,25 ± 1,91 (SB) 18,3 ± 2,54 (SBM) 19,17 ± 2,21 (CB) ($p<0,05$). No significant difference was found between the control and experimental groups, considering both types of nanoparticles, all concentrations used and both periods of time. The mode of failure analysis didn't show any correlation between the type of failure and the modification of the adhesives, except after three months of water storage for one adhesive (Clearfil SE Bond), which showed mostly mixed failure. The modification of dental adhesive systems with antibacterial nanoparticles at different concentrations and storage time for 3 months do not exert any influence on their micro-shear bond strength.

1.Introduction

Nanotechnology can offers the possibility of improvement in different areas of human activity, including industry, electronic technology, environmental issues and communication.[1] These effects may be particularly interesting when it comes to human health. The application of nanotechnology has been a reality in modern medicine in areas like drug delivery and imaging for a reasonable amount of time.[2,3] In restorative and operative dentistry this application is also under course and promises advances in many fields.[4] A wide range of dental materials containing nanotechnology is already being investigated, showing interesting results, which includes nanostructures like nanoparticles, nanorods, nanofibers, nanospheres, nanoshells and others.[5] Novel strategies using these mechanisms are being studied, revealing a potential to prevent and treat dental caries, as well as control and prevent secondary/recurrent caries.[6] Researchers also believe that the use of nanotechnology can have a significant impact on other areas of scientific research and dental practice, such as the remineralization of initial caries and on the formation of dental plaque.[6] Although the speed in which nanotechnology has been used in dentistry is slower than medicine, changes are already under way. Nanoparticles are defined as particles of a determined substance that usually have a maximum size of 100nm or 1 x 10^{-7}m.[7]

Dental caries remains as an infection disease and can have a negative impact on the human health.[6] Secondary caries or caries adjacent to restorations is the most frequent reason for replacement of dental restorations. The increase on the secondary caries at the margins of composite resins restorations can suggests that the sealing at the filling interface is not adequate to resist the physical, chemical, and mechanical stresses in the mouth. The failure of composite resin restorations has been linked to the degradation of the bond at the tooth surface–composite material interface and an increase in the concentration of the cariogenic bactéria, such as Streptococcus mutans around and over these materials.[8,9,10] Some antibacterial agents have been incorporated into dental materials, aiming to prevent the destructive effects of dental caries.[11] More recently, antibacterial agents based on nanoparticles have been used to modify dental composites and dental adhesive systems. Inorganic metals, such as zinc oxide, titanium dioxide (TiO_2) and silver (Ag), as well as other substances like monomers and releasable agents are used for this purpose. Some studies have shown that the incorporation of these nanoparticles can yielding potent antimicrobial properties to the dental adhesives they modify.[12,13] The same effect has been shown in composite resins, as well as other bonding and restorative products, such as orthodontic adhesives, glass- ionomer cements and resin cements.[14-17]

The antibacterial effect of TiO_2 has already been investigated in dentistry and medicine, ranging from dental implants to catheters.[18,19,20] Since studies have demonstrated that composite resins containing TiO_2 nanoparticles exhibit antibacterial properties, future researches to focus more on the effect to prevent secondary caries and enamel demineralization are needed. A previous study also reported that the modification of a glass ionomer cement with TiO_2 nanoparticles did not compromise the shear bond strength, as well as other important mechanical properties such as flexural strength, elasticity modulus and microhardness.[17] This modification seems to reinforce the glass ionomer cement.

Silver is another antibacterial agent that can be an alternative to control dental caries. The modification of dental adhesive systems with silver nanoparticles has been reported in the literature, showing that the novel bonding agents can successfully inhibit different bacteria related to the dental caries. A previous report showed that the shear bond strength of adhesive systems are not compromised by the addition of silver nanoparticles and no significant difference between control and experimental adhesive was detected.[21]

It is clear to the authors that there is scrutiny regarding the antibacterial properties of nanoparticles in dental products. However, more needs to be understood before a safe protocol can be achieved. Not enough knowledge has been attained regarding the effect that TiO_2 and Ag nanoparticles may have on the mechanical properties of dental adhesive systems. Furthermore, although the decoration of nanoparticles is a known subject and the aim of extensive research, the potentials of this mechanism are not yet fully comprehended in dentistry. Given the fact that the current literature lacks in those matters, the aim of this study was to evaluated the micro-shear bond strength of three commercially available dental adhesive systems modified with TiO_2 or TiO_2/Ag nanoparticles at three concentrations (1%, 2% and 5%) and test them in two distinct periods (immediately and after three months of water storage). The null hypothesis of the study was that the micro-shear bond strength values would be similar to all adhesive systems, at all concentrations and for both periods of time.

2. Materials and Methods:

2.1. Specimen’s preparations:

The methodology for the tests performed in this study is explained in appendix B. The present study was approved by the Ethics Committee in Animal Research of the Araraquara School of Dentistry - UNESP (Protocol number 28/2015 – Anex A). For each period of time tested (immediately and after three months) the bovine teeth (n=252) were stored in 5% thymol solution during 24 hours. After that, the specimens were cut using a cutting machine (IsoMet 1000, Buehler – ITW Company) (13mm lengthx7mm widthx2mm girth). To standardize dentin depth for all the specimens, each one was abraded with 120-grit silicon carbide sandpaper until the immediate dentin surface below the dentin enamel junction was exposed. After that, 0.5mm of dentin was removed using an 120-grit silicon carbide sandpaper to reach the intermediate dentin and to attain a standardized smear layer, each specimen was polished using a 600-grit silicon carbide sandpaper for 20 seconds. Each bovine tooth was then mounted in a polyvinyl chloride tube (2 x 2cm) with self-cured acrylic resin (VIPI Flash, VIPI Produtos Odontológicos, Pirassununga, SP, Brazil). The dental adhesive systems Adper^{TM} Single Bond (3M Dental Products, St.Paul, Minnesota, 55144, USA), Adper^{TM} Scotch^{TM} Bond Multi Purpose (3M Dental Products, St.Paul, Minnesota, 55144, USA) and Clearfil SE Bond (Kuraray Noritake Dental Inc. 1621 Sakazu, Kurashiki, Okayama 710-0801, Japan) were modified with 1, 2 and 5%wt of TiO_2 or TiO_2/Ag nanoparticles that were manually blended into them using a ultrasonic bath (1440 Plus, Odontobras, Ribeirão Preto, SP, Brazil). The Table 1 shows the composition of the adhesive systems used and also the nanoparticles used in this study. The TiO_2 and TiO_2/Ag were produced by the Crystals and Ceramics Materials Research Group (São Carlos Institute of Physics, University of Sao Paulo, São Carlos, Brazil).

