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**NANOPARTÍCULAS DE PRATA EM CIMENTOS RESINOSOS:
PROPRIEDADES ANTIBACTERIANA E FÍSICO-QUÍMICAS**

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**Nanopartículas de prata em cimentos resinosos: propriedades
antibacteriana e físico-químicas**

ANA PAULA RODRIGUES DE MAGALHÃES

**NANOPARTÍCULAS DE PRATA EM CIMENTOS RESINOSOS: PROPRIEDADES
ANTIBACTERIANA E FÍSICO-QUÍMICAS**

Dissertação apresentada ao Programa de Pós-Graduação em Odontologia da Faculdade de Odontologia da Universidade Federal de Goiás para obtenção do título de Mestre em Odontologia, área de concentração Clínica Odontológica.

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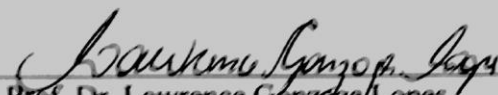
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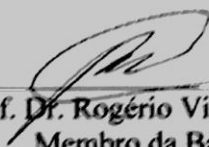
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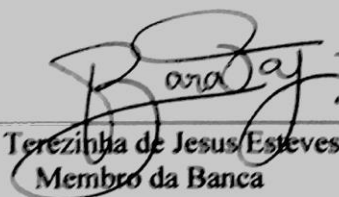
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*“Nobody said it was easy
No one ever said it would be so hard
Oh let’s go back to the start”
(Coldplay - “The Scientist”)*

RESUMO

O objetivo deste trabalho foi avaliar o efeito antibacteriano de cimentos resinosos com e sem adição de NAg, e a influência das mesmas na cor, sorção e solubilidade. Soluções de NAg foram incorporadas a dois cimentos resinosos de dupla polimerização (RelyX ARC - cor A1- e RelyX U200 – cor A2) em duas concentrações (0,05% e 0,07%) e os espécimes confeccionados em forma de discos foram testados quanto: à inibição de crescimento bacteriano (*Streptococcus mutans*) por meio de leituras de densidade ótica e análise em microscopia eletrônica de varredura (MEV); quanto à mudança de cor com um espectrofotômetro e quanto a sorção e solubilidade após armazenamento em água ou etanol 75% por 28 dias, de acordo com as normas da ISO 4049:2010. Os dados obtidos foram tabulados e submetidos a ANOVA e Tukey com $p=0,05$. A densidade ótica medida nos meios de cultura indicou crescimento bacteriano, mas a análise em MEV somente revelou a bactéria *Bacillus subtilis*. A NAg induziu mudança de cor significativa nos cimentos resinosos, especialmente no de cor mais clara (RelyX ARC). Os valores de solubilidade foram muito baixos para todos os grupos, enquanto a sorção aumentou com a adição de NAg. Os cimentos resinosos adicionados de NAg não apresentaram efeito antibacteriano contra *S mutans*. Também apresentaram mudança de cor perceptível e maior sorção do que os materiais sem NAg.

Palavras-chave: Prata. Cimentos resinosos. Produtos com ação antimicrobiana. Solubilidade. Testes de percepção de cor.

ABSTRACT

SILVER NANOPARTICLES IN RESIN LUTING CEMENTS: ANTIBACTERIAL AND PHYSIOCHEMICAL PROPERTIES

This study aims to evaluate the antibacterial effect of resin luting cements with and without NAg addition, and their influence on color, sorption and solubility. NAg were incorporated to two dual-cured resin luting cements (RelyX ARC - color A1 - and RelyX U200 - color A2 -) in two concentrations (0.05% and 0.07%) and the disc specimens obtained were tested for bacteria inhibition growth against *Streptococcus mutans* through optical density readings and scanning electron microscopy (SEM) analysis, for color changes with a spectrophotometer and for sorption and solubility after storage in water or 75% ethanol for 28 days, according to the ISO 4049:2010 specifications. Data were subjected to statistical analysis with ANOVA and Tukey ($p=0.05$). The optical density of the culture broth indicated bacterial growth, but on SEM the only species identified was *Bacillus subtilis*. NAg produced significant color change on the resin cements, especially in the lighter color (RelyX ARC). Solubility values were very low for all groups, while sorption values were raised with NAg addition. The resin luting cements with NAg addition did not show antibacterial activity against *S. mutans*. They also showed perceptible color change and higher sorption than the materials without NAg.

Keywords: Silver. Resin cements. Products with antimicrobial action. Solubility. Color perception tests.

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LISTA DE ABREVIATURAS E SIGLAS

Ag+	Íon prata
ATCC	American type culture collection
BHI	Brain-heart infusion
EDS	Espectroscopia de Energia Dispersiva
h	Hora
h	Altura
ISO	International standardization for organization
LabMic	Laboratório de Multiusuário de Microscopia de Alta Resolução
LED	Light-emitting diode
M1	Massa 1
M2	Massa 2
M3	Massa 3
MEV	Microscopia eletrônica de varredura
min	Minuto
mL	Mililitro
NAg	Nanopartículas de prata
°C	Grau Celsius
p	Nível de significância
pH	Potencial hidrogeniônico
r	Raio
ROS	Reactive oxygen species/ radicais livres
rpm	Rotações por minuto
SD	Desvio padrão
SEM	Scanning Electron Microscopy
UFG	Universidade Federal de Goiás
US FDA	United States Food and Drug Administration
UV	Ultravioleta
V	Volume

Wsl

Solubilidade

Wsp

Sorção

LISTA DE SÍMBOLOS

nm	Nanômetro
M	Molar
AgNO ₃	Nitrato de prata
NaBH ₄	Boridreto de sódio
Ag	Prata
H ₂	Hidrogênio
B ₂ H ₆	Diborano
NaNO ₃	Nitrato de sódio
mL	Mililitros
%	Porcentagem
mm	Milímetros
μl	Microlitros
mW/cm ²	Miliwatts por centímetro quadrado
CO ₂	Dióxido de carbono
L*	Luminosidade
a*	Saturação de cor no eixo vermelho-verde
b*	Saturação de cor no eixo azul-amarelo
ΔE	Mudança de cor
°	Graus
π	Pi

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1 INTRODUÇÃO

Os cimentos resinosos estão sendo cada vez mais utilizados na prática clínica para cimentação de facetas e lâminas de cerâmica odontológica, coroas, inlays e onlays (Gerdole *et al.*, 2008; Mese *et al.*, 2008). Isso se deve especialmente à sua propriedade de aderir, em conjunto com o sistema adesivo, à superfície dentária e às suas melhores propriedades mecânicas (Cekic-Nagas & Ergun, 2010; Marghalani, 2012). Atualmente, há uma variedade de cimentos resinosos disponíveis no mercado, considerando o modo de polimerização, a composição e o processo de adesão. Os cimentos resinosos de dupla polimerização são frequentemente utilizados, por terem componentes que promovem a polimerização química, especialmente sob restaurações indiretas estéticas de cerâmica ou resina (Cekic-Nagas & Ergun, 2010; Marghalani, 2012). Recentemente, os cimentos resinosos auto-adesivos foram criados para simplificar o processo de adesão e minimizar os passos operatórios envolvidos, como o tempo consumido durante os procedimentos de adesão, não havendo necessidade de tratamento prévio do tecido dentário (Marghalani, 2012).

Há séculos, a doença cárie dentária é considerada um sério problema de saúde pública (Espinosa-Cristóbal *et al.*, 2012). O início e o desenvolvimento dessa doença envolvem bactérias acidogênicas formadoras de biofilme, incluindo *Streptococcus mutans*, *Streptococcus sobrinus* e *Lactobacillus spp* (Allaker, 2010). Apesar da prevalência de lesões de cárie primária ter diminuído mundialmente desde o início da década de 80, a lesão de cárie secundária continua sendo um problema sem solução para a Odontologia (Bürgers *et al.*, 2009). O uso de agentes antimicrobianos pode, idealmente, prevenir a formação do biofilme dentário sem afetar significativamente o equilíbrio biológico da cavidade bucal (Allaker, 2010).

Em reabilitações com restaurações indiretas, ainda podem haver bactérias sob a restauração quando o tecido afetado pela cárie dentária não foi completamente removido ou quando há microinfiltração após a cimentação (Daugela *et al.*, 2008). Na área entre a cavidade preparada e a restauração aplicada, *S. mutans* pode crescer sem perturbações mecânicas, e a falta de oxigênio gradualmente favorece o crescimento dessa bactéria anaeróbica facultativa às custas das aeróbicas, cuja sobrevivência depende de adequado fornecimento de oxigênio (Daugela *et al.*, 2008). Com isso, um crescimento excessivo das colônias

de *S. mutans* sob a restauração induz o desenvolvimento de lesões cáries secundárias e reduz a longevidade da restauração (Daugela *et al.*, 2008). Portanto, o efeito de cimentos resinosos com propriedades anticariogênicas em microrganismos orais deve ser considerado.

Daugela *et al.* (2008) avaliaram a atividade antibacteriana de alguns cimentos para cimentação de peças protéticas sobre *S. mutans* e relatou que: os cimentos à base de fosfato de zinco apresentaram a mais forte atividade antibacteriana; que cimentos de ionômero de vidro, ionômero de vidro modificado por resina e cimentos de poliacrilato de zinco apresentaram moderada ação antibacteriana antes da geleificação; e os cimentos sem eugenol, com eugenol e resinosos não demonstraram nenhum efeito antibacteriano. Considerando a ampla indicação de cimentos resinosos, o desenvolvimento de um método para introduzir agentes antibacterianos nesses materiais seria interessante para tratamentos restauradores mais duradouros.