Table 1. Materials used in this study

Material	Composition	Manufacturer
Adper^{TM} Single Bond	Ethanol, Bis-GMA, Silica, HEMA, Water, UDMA, EDMAB, PAA	3M, Espe, USA
Adper^{TM} Scotch^{TM} Bond	Water, PAA, HEMA, Bis-GMA, Bis-GMA, HEMA,	3M, Espe, USA

Multipurpose	Triphenylantimony	
Clearfil SE Bond	HEMA, 10-methacryloyloxydecyl di-hidrogen phosphate, Bis-GMA Bis-GMA, HEMA, colloidal silica, dl-camphorquinone, hydrophobic aliphatic methacrylate, 10-methacryloyloxydecyl di-hidrogen phospate	Kuraray Inc, Japan
Filtek^{TM}Z250XT	Bis-GMA, UDMA, TEGDMA, bis-EMA	3M, Espe, USA
Acid Etchant 37% Phosphoric Acid CONDAC	-	FGM, Joinville, SC, Brazil
TiO_2 and TiO_2/Ag nanoparticles (average size 56nm)	-	The Crystals and Ceramics Materials Research Group (São Carlos Institute of Physics, University of Sao Paulo, São Carlos, Brazil)

The adhesive systems were then applied according to the manufacturer's instructions in a limited area of the specimens. The adhesive area was determined using a plastic mold (1.5 x 1.5mm) and the composite resin cylinders were build-up on the bonded area using Filtek^{TM}Z250XT (3M Espe Dental Products, St.Paul, Minnesota, 55144, USA) and a Teflon matrix (1.5mmx1.5mm) and light-cured for 40 seconds under 1500mW/cm² (LED Radii Plus, SDI Limited, Australia). The specimens were stored in distilled water (changed every week) at 37ºC in an incubator (Isotemp Fischer Scientific, Pittsburgh, PA, USA).

2.2. Assessment of microshear bond strength

An universal testing machine (EMIC 2000, EMIC, São José dos Pinhais, PR, Brazil) was used to evaluate the microshear bond-strength after 24 hours and 3 months storage in distilled water at 37°C (±1°C). The specimens were positioned in the lower cross-head, allowing the force to be applied in a parallel direction. The cross-head used (500Kg/F) moved at a constant speed of 0.5mm/min. A stainless steel wire (0,5mm) was used in the form of a loop to apply the force. The loop was positioned directly adjacent to the bonded area and the test was then performed. The statistical analysis was conducted using GraphPad (GraphPad Software Inc, La Jolla, CA, USA). The data was analyzed by three-way ANOVA and post-hoc Tukey's test at 95% confidence level.

2.3. Failure mode of the specimens after the microshear bond strength test:

All specimens, after the microshear bond strength test, were observed using a stereomicroscope at 40x (Olympics, SZX, Japan) in order to determine the failure mode. The failure modes were divided into three categories: cohesive, adhesive and mixed. The adhesive failure was defined as any kind of failure that happened along the adhesive/resin interface and/or the adhesive/dentin interface. The cohesive failure was defined as the failure that occurred inside of the resin/dentin substrate. The mixed failure was defined as combination of both failure modes.

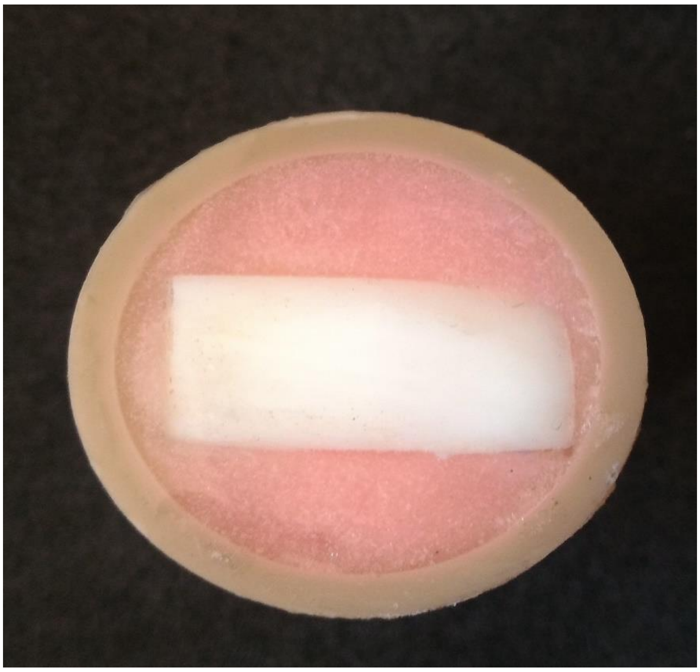

Figure 1. Bovine dentin specimen used in the study.

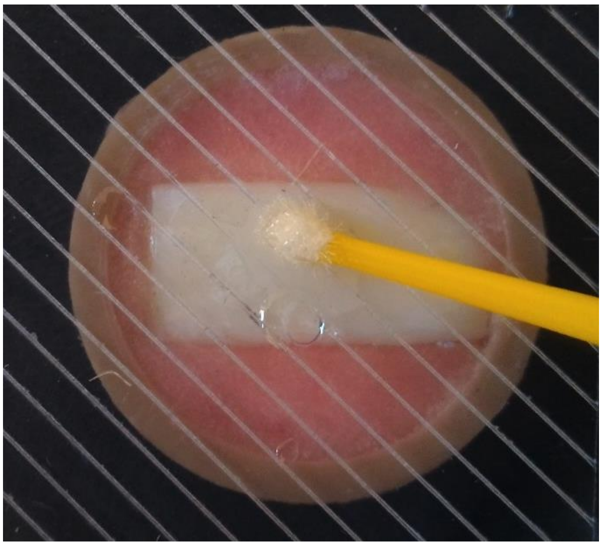

Figure 2. Application of the adhesive system on the limited adhesive area using a plastic mold.

Figure 3. Teflon matrix and metallic ring used to built-up the composite resin cylinders.