A prata tem um longo histórico de uso na medicina como um agente antimicrobiano e anti-inflamatório (Ahn *et al.*, 2009; Chaloupka *et al.*, 2010; Durner *et al.*, 2011; Peng *et al.*, 2012; Pérez-Tanoira *et al.*, 2012). Tem sido utilizada na purificação de água (Gangadharan *et al.*, 2010; Bao *et al.*, 2011; Dankovich *et al.*, 2011), implantes cardíacos, catéteres, cimentos ortopédicos, cicatrização de feridas e para tratamento de queimaduras (Chaloupka *et al.*, 2010; Durner *et al.*, 2011; Oei *et al.*, 2011; Pérez-Tanoira *et al.*, 2012). A prata tem sido cada vez mais utilizada devido a seu amplo espectro de atividade antimicrobiana, atuando sobre bactérias Gram-positivas e negativas, fungos, protozoários e alguns vírus (Monteiro *et al.*, 2009; Allaker, 2010). É também interessante por suas propriedades físico-químicas, baixo custo, baixa toxicidade e pouca resistência bacteriana (Peng *et al.*, 2012; Saengmee-anupharb *et al.*, 2013).

A prata tem sido incorporada a uma série de materiais em Medicina e Odontologia nas mais diversas formas: zeólitos de prata, íons, micropartículas e nanopartículas (Çinar *et al.*, 2009; Bürgers *et al.*, 2009; Peng *et al.*, 2012; Saengmee-anupharb *et al.*, 2013). As nanopartículas de prata (N_{Ag}) são aglomerados insolúveis de átomos de prata, menores do que 100 nm (Monteiro *et al.*, 2009; Cheng *et al.*, 2011; Kurek *et al.*, 2011). O seu tamanho é uma característica importante visto que quanto menores as partículas, maior a área da

superfície e portanto, menor a concentração de partículas necessária para eficácia (Cheng *et al.*, 2010; Oei *et al.*, 2011; Lu *et al.*, 2013; Zhang *et al.*, 2013). NAg menores do que 10 nm foram encontradas não só na superfície da membrana de células bacterianas, mas também dentro dessas bactérias (Morones *et al.*, 2005). O mecanismo de ação das NAg ainda não é totalmente elucidado (Allaker, 2010; Besinis *et al.*, 2012), mas está normalmente relacionado à liberação de íons prata (Ag^+), formação de radicais livres (ROS) e interação direta das partículas com as membranas microbianas (Morones *et al.*, 2005; Allaker, 2010; Chaloupka *et al.*, 2010; Durner *et al.*, 2011; Kurek *et al.*, 2011; Besinis *et al.*, 2012; Saengmee-anupharb *et al.*, 2013).

O efeito antibacteriano das NAg sobre *S. mutans* já foi relatado em alguns estudos nos últimos anos (Ahn *et al.*, 2009; Cheng *et al.*, 2011; Fan *et al.*, 2011; Nam, 2011; Melo *et al.*, 2012; Zhang *et al.*, 2013-a; Zhang *et al.*, 2013-b). Melo *et al.* (2012), Zhang *et al.* (2013-a) e Ahn *et al.* (2009) adicionaram NAg a adesivos e *primers*, mostrando um efeito antibacteriano promissor sobre *S. mutans*. Outra importante propriedade das NAg é a habilidade de reduzir a adesão de bactérias à superfície do material (Yoshida *et al.*, 1999-b; Bürgers *et al.*, 2009; Cheng *et al.*, 2011). Essa característica é ainda mais significativa quando consideramos o meio oral, os materiais resinosos e sua maior probabilidade de acumular biofilme quando comparados aos demais materiais dentários (Cheng *et al.*, 2012-b). NAg oferecem a possibilidade de controlar a formação desse e de outros biofilmes orais devido a suas propriedades biocidas e antiadesivas (Allaker, 2010). Apenas um estudo relatou a adição de NAg a cimentos resinosos (Magalhães *et al.*, 2012).

Entretanto, a adição de metais como a NAg em materiais resinosos deve ser feita na menor concentração de nanopartículas capaz de manter efeito antibacteriano suficiente sem afetar adversamente outras propriedades dos mesmos, como a cor e as propriedades mecânicas (Cheng *et al.*, 2011; Lu *et al.*, 2013; Fan *et al.*, 2011). Apesar da importância da estética para a Odontologia atual, há poucos relatos na literatura das mudanças de cor causadas pela adição de prata a materiais dentários (Cheng *et al.*, 2012; Fan *et al.*, 2011; Nam *et al.*, 2012; Uno *et al.*, 2013; Zhang *et al.*, 2013-a). Fan *et al.* (2011) relatou que ao desenvolver uma resina fotopolimerizável com efeito antimicrobiano, o aumento da concentração de prata diminuiu o grau de conversão. Quando materiais resinosos são imersos em

água, uma rápida liberação de monômeros não-reagidos acontece e simultaneamente, água é absorvida pela resina, ocupando os espaços deixados pelos monômeros, afetando adversamente suas propriedades mecânicas (Sideridou *et al.*, 2007; Fan *et al.*, 2010). Portanto, o estudo da sorção e solubilidade dos cimentos resinosos adicionados de NAg é importante do ponto de vista clínico.

Considerando a importância do desenvolvimento de um cimento resinoso com atividade antimicrobiana para a longevidade de restaurações indiretas, o objetivo desse estudo foi avaliar o efeito antibacteriano de cimentos resinosos com e sem adição de NAg e sua influência na cor, sorção e solubilidade desses materiais. As hipóteses nulas testadas foram: (1) não há diferença de efeito antibacteriano de cimentos resinosos com ou sem NAg; (2) não há diferença na cor obtida em cimentos com ou sem adição de NAg; (3) não há diferenças em solubilidade para os cimentos resinosos com ou sem NAg, e (4) não há diferença em sorção entre os cimentos resinosos com ou sem NAg.

2 MATERIAL E MÉTODOS

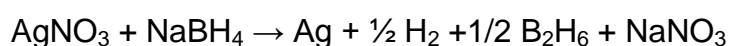
Dois cimentos resinosos de dupla polimerização foram testados nesse estudo: um convencional (RelyX ARC, 3M ESPE, St. Paul, MN, EUA; cor A1), e um auto-adesivo (RelyX U200, 3M ESPE, St. Paul, MN, EUA; cor A2) (Tabela 1). Em cada material, a NAg foi incorporada durante a manipulação do cimento. Os materiais foram manipulados de acordo com a ISO 4049:2010, no que diz respeito ao preparo dos espécimes.

Tabela 1. Cimentos resinosos utilizados nesse estudo (com lote e validade) e sua composição

Material	Fabricante	Composição
RelyX ARC (Lote: N47405 Validade: 01/2015)	3M ESPE, St. Paul, MN, EUA	Bis-GMA, TEGDMA, partículas de zircônia e sílica, pigmentos, amina, fotoiniciador, peróxido de benzoíla
RelyX U200 (Lote: 522958 Validade: 12/2014)		Monômeros de metacrilato com grupos de ácido fosfórico, metacrilato, partículas silanizadas e alcalinas, iniciadores, estabilizantes, aditivos reológicos, pigmentos

2.1 SÍNTESE E TESTE DAS NANOPARTÍCULAS DE PRATA

As NAg foram sintetizadas no Instituto de Física da Universidade Federal de Goiás (UFG) pela redução do nitrato de prata (0,001 M) (Sigma-Aldrich, St. Louis, MO, EUA) com o boridreto de sódio (0,002 M) (Sigma-Aldrich, St. Louis, MO, EUA) em baixas temperaturas controladas em agitador magnético (TE 080, Techal, São José dos Campos, SP, Brasil). Esse método pode ser descrito pela reação:



A solução obtida de NAg em água foi dividida em frascos de 45 mL e cada frasco foi adicionado de 3 mL de uma solução 0,75 M de cloreto de sódio (Sigma-Aldrich, St. Louis, MO, EUA) para desestabilizar a solução e estimular a precipitação das NAg. Então os frascos foram tampados e levados a uma centrífuga (Elektra, Laborline, Osasco, SP, Brasil) por 5 min a 3000 rpm; ao fim desse processo toda a prata estava depositada no fundo do frasco. A água foi removida e 1,5 mL de

hidroxietil metacrilato (HEMA, Sigma-Aldrich, St. Louis, MO, EUA) foi adicionado ao frasco. Esse processo foi repetido duas vezes para obter uma solução HEMA-NAg de 0,18% e três vezes para obter uma solução de 0,27% em concentração de NA_g. As soluções finais foram colocadas em agitador ultrassônico (USC-2800, Unique, Indaiatuba, SP, Brasil) por 3 ciclos de 10 minutos para completa dispersão do metal no material.