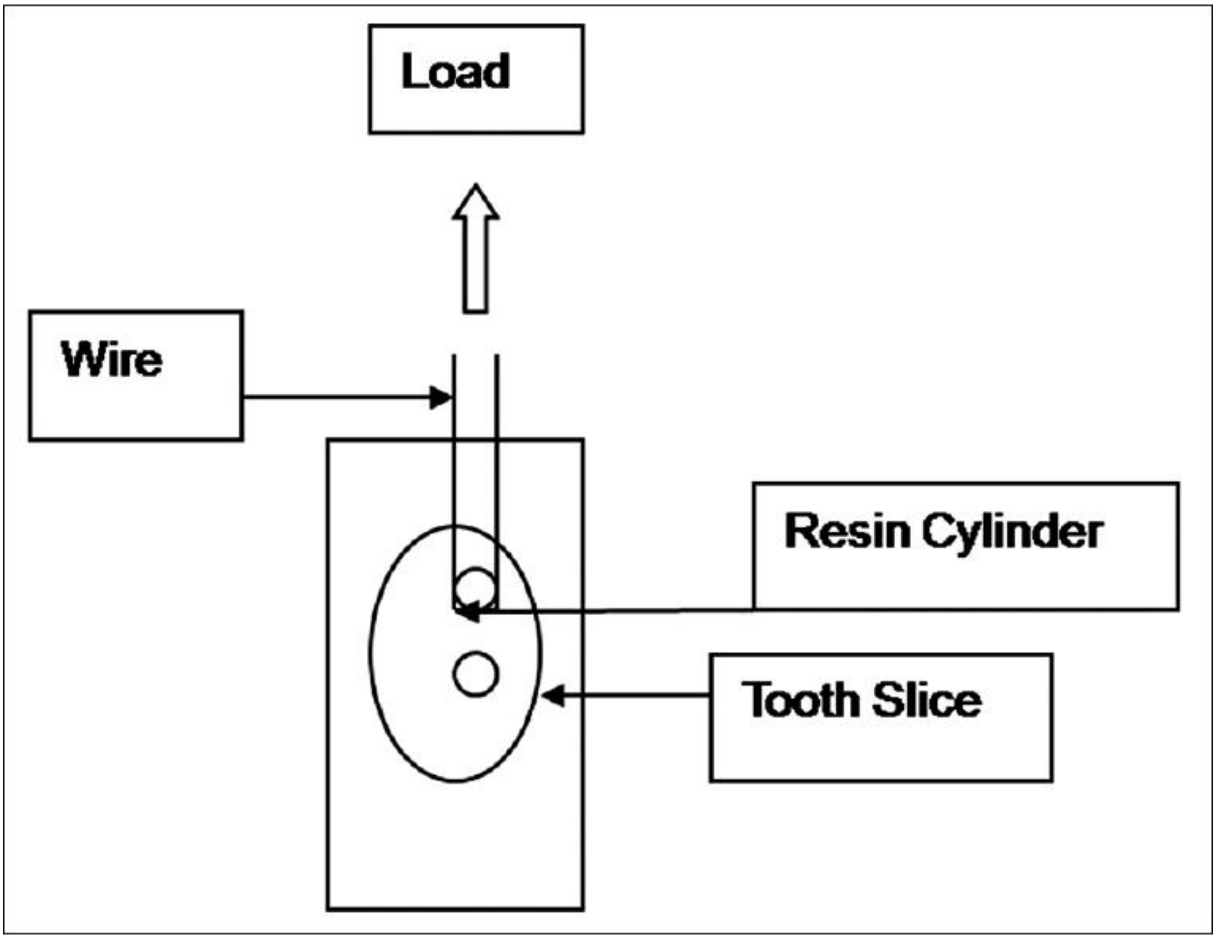

Figure 4. Micro-shear bond strength test being performed.

3. Results

3.1. Microshear bond strength after 24 hours

The results are shown in Tables 3 and 4. The statistical analysis showed significant difference among the control Groups of ScotchBond and the control Groups of Single Bond and Clearfil SE Bond, showing that ScotchBond had the highest mean values ($p<0,05$). Although this difference among the control Groups was observed, no statistically significant difference was found among the control and experimental Groups, considering any dental adhesive system, concentrations and types of nanoparticles tested. When comparing the mean values obtained between 24 hours storage test and after three months of water storage, the results showed a statistically significant difference among all control Groups ($p<0,05$). No difference was observed among control and experimental Groups for both storage times (p>0.05).

3.2. Microshear bond strength after three months of water storage

The results of microshear bond strength after three months of water storage showed difference among the three types of adhesive systems, however ScotchBond Multipurpose showed higher micro-shear bond strength values than the other two systems. When considering the results among the same adhesive systems, similar results were found. No statistical significant difference was observed between the control Groups of each adhesive system and the experimental Groups (p>0,05).

3.3 Determination of the mode of failure:

The failure modes of the microshear bond strength test are shown in Figure 5 and 6. The failure mode frequency was determined by the chi-square test (p<0.05). The failure mode frequency was not significantly influenced by the nanoparticles, adhesive system or nanoparticles concentration. For the specimens modified with Clearfil SE Bond and AgTiO_2 nanoparticles, the most frequent failure mode was the mixed and the groups modified with TiO_2 showed mostly adhesive or cohesive failure modes. For the Single Bond adhesive, the groups modified with TiO_2 nanoparticles at 5%, AgTiO2 at 1% showed mostly cohesive failure, and the control group showed more adhesive failure modes. The control group of ScotchBond Multipurpose had more specimens with an adhesive failure mode and the experimental groups showed more cohesive failure modes.

Table 2. Microshear bond strenght mean values and standard deviation of adhesive systems modified with TiO_2 or TiO_2/Ag after 24 hours water storage

Experimental Groups	Adper^{TM} Single Bond	Adper^{TM} Scotch^{TM} Bond	Clearfil SE Bond
Control	19,3 (± 2,7)^{A}	22,4(± 3)^{B}	16,6(± 2,9)^{A}
1% TiO_2	18,7 (± 2,4)^{A}	22,8(± 4,8)^{B}	15,2(± 2,7)^{A}
2% TiO_2	17,9(± 2,7)^{A}	21,5(± 2,8)^{B}	17,9(± 3,4)^{A}

5% TiO$_2$	18,2(± 3,01)^{A}	21,9(± 3,9)^{B}	17,5(± 3,9)^{A}
1% TiO$_2$/Ag	19,5(± 3,2)^{A}	23,5(± 3,8)^{B}	16,5(± 3,1)^{A}
2% TiO$_2$/Ag	18,1(± 3,1)^{A}	24,2(± 3,9)^{B}	14,8(± 3,2)^{A}
5% TiO$_2$/Ag	17,2(± 4,5)^{A}	23,3(± 4)^{B}	14,1(± 5)^{A}

Means followed by the same uppercase letters show no statistically significant difference ($p \leq 0,05$)

Table 3. Microshear bond strenght values and standard deviation of adhesive systems modified with TiO$_2$ or TiO$_2$/Ag after three months of water storage

Experimental Groups	Adper^{TM} Single Bond	Adper^{TM} Scotch^{TM} Bond	Clearfil SE Bond
Control	19,3 (± 2,7)^{A}	22,4(± 3)^{B}	16,6(± 2,9)^{A}
1% TiO$_2$	18,7 (± 2,4)^{A}	22,8(± 4,8)^{B}	15,2(± 2,7)^{A}
2% TiO$_2$	17,9(± 2,7)^{A}	21,5(± 2,8)^{B}	17,9(± 3,4)^{A}
5% TiO$_2$	18,2(± 3,01)^{A}	21,9(± 3,9)^{B}	17,5(± 3,9)^{A}
1% TiO$_2$/Ag	19,5(± 3,2)^{A}	23,5(± 3,8)^{B}	16,5(± 3,1)^{A}
2% TiO$_2$/Ag	18,1(± 3,1)^{A}	24,2(± 3,9)^{B}	14,8(± 3,2)^{A}
5% TiO$_2$/Ag	17,2(± 4,5)^{A}	23,3(± 4)^{B}	14,1(± 5)^{A}

Means followed by the same uppercase letters show no statistically significant difference ($p \leq 0,05$)

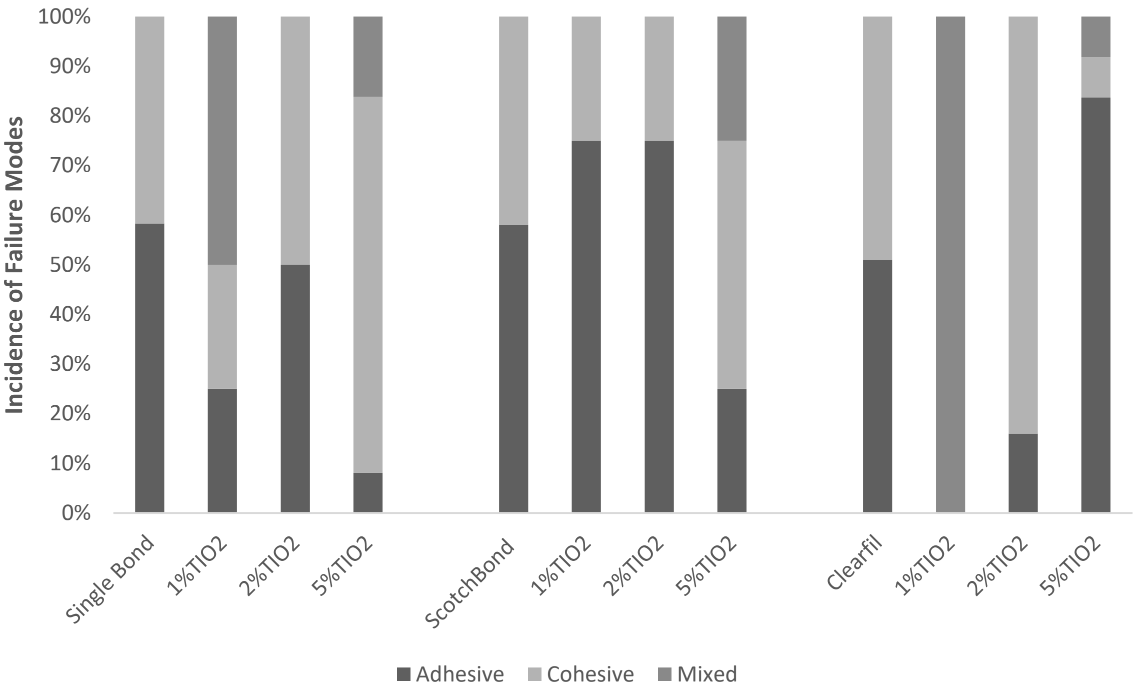

Figure 5. Incidence of mode of failure for the adhesive systems modified with TiO_2 nanoparticles.

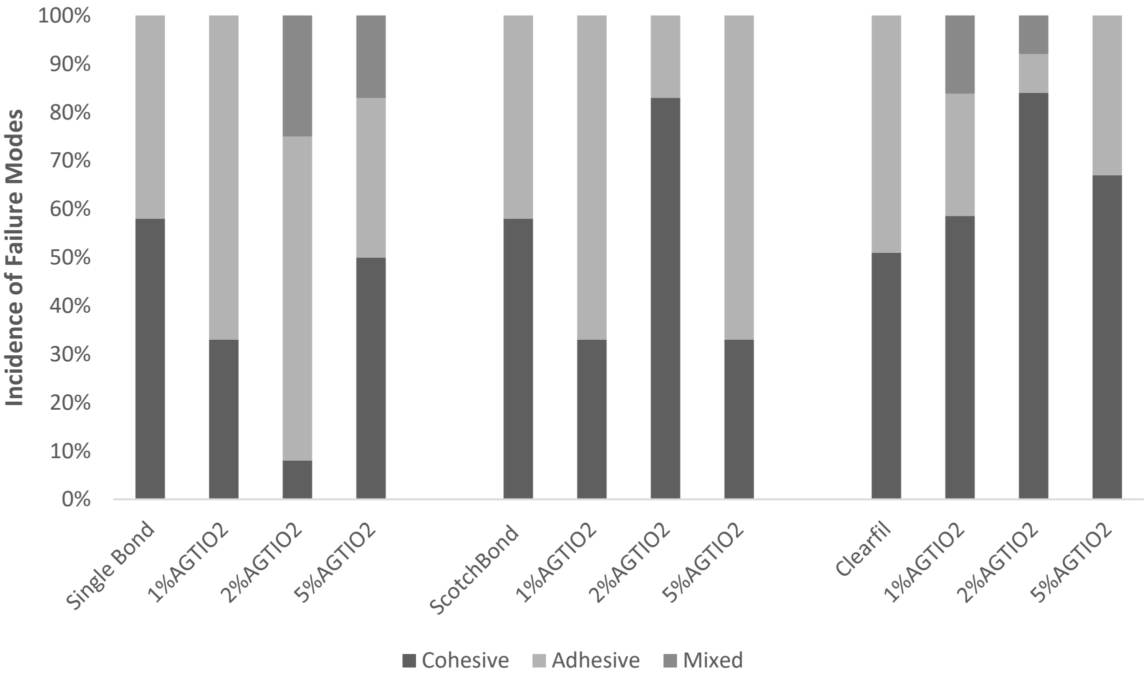

Figure 6. Incidence of mode of failure for the adhesive systems modified with $AgTiO_2$ nanoparticles.

4. Discussion

The current study aimed to evaluate the effects of modification of three adhesive systems using two different types of antibacterial nanoparticles (TiO_2 or TiO_2/Ag) at three concentrations on the micro-shear bond strength, at two different periods of time.

The results showed that the incorporation of a small amount (up to 5%wt) of nanoparticles did not cause any adverse effects on the bond strength mean values for all adhesive systems, even after three months of water storage. However, the mean values between the immediately test performed after specimen's preparation and the specimens stored in distilled water during three months showed statistically significant differences ($p<0.05$). This means that the microshear bond strength was significantly low for all adhesive systems, for both nanoparticles at all concentrations including also the control Groups after three months of water storage. Thus, the null hypothesis was partially rejected.

The micro-shear bond strength parameter is a widely used laboratory method to evaluate the quality of the bond strength between the dental substrate and the adhesive/composite resin restoration placed.[22,23] In order to fulfill the conditions necessary to obtain a successful composite restoration, many factors regarding the mechanical properties of the adhesive systems and composite resins need to be fully comprehended.[24,25] According to the literature, the acceptable microshear bond strength values using dentin as a substrate ranges from 17 to 20 MPa.[26,27]

Several studies have shown that the decrease on micro-shear bond strength values may be related to the performance of dental adhesive systems and the composite resin restoration.[28] Many factors can be related to this outcome, such as masticatory load, external forces, incorrect handling of the dental products, unexperienced operators, sensitive technique, temperature, humidity, possible previous treatments applied to the dentin surface and the fluids in the oral environment.[29-34] Therefore, the mechanical properties of adhesive systems should not be compromised, because this could provide many problems for both, direct and indirect restorations.