Para confirmar o efeito antibacteriano dessa solução de HEMA-NA_g, foi realizado um teste preliminar de Difusão em Ágar. Três placas de petri com 20 mL de Infusão Cérebro-Coração (BHI, Difco Laboratories, Detroit, MI, EUA) foram inoculadas com 0,1 mL de *S. mutans* (ATCC 25175). Três soluções foram testadas: HEMA sem prata, HEMA-NA_g 0,18% e HEMA-NA_g 0,27%, com discos de papel esterilizados embebidos nas mesmas (n=3). As placas foram incubadas a 37°C por 48h, e após esse período, a presença ou não de halos de inibição foi verificada. Halos foram identificados para as três soluções, sendo mais evidentes para os grupos com adição de NA_g, confirmando a inibição do crescimento bacteriano; enquanto o halo observado para a solução de HEMA foi apenas um halo de difusão, sem inibição de crescimento (Apêndice A).

2.2 CONFECÇÃO DOS ESPÉCIMES

Esse estudo foi realizado de acordo com as normas da ISO 4049:2010, no que se refere à confecção dos espécimes. Durante todo processo de confecção, temperatura ($25\pm 1^{\circ}\text{C}$) e umidade ($50\pm 2\%$) foram controlados pelo uso de um termo-higrômetro (Termo-Hygro Clock, Corel, São Paulo, SP, Brasil).

Cimentos resinosos pertencentes aos grupos sem NA_g foram proporcionados e manipulados de acordo com as especificações do fabricante, inseridos em uma matriz de aço inoxidável com geometria interna circular (1,0 mm espessura, 6 mm diâmetro) confinada entre duas tiras de poliéster (3M ESPE, St. Paul, MN, EUA). Aos espécimes com NA_g foram adicionados 10 µl de solução HEMA-NA_g previamente à manipulação e foram então manipulados e inseridos na mesma matriz de aço. Todos os espécimes foram fotopolimerizados com um aparelho à base de LED (Emitter, Schuster, Santa Maria, RS, Brazil) com a técnica de fotopolimerização contínua (600 mW/cm^2 , por 60 s). O aparelho foi ligado em um estabilizador de energia e a densidade de potência foi previamente verificada em um

radiômetro (Kerr Corporation, Orange, CA, EUA). Após a fotoativação, os espécimes foram retirados da matriz de aço com o auxílio de um instrumento esterilizado.

Os cimentos foram divididos em três grupos cada, totalizando 6 grupos experimentais: G1- RelyX ARC, G2- RelyX ARC com 0,05% de NAg, G3- RelyX ARC com 0,07% de NAg, G4- RelyX U200, G5- RelyX U200 com 0,05% de NAg e G6- RelyX U200 com 0,07% de NAg (Figura 1).

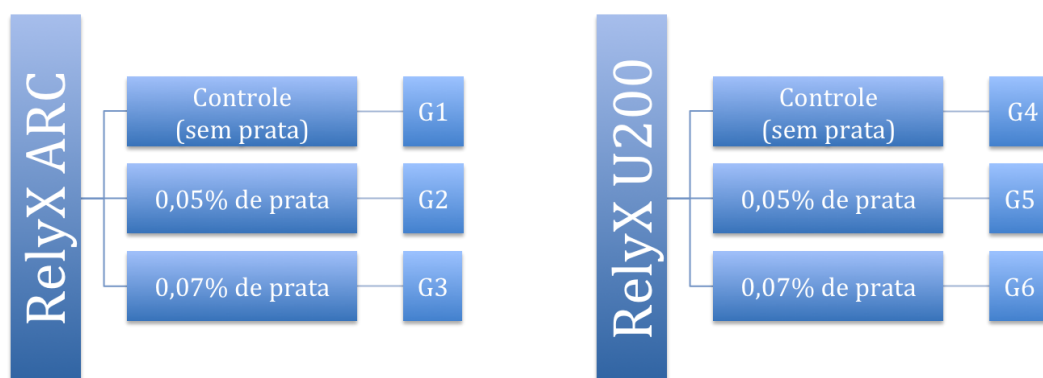


Figura 1 – Diagrama representando os grupos experimentais do estudo.

2.3 TESTE ANTIBACTERIANO

Foi utilizada a cepa padrão de *Streptococcus mutans* obtida da American Type Culture Collection (ATCC 25175), inoculada em 7 mL de caldo de BHI (Brain-heart infusion; Difco Laboratories, Detroit, MI, EUA) e incubada a 37°C, por 24 horas. Após o período de incubação, as células bacterianas foram ressuspensas em 4 frascos com 10 mL de BHI, em uma concentração final de aproximadamente 3×10^8 células/mL em cada frasco, ajustada para o padrão de turbidez 1 de McFarland.

Para o teste antibacteriano, foram utilizados 108 espécimes, armazenados em uma placa de Petri com tampa, esterilizada e devidamente identificada para cada grupo. Três espécimes de cada grupo foram separados antes da contaminação e transferidos para um tubo de ensaio com BHI esterilizado compondo o grupo controle negativo. Dez mililitros de suspensão bacteriana foram adicionados a cada placa para contaminação dos 15 espécimes restantes com *S. mutans*. A contaminação foi realizada nos períodos de tempo de 1 min, 5 min, 1 h, 6 h e 24 h, sendo n=3.

Após cada período de contaminação, os espécimes foram retirados das placas e colocados em frascos com 10 mL de água deionizada esterilizada para lavagem com agitação em vórtex (modelo AP 56, Phoenix, Araraquara, SP, Brasil), por 1 minuto. Foram, então, recondicionados em tubos de ensaio com BHI esterilizado e incubados por 48 horas, a 37°C, em estufa bacteriológica (modelo B2CBE, Deleo, Porto Alegre, RS, Brasil). Decorrido esse período, 2 mL de amostra de cada um dos meios foram coletados, colocados em frascos de acrílico transparentes e analisados quanto à densidade ótica (turvação), utilizando para isso o espectrofotômetro UV (Spectrophotometer Model Nova 1600 UV, Piracicaba, SP, Brasil) ajustado para leitura em comprimento de onda $\lambda=600$ nm, adotando-se como padrão a escala 1 de McFarland, que correspondeu à absorbância de 0,137 nm após leitura zero de BHI esterilizado. Outros 2 mL de meio de cultura foram recolhidos para teste de coloração de Gram para verificar a co-presença de outras bactérias além da espécie alvo, *S. Mutans*.

A seguir, os espécimes foram retirados dos meios de cultura e irrigados individualmente com 10 mL de água destilada esterilizada e armazenados em solução fixadora (2,5% glutaraldeído e tampão fosfato pH 7,2; 0,1 M), por 7 dias, para posterior análise superficial em microscopia eletrônica de varredura (MEV).

2.4 MICROSCOPIA ELETRÔNICA DE VARREDURA

Decorrido o período de fixação prosseguiu-se ao processo de desidratação dos espécimes. Eles foram submetidos a banhos de etanol em diferentes concentrações: 30 min em etanol a 70%, 30 min em etanol a 95% e, por fim, 30 min em etanol a 99%. Em seguida foi realizado o aquecimento em ponto crítico de CO₂ para completar o processo de desidratação (Autosamdri®, 815, Series A, Raleigh, NC. EUA).

Após a desidratação, os espécimes foram fixados em *stubs* metálicos e posicionados no metalizador (Denton Vacuum, Desk V, Moorestown, NJ, EUA), para deposição superficial de fina camada de ouro. Após a metalização, os espécimes foram examinados em MEV de pressão variável, em alto vácuo (Jeol, JSM – 6610,

equipado com EDS, Thermo scientific NSS Spectral Imaging. São Paula, SP, Brasil), para verificar a presença ou ausência de *S. mutans* na superfície de cada espécime. As amostras também foram analisadas por EDS (Espectroscopia de Energia Dispersiva) para confirmação da presença da NAg na superfície dos espécimes.

A leitura foi realizada em toda a superfície do espécime, por um único examinador e foram coletadas duas imagens de cada espécime, em diferentes aumentos, de x2500 e x5000.

2.5 AVALIAÇÃO DA ALTERAÇÃO DE COR

Para avaliar a alteração de cor foram utilizados 60 espécimes, sendo n=10 para cada grupo. A mensuração da cor dos espécimes foi realizada com espectrofotômetro (Easyshade, Vita, Alemanha) de forma que G1 e G4 serviram como controle, para comparação de cor com os cimentos resinosos experimentais G2 e G3; e com G5 e G6, respectivamente.

A cor foi determinada utilizando os parâmetros do sistema CIELab (L^* a^* b^*), no qual L^* indica a luminosidade em que a média varia de 0 (preto) a 100 (branco) e a^* e b^* indicam o matiz, sendo que o a^* representa a saturação no eixo vermelho-verde e o b^* no eixo azul-amarelo. Com este sistema todas as cores podem ser especificadas com as coordenadas L^* , a^* , b^* .

Os espécimes foram submetidos a três leituras da cor sobre bloco de fundo padrão branco (Standard For 45°, 0° Reflectance and Color Gardner Laboratory Inc., Maryland, EUA), e a cor final foi definida pela média das três medidas obtidas. As amostras sob a ponta do espectrofotômetro receberam a luz proveniente de 30 lâmpadas LED, com 10 cores diferentes, dispostas de forma circular, com incidência de 45° com a superfície do material. Esse feixe foi refletido em 0° de volta para o aparelho, que captou e registrou os valores de L^* , a^* e b^* de cada amostra.

A alteração de cor foi determinada pela diferença (ΔE^*) entre as coordenadas obtidas das amostras de G1 e G4 e as amostras de G2, G3 e G5, G6, respectivamente. A variação de cor, ΔE^* , foi usada para representar a diferença de cor e foi calculada a partir da fórmula:

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{0.5},$$

onde $\Delta L^* = L_1^* - L_0^*$;

$\Delta a^* = a_1^* - a_0^*$;

$\Delta b^* = b_1^* - b_0^*$.

2.6 TESTE DE SORÇÃO E SOLUBILIDADE

Os testes de sorção e solubilidade foram realizados de acordo com as especificações padrão da ISO 4049:2010, exceto para as dimensões do espécime, os tipos de permeantes e o tempo de armazenagem.

Inicialmente, a espessura de cada espécime foi medida em quatro pontos e no centro do espécime; e o diâmetro foi obtido em dois pontos com um paquímetro digital (Mitutoyo Corporation, Tokyo, Japão) para cálculo do volume dos espécimes segundo a fórmula: $V = \pi r^2 \cdot h$, onde r corresponde ao raio e h a altura do espécime.

Então, 60 novos espécimes foram confeccionados ($n=10$) e colocados em vidros de cor âmbar abertos (Saint Gabain, São Paulo, SP, Brasil) e armazenados em um dessecador (Vidrolabor, São Paulo, SP, Brasil) contendo sílica gel azul (Vetec®, Rio de Janeiro, RJ, Brasil) em uma estufa biológica (FANEM LTDA- modelo 002 CB, Guarulhos, SP, Brasil) a $37 \pm 1^\circ\text{C}$ por 22 h. Foram, então, removidos, mantidos a $23 \pm 1^\circ\text{C}$ por 2h e pesados em uma balança analítica (Marte AY 220, Santa Rita do Sapucaí, MG, Brasil) calibrada para 0,0001 g. Os espécimes foram em seguida recolocados no dessecador e esse ciclo foi repetido a cada 24 h até que uma massa constante M_1 fosse obtida, ou seja, até que a perda de massa total de cada espécime não fosse superior a 0,1 mg a cada período de 24 h.

Após obtenção de M_1 , os espécimes foram devolvidos a seus vidros identificados e 15 ml de água deionizada ou etanol 75% foram adicionados com pipetas manuais (uma para cada permeante), totalizando 5 espécimes de cada grupo para cada permeante. Os vidros foram tampados e recolocados na estufa, onde foram mantidos a $37 \pm 1^\circ\text{C}$ por 28 dias.

Após esse período, os espécimes foram novamente removidos do dessecador, mantidos a $23 \pm 1^\circ\text{C}$ por 2 horas, removidos dos vidros, lavados em água corrente por 15 s, colocados numa cubeta estéril (Duflex, Rio de Janeiro, RJ, Brasil) por 1 min e pesados para obtenção de M_2 . Finalmente os espécimes foram

devolvidos aos vidros e ao dessecador, sem permeantes, e pesados até obtenção de massa constante M3 sob o mesmo ciclo descrito para obtenção de M1.

Os valores de sorção e solubilidade foram calculados em microgramas por milímetro cúbico ($\mu\text{g}/\text{mm}^3$) usando as seguintes equações (ISO 4049:2010):

$$W_{sp} = \frac{M2 - M3}{V}$$

$$W_{sl} = \frac{M1 - M3}{V}$$

Onde W_{sp} é sorção, W_{sl} é solubilidade, M1 é a massa condicionada antes da imersão em permeantes, M2 é a massa do espécime após imersão nos permeantes, M3 é a massa recondicionada e V é o volume do espécime.

2.7 TRATAMENTO E INTERPRETAÇÃO DOS DADOS

Os resultados encontrados foram descritos pelos parâmetros de média e desvio-padrão. Devido à distribuição normal das variáveis (Kolmogorov-Smirnov e Shapiro-Wilk), para verificar o efeito das diferentes variáveis, foi utilizada a Análise de Variância (ANOVA) e para as comparações múltiplas foi usado o teste de Tukey. As análises foram realizadas no programa IBM SPSS Statistics 19.0 for Windows (SPSS Inc., Chicago, IL, EUA), com um nível de significância de 5% ($p < 0,05$).

3 ARTIGO

Silver nanoparticles in resin luting cements: antibacterial and physiochemical properties

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Short title: Silver nanoparticles in resin luting cements

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Silver nanoparticles in resin luting cements: antibacterial and physiochemical properties

Abstract

Objective: This study aims to evaluate the antibacterial effect of resin luting cements with and without silver nanoparticles (NAg) addition, and their influence on color, sorption and solubility. Materials and methods: NAg were incorporated to two dual-cured resin luting cements (RelyX ARC - color A1 - and RelyX U200 - color A2 -) in two concentrations (0.05% and 0.07%) and the disc specimens obtained were tested for bacteria inhibition growth against *Streptococcus mutans* through optical density readings and scanning electron microscopy (SEM) analysis, for color changes with a spectrophotometer and for sorption and solubility after storage in water or 75% ethanol for 28 days, according to the ISO 4049:2010 specifications. Data were subjected to statistical analysis with ANOVA and Tukey ($p=0.05$). Results: The optical density of the culture broth indicated bacterial growth, but on SEM the only species identified was *Bacillus subtilis*. NAg produced significant color change on the resin cements, especially in the lighter color (RelyX ARC). Solubility values were very low for all groups, while sorption values were raised with NAg addition. Significance: The resin luting cements with NAg addition did not show antibacterial activity against *S. mutans*. They also showed perceptible color change and higher sorption than the materials without NAg.

Keywords: Silver. Resin cements. Products with antimicrobial action. Solubility. Color perception tests.

Introduction

Dental caries has been a serious health problem for centuries. [1] The disease initiation and development involves acidogenic bacteria, including *Streptococcus mutans*, *Streptococcus sobrinus*, and *Lactobacillus spp.* [2] Although the prevalence of primary caries has been on decline worldwide since early 1980s, secondary caries remains an unresolved problem in Dentistry. [3] The use of antimicrobial agents should ideally prevent biofilm formation without markedly affecting the biological equilibrium within the oral cavity. [2]

When applying indirect restorations, bacteria may still be present under the restoration when the tissue affected by caries is not fully removed or if there is microleakage present after cementing. [4] This may cause an increase of bacterial colonies, especially *S. mutans*, under the restoration inducing secondary caries and particularly reducing its longevity. [4] Thus, the effects of luting cements with anticariogenic properties on oral microorganisms have to be considered.

Silver has a long history of use in medicine as an antimicrobial and anti-inflammatory agent. [5-9] It has been incorporated to several materials in Medicine and Dentistry in different forms: silver zeolites, ions, microparticles, and nanoparticles. [3,8,10,11] Silver nanoparticles (NAg) are clusters of silver atoms that are insoluble and smaller than 100 nm in size. [12-14] Their size is an important characteristic because smaller particles give rise to higher specific surface areas, and therefore reduce the particle concentration necessary for efficacy. [12,15-17] NAg offers the possibility to control the formation of these and other oral biofilms through the use of nanoparticles with biocidal, anti-adhesive, and delivery abilities. [2] Only one study has been reported so far with NAg addition to resin luting cements. [18]

However, the addition of a material as NAg in dental composites has to be done in the lowest concentration of nanoparticles capable of maintaining sufficient antibacterial effect without adversely affecting other material properties, like color and mechanical properties.

[12,17,19] In spite of the importance of esthetic for today's Dentistry, there are few reports in the literature of the color changes caused by silver addition in dental materials. [19-21] Fan *et al.* [19] reported that when developing an antimicrobial light-cured resin, as the concentration of NAg increased the degree of cure decreased. When composites are immersed in water, a rapid elution of the unreacted monomers takes place and simultaneously water is absorbed by the resin, occupying the holes left by the monomers, and affecting adversely their mechanical properties. [19,22] Therefore, the water sorption and solubility behavior of resin luting cements added by NAg is very important from the clinical point of view.

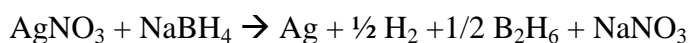
Considering the importance of the resin luting cements antibacterial activity for the indirect restorations longevity, this study aimed to evaluate the antibacterial effect of resin luting cements with NAg addition, and their influence on color, sorption and solubility. The null hypotheses were: (1) there is no difference in antibacterial effect of resin luting cements with or without the addition of NAg (2) there is no difference in the color obtained in luting cements added or not with NAg; (3) there is no difference in solubility for resin luting cements with or without NAg addition, and (4) there is no difference in sorption between luting cements with and without NAg.

Materials and Methods

Two dual-cured resin luting cements were tested in this study: one conventional (RelyX ARC, 3M ESPE, St. Paul, USA; color A1), one self-adhesive (RelyX U200, 3M ESPE, St. Paul, USA; color A2) (Table 1). Each material had silver nanoparticles incorporated during manipulation. The materials were handled according to the guidelines of ISO 4049:2010, in regards to specimens preparation.