Studies regarding the mechanical properties analysis of dental adhesive systems modified with metallic oxide nanoparticles are still scarce.[35] The investigations found in the contemporary literature are conflicting. According to Barcellos et al (2016), adhesive systems containing ZnO did not show decreased on micro-tensile bond strength values under concentrations.[36] In fact, in some cases this modification seemed to improve mechanical properties, showing that this technique might be safe for future clinical trials.[37,38] However, another study by Hojati et al (2013) claimed that the increase of ZnO concentration led to a decrease on depth of cure.[39]

Modified orthodontic bonding agents have also been studied, showing that the incorporation of ZnO, silver and TiO_2 nanoparticles can significantly reduce the shear bond strength (SBS) values at a concentration of 1% in weight.[40] Nonetheless, another study using a different orthodontic bonding agent found that TiO_2 nanoparticles do not seem to affect the SBS values.[41] Furthermore, another study using a composite resin found similar results[18]. Just one study found in the literature assessed the mechanical property of a modified dental adhesive, using a micro-tensile bond strength test. The authors concluded that TiO_2 nanoparticles did not exert any influence on the SBS, which also supports our findings.[42] Although some results may differ, the explanation for the positive findings could be related to the possibility that the metallic oxide nanoparticles may reinforce the adhesive systems and in this way, provide a stronger and more reliable dental product.[43]

The storage time has been shown to have no effect on the SBS values of dental adhesive systems, regardless of the adhesive strategy implemented.[44] The correlation seems to be that as the storage time increases, the SBS values do not seem to significantly reduced. It is interesting to know that the current literature lacks to present a reasonable amount of studies that have evaluated the influence of nanoparticles in SBS, considering a long storage time, whether in artificial saliva or distilled water. Some studies on SBS did not show significant changes for Single Bond and ScotchBond Multipurpose after storage time. [45,46,47] Although the overall values in these studies decreased over time, these changes were not significant. This reduction may be a result of the water sorption to which the bonded areas are exposed, leading to the establishment of passive hydrolysis and a leaching effect.[48] The decrease of SBS values can result in micro-gaps formation in the adhesive interface, leading to micro-leakage and ultimately secondary caries and restoration's replacement.[49] It is known that the bonding mechanism of Single Bond relies on the penetration of resin monomers into the collagen matrix and the dentin tubules, which depends on the presence of moisture.[50,51] It is possible to rationalize that the lack of change on the SBS values may depend on the bonding procedure in order to the matrix does not collapse and the bond remains stable. A study performed by Abdalla et al (2007) showed that the bond strength remained stable after 12 months of water storage. Our findings are in according to these results.[52] The lack of changes on the bond strength can be explained by the formation of insoluble calcium salts that may prevent the loss of resin and deterioration of the adhesive interface overtime and the use of a natural aging mechanism, instead of thermocycling.[53] In addition, the presentation of the adhesive systems in separate flasks (primer and bond) may be related to the lack of loss of SBS values, as the use of a hydrophobic bond, following the use of a hydrophilic primer, may prevent the passage of water, helping the adhesive layer remain unaltered after water storage.

The failure modes in this study were divided into: adhesive, cohesive and mixed. In general, the most of specimens after microshear bond strength showing a cohesive/mixed failure mode. Additionally, no difference was found among the control and experimental Groups, considering both types of nanoparticles, all the concentrations (1, 2 and 5%) and the three types of dental adhesive systems, which corroborates to the results found in a study performed previously by Tedesco et al (2013).[57] The adhesive failure was not as frequent as the other two types and few cracks and alterations were seen on the dentin after the test performed on the specimens, which leads us to believe that the mechanical load used during the test was correctly applied.[58] The high bond strengths showed in the study were generally linked to a cohesive failure mode, which is in agreement with previous studies, including one that contained the determination of the bonding area .[58,59] It has been showed that when a high bond strength between the dental substrate and the composite is obtained, more frequent is the cohesive type of failure.[60] The frequency of cohesive failure can also be explained by the high bond strength shown by these adhesive systems and the unreliability of the method used to determine restricted bonding areas.[58]

The modification of dental adhesive systems with pure and doped antibacterial nanoparticles did not exert a negative influence on the mechanical property of the adhesive systems tested. Moreover, the water storage for three months also did not exert any influence on the micro-shear bond strength values observed.

A dental adhesive system containing antibacterial monomers is already commercially available and studies have shown a strong antimicrobial action, but also changes on their mechanical properties have been shown.[54,55] The clinical impact shown by this adhesive, on secondary caries, is still unprecise and uncertain.[56] Thus, new studies should be encouraged.

In this current study, the adhesive systems were modified by nanoparticles and the monomer composition was not changed. Maybe, this modification can become an alternative to control the development of secondary caries without compromise the adhesive bond strength, although we just evaluate the bond strength after three months.

5. Conclusion:

Considering the limitations of this study, the antibacterial nanoparticles, at three concentrations, did not promote statistically significant changes on the micro-shear bond strength values for the three commercially available dental adhesive systems. Water storage during three months also did not promote any further influences on their mechanical property. The modification of these dental adhesive systems can be a new perspective for the inhibition of secondary caries.

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5 CONCLUSÃO

De acordo com os resultados constatados em ambos os artigos contemplados nesta dissertação, podemos concluir que:

- todos os sistemas adesivos modificados com nanopartículas mostraram atividade antibacteriana estatisticamente significativa, para todas as concentrações e para ambos os tipos de partículas;
- o aumento da concentração e o tipo de sistema de adesivo não exerceu influência no poder da atividade antibacteriana;
- a modificação dos sistemas adesivos não comprometeu a resistência adesiva (imediata e após armazenamento) ao microcisalhamento, tampouco o grau de conversão;
- nenhuma relação ficou estabelecida entre a modificação de sistemas adesivos e o tipo de falha apresentada pelos espécimes.

Após a análise dos resultados, é possível afirmar que a associação entre materiais restauradores adesivos e nanopartículas inorgânicas metálicas podem consistir em uma possibilidade viável para a redução da contaminação bacteriana residual e ajudar a diminuir a prevalência da necessidade de troca de restaurações adesivas. Por mais que os estudos e a literatura atual nos deem um bom entendimento dessa área de estudo, diversas novas pesquisas devem ser conduzidas, especialmente na área clínica, para determinar se esses sistemas são utilizáveis corriqueiramente na prática clínica e se essa manutenção das propriedades mecânicas se mantém, principalmente em estudos com acompanhamento clínico mais longo.