Silver nanoparticles production and test

The NAg were prepared in the Physics Institute of Federal University of Goiás (UFG), by reduction of silver nitrate (0.001 M) (Sigma-Aldrich, St. Louis, USA) with sodium borohydride (0.002 M) (Sigma-Aldrich, St. Louis, USA) in controlled low temperature and magnetic stirrer (TE 080, Techal, São José dos Campos, Brazil). This method can be described by the reaction:



The solution obtained was added with 3 mL of a 0.75 M sodium chloride solution (Sigma-Aldrich, St. Louis, USA), then they went through a centrifugation process (Elektra, Laborline, Osasco, Brazil) for 3000 rpm for 5 minutes, until the NAg was in the bottom of the flask. The water was removed and 1.5 mL of hydroxy-ethyl methacrylate (HEMA, Sigma-Aldrich, St. Louis, USA) was added to the flask. This process was repeated twice to obtain a HEMA-NAg solution of 0.18% and three times to obtain a solution of 0.27% in NAg concentration. The final solutions were shaken in an ultrasonic apparatus (USC-2800, Unique, Indaiatuba, Brazil) for 3 cycles of 10 minutes for complete dispersion of the metal in the material.

To confirm the antibacterial effect of this HEMA-NAg solution the Agar Diffusion Test was performed. Three Petri plates with 20 mL of Brain Heart Infusion (BHI, Difco Laboratories, Detroit, USA) were inoculated with 0.1 mL of *S. mutans* (ATCC 25175). Three

solutions were tested: HEMA without NAg, HEMA-NAg 0.18% and HEMA-NAg 0.27%, with three sterile paper discs immersed in this solution. The plates were incubated at 37°C for 48 h, after that the presence or absence of bacterial inhibition halos were observed. Halos were identified for all three solutions tested, being more evident for the groups with NAg added, confirming the inhibition of bacterial growth; while only a diffusion halo was observed for the HEMA without NAg solution, not representing inhibition.

Specimens preparation

This study was performed in compliance with ISO 4049:2010 [23] standard specifications.

Resin luting cements that belonged to the groups without NAg were manipulated according to manufacturer's specifications, placed in a circle shaped stainless steel mold (1.0 mm thick, 6.0 mm diameter), and confined between two opposing polyester strips (3M ESPE, St. Paul, MN, USA). The ones with NAg addition had 10 µl of HEMA-NAg solution added prior to manipulation, and were then manipulated and placed in the same mold. All specimens were cured using a light-curing unit based on light emitting diodes (LED) (Emitter, Schuster, Santa Maria, Brazil) with continuous polymerization technique (600 mW/cm², for 60 s). Light irradiance was checked before each photopolymerization with a radiometer (Kerr Corporation, Orange, USA) to ensure consistency of light output.

The cements were divided in three groups each, a total of 6 groups: G1- RelyX ARC, G2- RelyX ARC with 0.05% of NAg, G3- RelyX ARC with 0.07% NAg, G4- RelyX U200, G5- RelyX U200 with 0.05% NAg, and G6- RelyX U200 with 0.07% NAg.

Antibacterial test

Standard strain of *Streptococcus mutans* (ATCC 25175) was obtained from American Type Culture Collection. The strain was inoculated into 7 mL of BHI broth (Brain-heart infusion, Difco Laboratories, Detroit, USA) and incubated at 37°C for 24 h. After the

incubation period, the bacterial cells were resuspended in four test-tubes with 10 mL of BHI broth in a final concentration of approximately 3×10^8 cells/ml in each tube in accordance to the turbidity standard 1 of McFarland scale.

One hundred and eight (108) specimens were used to the antibacterial test,. The specimens belonging to each group were stored in a Petri dish appropriately identified. Three specimens of each group were kept without contamination with the bacteria, the negative control. A quantity of 10 mL of bacterial suspension was added to each dish for contamination of the different groups specimens with *S. mutans*. Contamination was carried out at different time periods for each group: 1 min, 5 min, 1 h, 6 h, and 24 h (n = 3). Then, the specimens were placed in test tubes with 10 mL of deionized sterile water for washing with vortexing (Model AP 56, Phoenix, Araraquara, Brazil) for 1 minute. In sequence, they were reconditioned to test tubes with sterile BHI broth and incubated for 48 hours at 37°C under 5% CO₂ in a bacteriological incubator (model B2CBE , Deleo, Porto Alegre, Brazil). After that, 2 mL of broth sample were collected from each tube and their optical density (turbidity) were read by UV spectrophotometer (UV Spectrophotometer Model 1600 New, Piracicaba, Brazil) at $\lambda = 600$ nm, adopting as a standard the level 1 of McFarland scale, which corresponded to the absorbance of 0.137 nm after reset the device for BHI sterilized reading. Another 2 mL of culture broth was collected for Gram stain test to verify the co-presence of non-targeted bacteria along with the targeted one, *S. mutans*.

After collecting the culture broth, the specimens were individually irrigated with 10mL of sterile distilled water and stored in fixative solution (2.5% glutaraldehyde and buffer phosphate pH 7.2, 0.1M) for 7 days for subsequent analysis of presence/absence of bacteria on the specimens surface by Scanning Electron Microscopy (SEM) and Energy Dispersive Spectroscopy (EDS) (Jeol, JSM – 6610, Thermo scientific NSS Spectral Imaging, São Paulo, Brazil) in the Microscopy Laboratory of UFG (LabMic).

Evaluation of color changes

Sixty specimens were used to evaluate the color change, being n=10 for each group. The measurement of the color of the specimens was performed with an Easyshade spectrophotometer (Vita, Germany). For each specimen three readings were made using the parameters CIELab system (L* indicates lightness, a* represents the color saturation in red-green axis b* means color and saturation in blue-yellow axis). The reading for the determination of color parameters was always performed at the central point of the specimen in the same environment with the same lighting conditions, against a standard white background (Standard for 45o, 0o Reflectance and Color Garder Laboratory Inc., Maryland, USA).

The influence of NAg in color was directly expressed by ΔE^* values that indicate the color difference between an initial condition collected on G1 and G4 specimens, and the other evaluated condition collected on G2 and G5 (ΔE_1^*) and also in G3 and G6 (ΔE_2^*) specimens, respectively. The ΔE^* represents the total color change and was calculated from the formula:

$$\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{0.5},$$

$$\text{where } \Delta L^* = L_1^* - L_0^*,$$

$$\Delta a^* = a_1^* - a_0^*,$$

$$\Delta b^* = b_1^* - b_0^*.$$

Sorption and solubility test

This test was done in compliance with ISO 4049:2010. [23] Sixty specimens were placed in opened glass bottles (Saint Gabain, São Paulo, Brazil) and stored in a desiccator (Vidrolabor, São Paulo, Brazil) containing freshly dried blue silica (Vetec, Rio de Janeiro, Brazil) in a model 002 CB oven (Fanem LTDA- modelo 002 CB, Guarulhos, SP, Brazil) at $37 \pm 1^\circ\text{C}$ for 22 hours. They were then removed, maintained at $23 \pm 1^\circ\text{C}$ for two hours, and then weighed in an analytical balance (Marte AY 220, Santa Rita do Sapucaí, Brazil) accurate to

0.0001 g, and returned to the desiccator. The complete cycle was repeated until a constant mass (M1) was obtained, i.e., until the mass loss for each specimen was no more than 0.1mg per 24h cycle. Thereafter, the specimens were carefully placed back in their labeled bottles, and 15 mL of permeants, either deionized water or 75% ethanol, were added using manual pipettes (one for each permeant). The bottles were capped, brought back into the oven and kept at a temperature of $37^{\circ}\text{C}\pm 1^{\circ}\text{C}$ for twenty-eight days.

After this time period, all the bottles were removed from the oven and kept at room temperature ($23^{\circ}\text{C}\pm 1^{\circ}\text{C}$) for two hours. The specimens were removed from the bottles, washed in tap water for 15 seconds and left in a sterile bucket (Duflex, Rio de Janeiro, Brazil) for 1 minute. They were then weighed again to obtain M2. After weighting, the specimens were reconditioned in the desiccators until they reached a constant weight (M3) using the cycle describe for M1.

The values for water sorption (W_{sp}) and solubility (W_{sl}) in micrograms per cubic millimeter were calculated using the following equations:

$$W_{sp}=(M_2-M_3)/V; W_{sl}=(M_1-M_3)/V;$$

$$V= \pi r^2 \cdot h;$$

Where: V is the volume, r is the radius, and h is the height. These measurements were obtained using a digital electronic caliper (Mitutoyo America, Tokyo, Japan), measuring the thickness of each specimen at four points and at its center, and the diameter in two points.

Statistical analysis

Results obtained for all tests were described by mean and standard deviation. Due to the normal distribution of the variables (Kolmogorov-Smirnov and Shpauro-Wilk), ANOVA was performed to verify the effect of the different variables and Tukey test for multiple comparisons. Statistical analysis was carried out in software IBM SPSS Statistics 19.0 for Windows (SPSS Inc., Chicago, USA), with a significance of 5% ($p<0.05$).

Results

The results obtained for the optical density test are shown in Table 2. Besides the control groups that did not have contact with the bacteria, all groups showed optical density values greater than 0.137nm (standard 1 of McFarland scale). There were no statistically significant differences between the cements at different periods of contamination ($p>0.05$), except for G2 and G3 in 6h period. These groups showed statistically significant differences ($p<0.05$) between each other, being the 0.05% concentration the highest value. The SEM analysis confirmed the absence of bacteria in the control groups, the uncontaminated specimens. In the other groups, all specimens revealed the presence of bacillus in the specimens surface, which were not expected (Fig. 1 and 2). As *S. mutans* are coccus-shaped and no coccus were identified in the specimens, this finding was further investigated with a Gram test. The results revealed the presence of Gram-positive bacteria that were identified with further investigation as *Bacillus subtilis*. The EDS analysis confirmed the presence of silver agglomerates in the surface of the specimens (Fig. 3).

The results obtained for the color evaluation are presented in Table 3 and a picture of the specimens is showed in Fig. 4. After adding silver nanoparticles in their composition, both cements exhibited significant color changes. For the L^* value, test groups showed statistical significant difference ($p<0.05$) from the control group. The a^* value did not show significant difference between G1 and G2 ($p>0.05$), but were significant lower than G3 ($p<0.05$); G4, G5 and G6 showed significant changes in a^* value ($p<0.05$), being G6 the highest. For b^* there were significant differences between G1, G2 and G3 ($p<0.05$), and there was no difference between G4 and G6 ($p>0.05$), although they showed significant differences from G5 ($p<0.05$). Considering the color change (ΔE), the values observed for RelyX ARC were significantly higher ($p<0.05$), when compared to U200 with the same NAg content. The ΔE_1

for RelyX ARC showed no statistical significant difference from the ΔE_2 for RelyX U200 ($p>0.05$).

The results obtained for solubility and sorption are presented in Tables 4 and 5, respectively. No statistical significant differences were observed for solubility among all the groups stored in water, independent of cement or NAg content ($p>0.05$). For the ones stored in ethanol, G5 was statistically different from the other groups ($p<0.05$), except from G6 ($p>0.05$). Among all other ethanol groups there were no significant statistical differences ($p>0.05$). Considering the solvents, no statistical difference was observed between specimens stored in water or ethanol ($p>0.05$), but for groups G5 and G6 ($p<0.05$) where ethanol storage led to higher solubility.

For sorption in water, both control groups showed no statistical difference between each other ($p>0.05$), but were statistically different from all the other groups tested for the same cement ($p<0.05$), except for G4 and G6, that showed no statistical difference ($p>0.05$). There were no statistical difference also between G2 and G3; also for G5 and G6 in water ($p<0.05$). For the ethanol groups, the two control groups also showed no statistical difference from each other ($p>0.05$), but were statistically different from all the other groups tested for the same cement ($p<0.05$). G1 and G3 showed statistical significant differences ($p<0.05$), while G2 was similar only to G3 ($p>0.05$). G5 and G6 showed no significant difference between each other ($p>0.05$). Considering the solvents, there were no statistical significant differences between the groups stored in water and the ones in ethanol ($p>0.05$) but for G2, where ethanol storage led to higher sorption values ($p<0.05$).

Discussion

NAg application in Medicine and Dentistry has been encouraged by the broad-spectrum antimicrobial effect in low concentrations, and the ability to not cause resistant bacterial strains to develop. [2,12,14,16,19,20] This is probably because the metals may act on a broad range of microbial targets, and many mutations would have to occur for microorganisms to resist their antimicrobial activity. [2] The bactericidal mechanism of silver is only partially understood. One of the mechanisms is based on oxygen changing into active oxygen (ROS and hydroxyl radicals) as a result of catalytic action of silver, and the active oxygen causes structural damage in the bacteria. [5,11,18,24] Other explanation is based on the release of silver ions that are biologically active and can interact with proteins, especially those with thiol groups (sulfur), amino acid residues, enzymes, free anions, and receptors in the cell membranes; all these damage may cause the DNA to lose its ability to replicate. [6-8,11] Other possible explanation that is being reported by recent studies is the direct contact of the particles with the cell wall, releasing silver ions in a very high concentration in a small area, killing the cell. [5,24-27] This is important to explain the antibacterial activity of NAg entrapped in resin materials, as bone cement, denture basis and also composite materials for Dentistry, as resin luting cements.

Although most authors believe silver is an alternative to bacterial resistance, Gunawan *et al* [28] reported a change in that panorama. The authors observed, when studying the target bacteria *Escherichia coli*, the occurrence of a resistant non-targeted environmental *Bacillus* species, of which growth under the cytotoxicity of nanosilver leads to its ultimate domination in the microbiota. [28] The authors believe that natural selection favoring the more resilient members of the microbiota has taken place, which may occur in reality as a result of free-exposure of consumer products containing NAg, including the disposal stage. [28] This was also observed in this study where the target bacteria *S. mutans* was not

observed by SEM analysis in the resin cement specimens by the end of the experiment, though *Bacillus* were found in the surface of all specimens. The *Bacillus* presence was confirmed by Gram stain test. This may be the reason why there was no different optical density of the culture broths where the specimens were stored, as the growth of either *S. mutans* or *Bacillus* would lead to higher turbidity of the flasks. The HEMA-NAg, according to the Agar Diffusion Test, showed bactericidal effect on the target *S. mutans*, inhibiting bacterial growth. All these findings lead to the belief that the *Bacillus*, as in Gunawan *et al* [28] study, had dominated the microbiota in that environment. This inherent ability of the ubiquitously-occurring *Bacillus* sp. reported may represent adverse implications to the increasingly wide use of NA_g as antibacterial agents, and the environment. [28] Considering this discussion, the first null hypothesis cannot be accepted.

The two resin luting cements tested in this study were chosen because they are dual-cured materials, indicated for cementation of ceramic or porcelain crowns, onlays, inlays, and bridges, and widely applied in Dentistry. [29,30] Nowadays, esthetics is very important, and specially the color matching is imperative for the clinical acceptance of the patient. One probable disadvantage for the use of silver in Dentistry that has been reported is the color, because of oxidation reaction. [5,19-21,24,31] Cheng *et al* [20] found that a dental composite with a mass fraction of silver higher than 0.042% showed a brownish color and lower mechanical strength. Nam *et al* [24] reported color changes of denture base materials ranging from $\Delta E=15.6$ to $\Delta E=28.6$, which were considered unacceptable. Zhang *et al* [31] found that adhesives added of 0.05-0.15% of silver showed no color change. ΔE values ≥ 3.3 are considered noticeable by a non-trained person and because of that, clinically unacceptable. [32] In this study, significant color changes were found between the cements without NA_g and the ones with NA_g, especially for RelyX ARC, being the ΔE_1 found for RelyX ARC (12.91) similar to the ΔE_2 calculated for RelyX U200 (17.33). That was probably more

evident for the first because its color was A1, while the RelyX U200 was A2. The cements were used in different colors because they do not have a match color between each other, but that allowed the observation that the lighter the resin cement color the higher probability of noticeable color change to occur. This finding leads to rejecting the second null hypothesis but it does not inhibit the use of NAg in these cements due to its applications, as most of the crowns, onlays, inlays, and bridges are not highly influenced by the background or cement color. Also, Uno *et al* [21] reported that silver added porcelains showed L^* (65.13 to 67.66), a^* (0.76 to 1.82) and b^* (9.10 to 12.17) values similar to those of natural teeth for the anterior areas of maxilla and mandible, as demonstrated by Ueda [33] (L^* (56.3 to 77.1), a^* (-0.7 to 9.1), and b^* (1.4 to 27.6)). These values were very similar to the ones found for the cements with NAg addition in this study.

It is known that inadequate polymerization is usually associated with poor mechanical and biological properties because the unreacted monomers and fillers can be released by the resin based-materials in the aqueous environment, resulting in deterioration of the mechanical properties. [34,35] A few studies have been made evaluating the influence of NAg addition in the polymerization process. [7,19,36] Based on previous studies, Durner *et al* [7] suggested that NAg can influence the amount of elutable substances from light hardened composite specimens, as the NAg interact even in low concentrations with the polymerization process through interaction with the light from the curing lamp and the photoinitiator system. Fan *et al* [19] reported that NAg might form agglomerates around the polymer, preventing the cross-linking formation. The phenomena of sorption and solubility are closely related to the polymerization quality. [37] In this study sorption and solubility were clearly influenced by NAg addition and concentration.

The third null hypothesis was accepted for water storage but partially rejected for ethanol storage, as there were significant differences in solubility between RelyX U200

groups with and without NAg. The solubility values calculated were mostly negative. This has been reported in previous studies [35,38-40] and is usually indicating that these materials were more susceptible to sorption, masking the solubility values. In accordance with these studies, it can be implicated that this does not mean that no solubility occurred, rather, that there was mass gain. [38-40] Besides this, all solubility values are lower than the maximum value established by the ISO 4049:2010 [23] for polymer-based materials ($7.5 \mu\text{g}/\text{mm}^3$).