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APÊNDICE A – METODOLOGIA ARTIGO 1

1. Teste Antibacteriano por Contato Direto

Em função da variabilidade que pode ocorrer na contagem das unidades formadoras de colônia (UFC) durante o teste do contato direto, para avaliar a confiabilidade das medidas obtidas intra e inter-examinador, foi realizado o estudo de reprodutibilidade, onde os pesquisadores 1 e 2 examinarão em duplicata 2 placas de petri após experimento de atividade antibacteriana pelo contato direto. As mesmas placas foram avaliadas imediatamente após a retirada da estufa (período de 48 horas), 1, 4 horas e 1 dia após a retirada da estufa. A concordância intra e inter-examinador foi estimada por meio do Coeficiente de Correlação Intraclasse. Em função do valor obtido, o grau de concordância entre os dados foi classificado segundo a proposta de Fermanian, em 1984 (35). Neste estudo foi considerado adequado um nível de concordância classificada como "Boa" (0,71 0,91).

A capacidade antibacteriana dos sistemas adesivos não modificados (sem nanopartículas, grupo controle) e com 1, 2 e 5% de nanopartículas de TiO_2 e TiO_2/Ag foi analisada por meio do teste do contato direto. Após reativar e realizar cultura inicial, em tubo Falcon contendo 5mL de BHI caldo (Brain Heart Infusion, Difco Laboratories, Becton Dickinson and Company, USA) + 1% de sacarose, 3 a 5 colônias de *Streptococcus mutans* foram inoculadas separadamente, sendo incubadas em atmosfera de microaerofilia por 18 horas a 37ºC (±1°C) para ser usado nos experimentos do contato direto; foi dessa forma preparada a suspensão bacteriana com absorbância padronizada. Após o crescimento, a cultura dos tubos com BHI caldo foi centrifugada a 3000rpm por 15min, proporcionando a obtenção de precipitado da biomassa. O sobrenadante foi descartado e a biomassa resuspensa em PBS (solução salina tamponada fosfatada) até atingir a absorbância padronizada de 0.08-1.0 lida em 660nm, turvação aproximada a 0.5 da escala de Mac Farland, com quantidade de células da ordem de 10^8 células bacterianas/mL. Coloração de Gram e observação em microscopia óptica foi feitas previamente ao experimento com o objetivo de confirmar a presença apenas das bactérias acima mencionadas observando-se a morfologia das mesmas.

Para a realização do teste do contato direto (n=108), corpos-de-prova circulares (4mm de diâmetro e 2mm de espessura) foi preparados utilizando-se matriz metálica e fotoativados por 40s. Posteriormente, 0.1mL dos sistemas adesivos foi dispensados em tubos Eppendorf (Tubes ® 3810X (Eppendorf AG, Hamburgo, Alemanha) envoltos em papel alumínio com auxílio de seringa hipodérmica (BD Plastipak^{TM}, BD, Franklin Lakes, NJ, EUA) e as nanopartículas (TiO_2/TiO_2Ag) foi adicionadas e misturadas em suas diferentes concentrações por peso (Nos sistemas Clearfil SE Bond e Scotch Bond Multipurpose somente o *bond* foi modificado). Após a fotoativação da resina composta, os diferentes sistemas adesivos foram aplicados na superfície dos corpos-de-prova com auxílio de *micro brush* Single Tim (Voco GmbH, Cuxhaven, Alemanha) em duas camadas e em volume de 8µL proporcionado com micropipeta (38), de acordo com as instruções dos fabricantes.

Após serem esterilizados em autoclave, os corpos-de-prova foi colocados em placas de poliestireno com 24 poços (Corning®, USA) e colocado 0,01mL da suspensão bacteriana (5µL da suspensão bacteriana de cada bactéria) sobre a superfície de cada corpo-de-prova e levados à e colocado 0,01mL da suspensão bacteriana (5µL da suspensão bacteriana de cada bactéria) sobre a superfície de cada corpo-de-prova e levados à estufa a 37ºC por 1 hora, para evaporação da água. Então, foram colocados 1000 µL de BHI caldo e incubação em atmosfera de microaerofilia em estufa a 37ºC (±1°C) por 12 horas.

Decorrido este período, foi pipetado 0,01mL de cada meio de cultura e semeado uniformemente sobre placas de Petri com BHI + ágar +1 % de sacarose, sendo incubadas por 48 horas a 37ºC e, em seguida, contadas as unidades formadoras de colônias (UFC/mL). O experimento foi realizado em triplicata.

2. Grau de Conversão

O teste de grau de conversão (GC) foi realizado para ambas as nanopartículas nas três diferentes concentrações (1, 2 e 5%) para os três sistemas adesivos, incluindo espécimes curados e não-curados. Oito espécimes foram feitos para cada grupo (n=64). Os sistemas adesivos foram modificados como descrito anteriormente e 10µL das amostras foram colocados sobre placa de vidro e fotoativados de acordo com as instruções dos fabricantes. A ponta da unidade de fotopolimerização foi colocada a 2mm de distância da superfície dos espécimes. Os espécimes foram então incubados em estuda por 24 horas a 37ºC. Após o tempo de armazenamento, a análise de FTIR foi feita utilizando uma unidade de reflectância total atenuada, usando as seguintes condições: 64 escaneamentos a uma resolução de $4cm^{-1}$. O grau de conversão de monômeros foi determinado pela comparação entre a intensidade de absorção das ligações carbono-carbono alifáticas (pico a $1638cm^{-1}$) e o componente aromático (pico a $1608cm^{-1}$), antes e após a polimerização dos espécimes. A porcentagem final foi calculada após subtrair a porcentagem final de ligações duplas de carbono remanescentes de 100%, de acordo com a seguinte formula:

$$GC\ (\%)\,\frac{1-\left(\frac{C=C_{alif\acute{a}tico}}{C-C_{arom\acute{a}tico}}\right)curado}{\frac{C=C_{alif\acute{a}tico}}{C-C_{arom\acute{a}tico}}\,n\tilde{a}o-curado}$$