Considering sorption, the lowest values observed were the groups without NAg, this is expected as the resin luting cement without NAg and manipulated as manufacturers recommendation is supposed to deliver the best properties, rejecting the fourth null hypothesis. The self-adhesive cements contain hydrophilic acidic monomers, such as phosphoric acid, that may result in higher solvent uptake [41], thus higher sorption than the conventional cements, as showed in this study. The NAg content led to numerically different values, as the 0.07% concentration of NAg led to lower sorption than 0.05%. This can be explained by the two components added to these cements: NAg and HEMA. The increase in NAg content might contribute to a successive increase in the material hydrophobicity, leading to less water sorption when more NAg is present in the polymer matrix. [3] Also NAg may act as filler particles, increasing the filler content of the cement and contributing to lower sorption. [30] The hydrophobic nature of the monomers in the composites influence the sorption and solubility behavior of the cements, and more HEMA content is known to enhance water sorption and promote hygroscopic expansion of a material. [29,30] The higher amount of HEMA in the 0.05% solution and the lower content of NAg when compared to the 0.07% one might have led to these values of sorption observed in this study. Sorption was numerically higher for 75% ethanol when compared to water, this might be due to the enhanced ability of ethanol to penetrate and swell the crosslinked polymer network in comparison to water, promoting then an accelerated aging effect. [22,30] Also, according to

US FDA (United States Food and Drug Administration) [42] guidelines, 75% ethanol-water is an important clinical simulating liquid, and considering that, it was used in this study.

To be accepted clinically, the new materials added with NAg must provide superior antimicrobial activity and display comparable physical properties when compared with conventional ones. [5] Thus, the resin luting cement proposed by this study has to be further evaluated, especially for its toxicity, antibacterial and mechanical properties to be securely applied in clinical practice.

Conclusion

The resin luting cements with NAg addition did not show antibacterial effect against *S. mutans*. This resin cements with NAg showed perceptible color change and higher sorption than the materials without NAg.

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Tables

Table 1. Resin cements used in this study and their composition

Material	Manufacturer	Composition
RelyX ARC	3M ESPE, St. Paul, MN, USA	Bis-GMA, TEGDMA, zirconia/silica filler (67,5%), amine pigments, photoinitiador, benzoyl peroxide
RelyX U200		Methacrylate monomers containing phosphoric acid groups, methacrylate, silanated and alkaline (basic) fillers, initiator componentes, stabilizers, pigments, rheological additives

Table 2. Values of mean and standard deviation (\pm SD) for the optical density of the specimens culture broths contaminated

Groups	Times					
	control	1min	5min	1h	6h	24h
G1	0.000 \pm	0.886 \pm	0.956 \pm	0.831 \pm	0.949 \pm	1.058 \pm
	0.000 ^{A,a}	0.054 ^{B,a}	0.211 ^{B,a}	0.018 ^{B,a}	0.056 ^{B,a,b}	0.052 ^{B,a}
G2	0.000 \pm	0.847 \pm	0.925 \pm	0.951 \pm	1.032 \pm	0.977 \pm
	0.000 ^{A,a}	0.031 ^{B,a}	0.042 ^{B,a}	0.047 ^{B,a}	0.054 ^{B,a}	0.101 ^{B,a}
G3	0.000 \pm	0.859 \pm	0.898 \pm	0.884 \pm	0.582 \pm	0.944 \pm
	0.000 ^{A,a}	0.092 ^{B,a}	0.053 ^{B,a}	0.080 ^{B,a}	0.407 ^{B,b}	0.127 ^{B,a}
G4	0.000 \pm	0.811 \pm	0.982 \pm	0.717 \pm	0.892 \pm	0.955 \pm
	0.000 ^{A,a}	0.049 ^{B,a}	0.256 ^{B,a}	0.182 ^{B,a}	0.082 ^{B,a,b}	0.052 ^{B,a}
G5	0.000 \pm	0.736 \pm	0.881 \pm	0.855 \pm	0.981 \pm	1.062 \pm
	0.000 ^{A,a}	0.251 ^{B,a}	0.013 ^{B,a}	0.044 ^{B,a}	0.070 ^{B,a,b}	0.067 ^{B,a}
G6	0.000 \pm	0.731 \pm	0.852 \pm	0.772 \pm	0.897 \pm	1.076 \pm
	0.000 ^{A,a}	0.183 ^{B,a}	0.128 ^{B,a}	0.180 ^{B,a}	0.023 ^{B,a,b}	0.335 ^{B,a}

Values followed by the same uppercase letters (A,B) denote no statistical significant difference ($p>0.05$) among columns (different storage times).

Values followed by the same lowercase letters (a,b) denote no statistical significant difference ($p>0.05$) among lines (different silver concentrations and cements).

Table 3. Values of mean and standard deviation (\pm SD) for L*, a* and b* measured for each group and values of ΔE^* calculated between G1 and G2, and G4 and G5 (ΔE_1^*) and between G1 and G3, and G4 and G6 (ΔE_2^*)

Groups	CIELab System Coordinates				ΔE_1	ΔE_2
	L	a	b			
G1	100.00 \pm 0.00 ^A	0.25 \pm 0.53 ^A	19.50 \pm 1.57 ^A			
G2	90.66 \pm 4.19 ^B	-0.53 \pm 0.95 ^A	28.06 \pm 3.49 ^B	12.91 \pm 5.48 ^{A,B}	26.64 \pm 2.71 ^C	
G3	76.47 \pm 2.69 ^D	2.14 \pm 0.65 ^C	31.63 \pm 1.56 ^C			
G4	100.00 \pm 0.00 ^A	-2.10 \pm 0.54 ^B	25.26 \pm 2.20 ^B			
G5	93.24 \pm 2.26 ^B	-0.30 \pm 0.59 ^A	21.14 \pm 2.67 ^A	8.33 \pm 3.09 ^A	17.33 \pm 3.83 ^B	
G6	83.46 \pm 3.98 ^C	1.74 \pm 0.81 ^C	27.00 \pm 2.17 ^B			

Values of L, a and b followed by the same uppercase letters (A-D) denote no statistical significant difference ($p>0.05$) among lines (different silver concentrations and cements).

Values of ΔE followed by the same uppercase letters (A-C) denote no statistical significant difference ($p>0.05$) among lines and columns (between cements and the different ΔE).

Table 4. Values of mean and standard deviation (\pm SD) for solubility found for the different cements and silver concentrations tested

Groups	Solvents	
	Water	75% Ethanol
G1	4.75 \pm 6.10 ^{A,a}	-6.56 \pm 4.46 ^{A,a}
G2	0.78 \pm 5.10 ^{A,a}	-3.85 \pm 9.18 ^{A,a}
G3	3.12 \pm 4.82 ^{A,a}	-11.15 \pm 6.85 ^{A,a}
G4	-7.21 \pm 2.41 ^{A,a}	-6.36 \pm 5.97 ^{A,a}
G5	2.12 \pm 8.92 ^{A,a}	-30.62 \pm 9.45 ^{B,b}
G6	-7.44 \pm 3.10 ^{A,a}	-18.70 \pm 10.23 ^{B,a,b}

Values followed by the same uppercase letters (A,B) denote no statistical significant difference ($p>0.05$) among columns (different solvents).

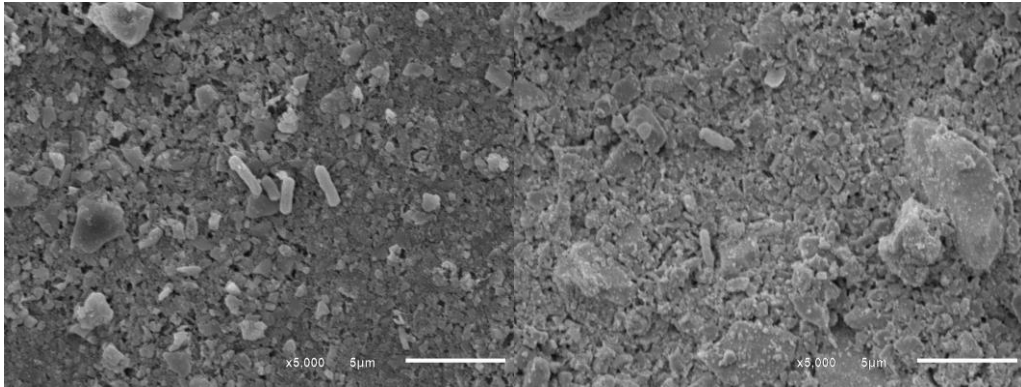
Values followed by the same lowercase letters (a,b) denote no statistical significant difference ($p>0.05$) among lines (different silver concentrations and cements).

Table 5. Values of mean and standard deviation (\pm SD) for sorption found for the different cements and silver concentrations tested

Groups	Solvents	
	Water	75% Ethanol
G1	14.78 \pm 6.89 ^{A,a}	31.46 \pm 6.69 ^{A,a,b}
G2	47.43 \pm 11.46 ^{A,b,c}	86.37 \pm 9.66 ^{B,d}
G3	54.05 \pm 10.56 ^{A,b}	68.46 \pm 9.26 ^{A,c,d}
G4	28.17 \pm 9.10 ^{A,a,c}	20.27 \pm 11.56 ^{A,a}
G5	67.81 \pm 20.93 ^{A,b}	46.05 \pm 10.64 ^{A,b,c}
G6	49.75 \pm 5.06 ^{A,b,c}	28.55 \pm 8.13 ^{A,b}

Values followed by the same uppercase letters (A,B) denote no statistical significant difference ($p>0.05$) among columns (different solvents).

Values followed by the same lowercase letters (a-d) denote no statistical significant difference ($p>0.05$) among lines (different silver concentrations and cements).



Figures 1 and 2 – SEM images from groups G2-1h and G5-1h (x5000) showing the presence of bacillus in the specimens surface, no coccus are observed in the figures.