Esquema 1 - Confecção de corpos-de-prova em resina composta

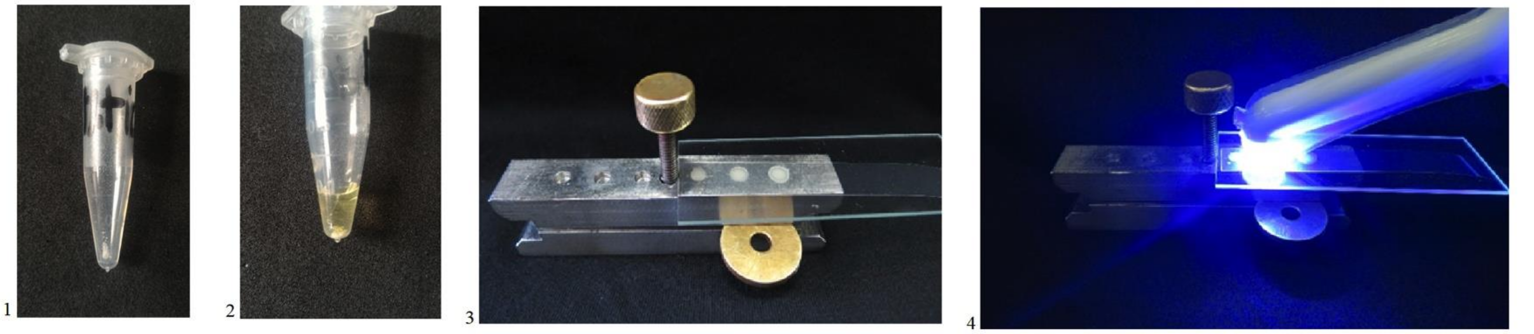

Figura 1. Nanopartículas de TiO2 em Eppendorf; Figura 2. Modificação dos sistemas adesivos com nanopartículas de TiO2; Figura 3. Confecção de espécime de resina com matriz metálica; Figura 4. Fotopolimerização do espécime de resina por 40 segundos.

Fonte: Arquivo pessoal do autor

Esquema 2 - Teste antibacteriano por contato direto

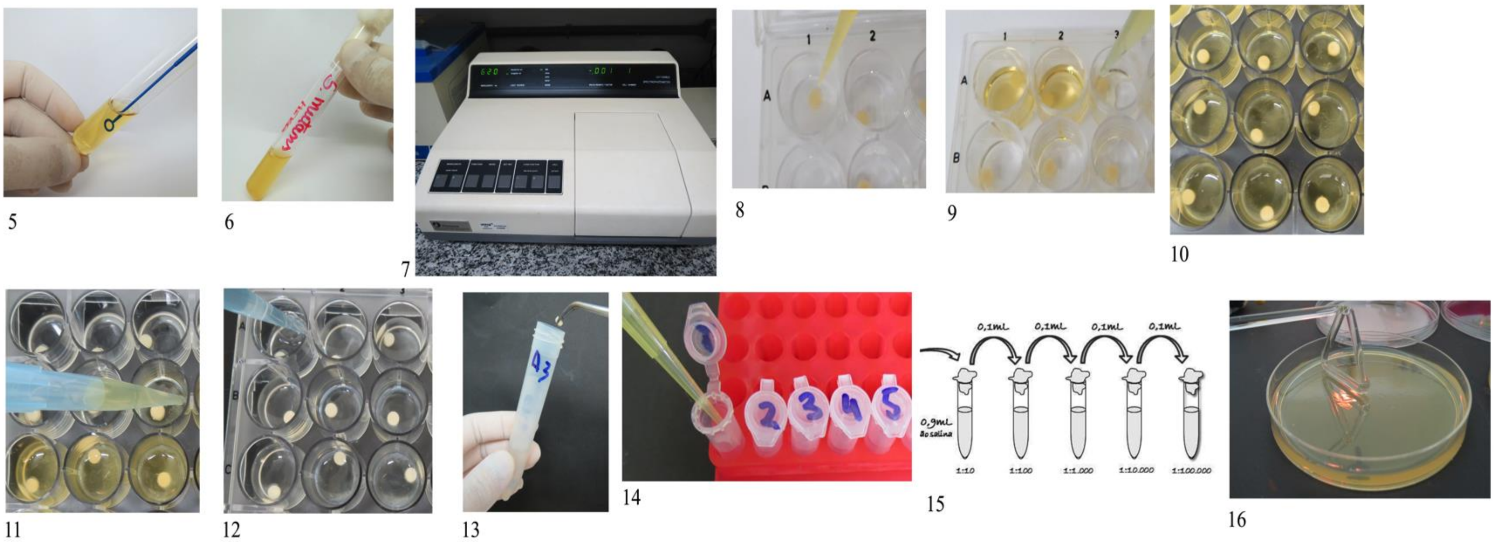

Figura 5. Colônias de S.mutans com 5mL de BHI Caldo; Figura 6. Biofilme após incubação por 24 horas a 37ºC/10%CO2; Figura 7. Padronização da suspensão bacteriana com espectofotômetro; Figura 8. 100uL da suspensão bacteriana sobre os espécimes e incubação por 1 hora; Figura 9. 900uL de BHI caldo e incubação por 18 horas; Figura 10. Biofilme formado sobre os espécimes; Figura 11. Remoção da solução bacteriana; Figura 12. Lavagem com PBS (100uL/3x); Figura 13. Espécimes transferidos para Tubo Falcon com 5mL de PBS; Figura 14. Adição de 100uL da suspensão em Eppendorf com 900uL de PBS; Figura 15. Diluição Seriada; Figura 16. Semeadura de 50uL de cada diluição em Placas de Petri e incubação por 24 horas.

Fonte: Arquivo pessoal do autor

APÊNDICE B – METODOLOGIA ARTIGO 2

1. Confecção dos espécimes e teste de microcisalhamento

Os corpos-de-prova foram confeccionados utilizando-se dentes bovinos, armazenados em solução de timol (5%) até o momento do preparo. Foram polidos com taça de borracha (KG Sorensen, São Paulo, SP, Brasil) e pasta de pedra pomes (SS White, Rio de Janeiro, RJ, Brasil) e água e armazenados em água destilada até o momento da utilização. As raízes foram removidas com auxílio de disco diamantado de dupla face (KG Sorensen, São Paulo, SP, Brasil) e micro motor em baixa rotação (Kavo do Brasil, Joinville, SC, Brasil), sendo a superfície de esmalte das coroas planificada com lixa de carbeto de silício de granulação 600 por 30 segundos em politriz (DP-10 Panambra, Struers, Ballerup, Dinamarca). Após, fragmentos da coroa (13 mm de comprimento x 7 mm largura) foram obtidos com o auxílio de cortadeira (IsoMet 1000, Buehler) sendo inseridos em base de resina acrílica autopolimerizável (1,2cm de altura x 2cm largura). A área adesiva foi delimitada com fita adesiva dupla face ácido resistente. A fita foi posicionada sobre papel contact preto e fragmentos de dimensões suficientes para recobrir os corpos-de-prova foram recortados. Em seguida, cada fragmento receberá 4 perfurações circulares com cerca de 0,7mm de diâmetro, realizadas com perfurador de borracha para isolamento absoluto, sendo a face adesiva aderida ao espécime e delimitando quatro superfícies de união nos corpos-de-prova. Finalmente, os espécimes foram feitos aplicando-se aos fragmentos os sistemas adesivos nas áreas delimitadas previamente, em seguida o compósito resinoso, em matrizes de Tygon (Tygon tubing, TYG-030, Saint-Gobain Performance Plastic, Maime Lakes, FL, USA) e fotoativados (LED Radii Plus, SDI, Australia), de acordo com as recomendações dos fabricantes. Os espécimes que foram testados após três meses foram armazenados em estufa a 37ºC com água destilada trocada semanalmente.

O estudo da resistência de união empregou a metodologia de ensaio de microcisalhamento. Após a remoção da matriz, cada cilindro de resina composta foi envolvido por fio de aço (0,2mm de diâmetro) acoplado à máquina de ensaio (EMIC DL2000, São José dos Pinhais, PR, Brasil), equipada com célula de carga de 500Kgf e alinhada cuidadosamente para permitir que a carga fosse aplicada o mais próximo possível da interface de união. A carga foi aplicada com velocidade de 0,5mm/min até a falha dos espécimes. O resultado foi dado pela força máxima (N) dividida pela área de união (mm^2) e expresso em MPa.

2. Análise de Falha

Após o teste de microcisalhamento, todas as interface resina/adesivo/dente foram avaliadas por microscópio estereoscópico. Isso com o objetivo de determinar o modo de fratura e classificá-lo dentre as seguintes formas: 1) adesiva, ao nível da interface de união, podendo haver presença de falha coesiva no adesivo. 2) coesiva em dentina, ruptura deste substrato. 3) coesiva no compósito, ruptura da resina composta. 4) mista, associação de falha adesiva e coesiva na dentina e ou na resina composta. Para a realização da análise, os espécimes foram seccionados com auxílio de lâmina diamantada, polidos com lixas de carbeto de silício de granulação decrescente (1000, 1200 e 1500) e limpos ultrasonicamente com água deionizada por 10 minutos. Após isso, foram descalcificados (ácido fosfórico a 50% por 5 segundos e lavados com água destilada) e desproteinizados (NaOCl a 2,5% por 10 minutos e lavados em água destilada).

Esquema 3 – Confecção de espécimes e teste de microcisalhamento

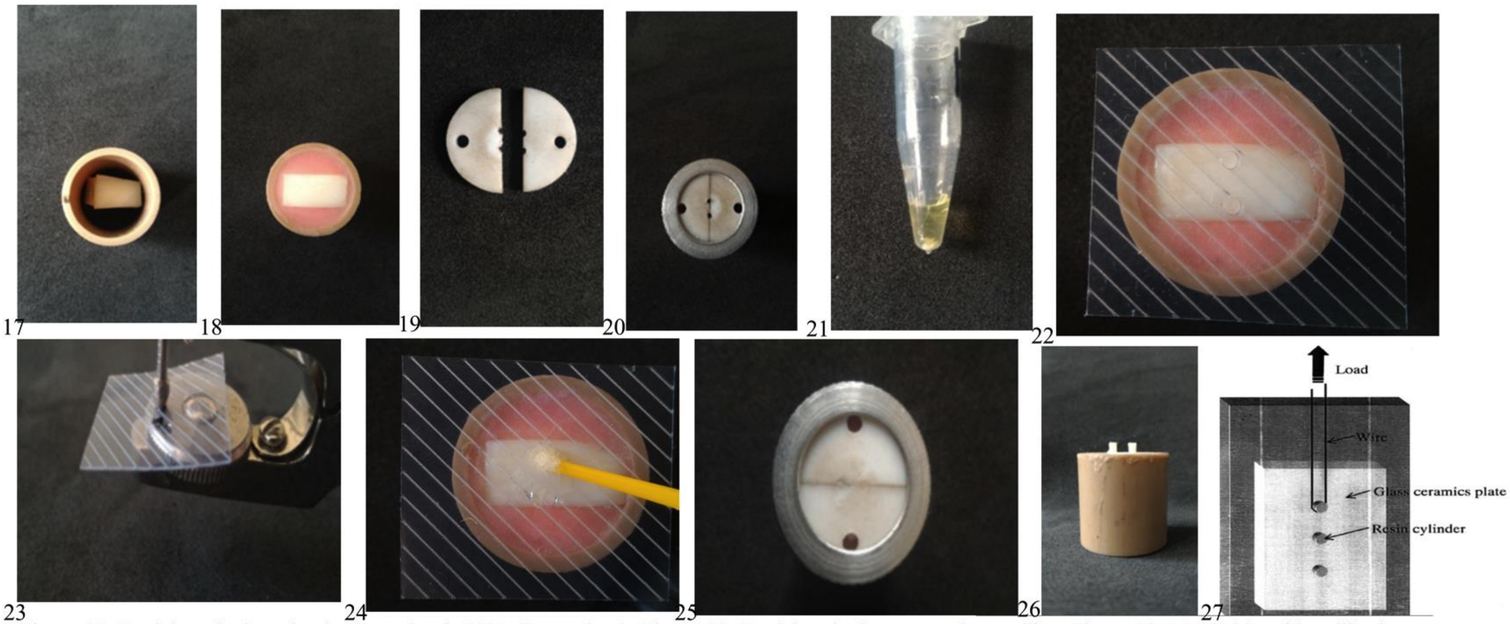

Figura 17. Espécime de dente bovino em tubo de PVC (2cm x 2cm); Figura 18. Espécime incluso em resina acrílica; Figura 19. Matriz bipartida utilizada na confecção de espécimes resina; Figura 20. Matriz bipartida e anel metálico estabilizante; Figura 21. Sistema adesivo modificado; Figura 22 e 23. Delimitação da área adesiva; Figura 24. Aplicação dos sistemas adesivos modificados nas áreas determinadas; Figura 25. Resina composta posicionada na matriz; Figura 26. Espécime finalizado; Figura 27. Esquema do teste de microcisalhamento realizado (Shimada, Y 2002)

Fonte: Arquivo pessoal do autor

ANEXO A – PROTOCOLO CEUA

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de Araraquara
FACULDADE DE ODONTOLOGIA

CERTIFICADO

Certificamos que o projeto intitulado ***"EFEITO DE ADESIVOS MODIFICADOS POR NANOPARTÍCULAS DE TIO2 DECORADAS OU NÃO POR PRATA EM DIFERENTES PROPRIEDADES"***, protocolo nº 28/2015, sob a responsabilidade do(a) **Prof(a). Dr(a). Alessandra Nara de Souza Rastelli** – que envolve a utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela **COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DA FACULDADE DE ODONTOLOGIA DE ARARAQUARA** em reunião de 29/02/2016.

Vigência do Projeto	Março/2017
Espécie/linhagem	Gado Nelore
Nº de animais	250 dentes
Peso/Idade	250 Kg – 5 anos
Sexo	
Origem	Mondelli Indústria de Alimentos S/A.

Paulo Sergio Cerri

Prof. Dr. PAULO SÉRGIO CERRI
Coordenador da CEUA

Araraquara, 21 de Setembro de 2017

VICTOR TRASSI FERNANDES SILVA DE SOUZA