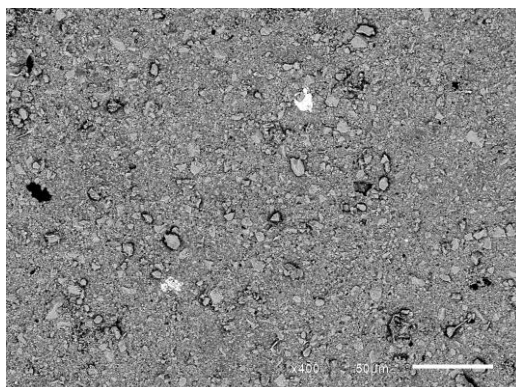


Figure 3 – EDS image of the same G2-1h specimen (x400) showing, in white, the silver agglomerates in the specimen surface.



Figure 4 – Picture of the specimens of each group, in order: control groups (G1 and G4), 0.05% silver groups (G2 and G5) and 0.07% silver groups (G3 and G6). The color change caused by silver can be noticed in white and black background.

4 CONSIDERAÇÕES FINAIS

Os cimentos resinosos com adição de NAg parecem não ter apresentado efeito antimicrobiano sobre *S. mutans*. Também apresentaram mudança de cor perceptível e maior sorção e solubilidade do que os materiais sem adição de NAg.

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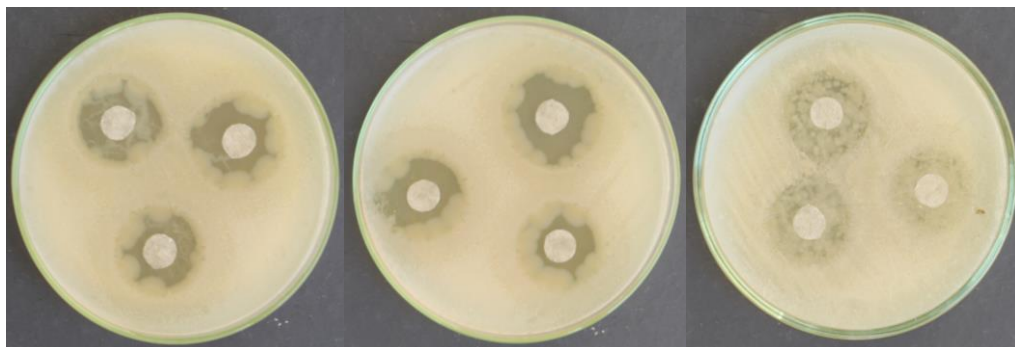
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APÊNDICE A

Resultado do teste de Difusão em Ágar feito com as soluções de HEMA-NAg obtidas:



Figuras 5, 6 e 7 – Fotografias dos halos obtidos no pré-teste de Difusão em Ágar para HEMA-NAg 0,07%, HEMA-NAg 0,05% e HEMA puro, respectivamente.

ANEXO A

Guide for Authors – Dental Materials

Authors are requested to submit their original manuscript and figures via the online submission and editorial system for Dental Materials. Using this online system, authors may submit manuscripts and track their progress through the system to publication. Reviewers can download manuscripts and submit their opinions to the editor. Editors can manage the whole submission/review/revise/publish process. Please register at: <http://ees.elsevier.com/dema>. Dental Materials now only accepts online submissions. The Artwork Quality Control Tool is now available to users of the online submission system. To help authors submit high-quality artwork early in the process, this tool checks the submitted artwork and other file types against the artwork requirements outlined in the Artwork Instructions to Authors on www.elsevier.com/artworkinstructions. The Artwork Quality Control Tool automatically checks all artwork files when they are first uploaded. Each figure/file is checked only once, so further along in the process only new uploaded files will be checked.

Manuscripts

The journal is principally for publication of Original Research Reports, which should preferably investigate a defined hypothesis. Maximum length 6 journal pages (approximately 20 double-spaced typescript pages) including illustrations and tables. Systematic Reviews will however be considered. Intending authors should communicate with the Editor beforehand, by email, outlining the proposed scope of the review. Maximum length 10 journal pages (approximately 33 double-spaced typescript pages) including figures and tables. Three copies of the manuscript should be submitted: each accompanied by a set of illustrations. The requirements for submission are in accordance with the "Uniform Requirements for Manuscripts Submitted to Biomedical Journals", *Annals of Internal Medicine*, 1997,126, 36-47. All manuscripts must be written in American English. Authors are urged to write as concisely as possible. The Editor and Publisher reserve the right to make minimal literary corrections for the sake of clarity. Authors for whom English is not the first

language should have their manuscripts read by colleagues fluent in English. If extensive English corrections are needed, authors may be charged for the cost of editing. For additional reference, consult issues of *Dental Materials* published after January 1999 or the Council of Biology Editors Style Manual (1995 ed.). All manuscripts should be accompanied by a letter of transmittal, signed by each author, and stating that the manuscript is not concurrently under consideration for publication in another journal, that all of the named authors were involved in the work leading to the publication of the paper, and that all the named authors have read the paper before it is submitted for publication. Always keep a backup copy of the electronic file for reference and safety. Manuscripts not conforming to the journal style will be returned. In addition, manuscripts which are not written in fluent English will be rejected automatically without refereeing.

Format

General

- number all pages consecutively.
- type double-spaced on A4 or 8.5 x 11-inch bond paper, with margins of 30 mm.
- double-space references.
- indent or space paragraphs.
- arrange article in the following order: Title, Abstract, Introduction, Materials and Methods, Results, Discussion, Conclusion, Acknowledgements, References, Tables, Figures, Captions.
- start each section on a separate page.

Title page

- Title (capitalize the first letter of the first word) e.g. Comparison of the color stability of ten new composites.
- Authors (first name, middle initial, surname) e.g. Kenneth J. Anusavice 1, Victoria Marker 2

- Authors' addresses (abbreviated) e.g. 1 Department of Biomaterials, University of Florida, Gainesville, Florida, USA 2 Department of Biomaterials Science, Baylor College of Dentistry, Dallas, Texas, USA

- Short Title (45 characters) e.g Color stability of composites
- Corresponding Author details (essential): Name, complete address, phone, fax, and E-mail numbers

Abstract (structured format)

- 250 words or less.
- subheadings should appear in the text of the abstract as follows: Objectives, Methods, Results, Significance. (For Systematic Reviews: Objectives, Data, Sources, Study selection, Conclusions). The Results section may incorporate small tabulations of data, normally 3 rows maximum.

Keywords

Up to 10 keywords should be supplied e.g. dental material, composite resin, adhesion.

Introduction

This must be presented in a structured format, covering the following subjects, although actual subheadings should not be included:

- succinct statements of the issue in question;
- the essence of existing knowledge and understanding pertinent to the issue (reference);
- the aims and objectives of the research being reported relating the research to dentistry, where not obvious.

Materials and methods

- describe the procedures and analytical techniques.
- only cite references to published methods.
- include at least general composition details and batch numbers for all materials.

- identify names and sources of all commercial products e.g. "The composite (Silar, 3M Co., St. Paul, MN, USA)..." "... an Au-Pd alloy (Estheticor Opal, Cendres et Metaux, Switzerland)."

- specify statistical significance test methods.

Results

- refer to appropriate tables and figures.
- refrain from subjective comments.
- make no reference to previous literature.
- report statistical findings.

Discussion

- explain and interpret data.
- state implications of the results, relate to composition.
- indicate limitations of findings.
- relate to other relevant research.
- suggest directions for future research.

Conclusion (if included)

- must NOT repeat Results or Discussion
- must concisely state inference, significance, or consequences

Acknowledgements

As appropriate, e.g.: "Based on a thesis submitted to the graduate faculty, University of Virginia, in partial fulfilment of the requirements for the M.S. degree." "This investigation was supported in part by Research Grant DE 00000 from the National Institute of Dental Research, Bethesda, MD 20892."

References - must now be given according to the following numeric system:

Cite references in text in numerical order. Use square brackets: in-line, not superscript e.g. [23]. All references must be listed at the end of the paper, double-spaced, without indents. For example:

1. Moulin P, Picard B and Degrange M. Water resistance of resin-bonded joints with time related to alloy surface treatments. *J Dent*, 1999; 27:79-87.
2. Taylor DF, Bayne SC, Sturdevant JR and Wilder AD. Comparison of direct and indirect methods for analyzing wear of posterior composite restorations. *Dent Mater*, 1989; 5:157-160.

Avoid referencing abstracts if possible. If unavoidable, reference as follows:

3. Demarest VA and Greener EH . Storage moduli and interaction parameters of experimental dental composites. *J Dent Res*, 1996; 67:221, Abstr. No. 868.

Tables and figures

All tables and figures must be thoroughly discussed in the text of the manuscript.

Tables

- one table to a page, each with a title.
- number tables in order of mention using Arabic numerals.
- must be able to "stand alone" apart from text.
- when appropriate, standard deviations of values should be indicated in parentheses; (do NOT use \pm notation).
- results of statistical analysis must be included, use superscript letters to indicate significant differences.
- for explanatory footnotes, use symbols (*, #, **, ##).

Figures

- Do not import the figures into the text file but, instead, indicate their approximate locations directly in the electronic text. Images to be supplied separately in jpg, gif or other graphics file.

- only black and white photographs for print publication.

- omit titles and other information contained in the figure caption.

- maximum of 6 figures per manuscript.

- figures grouped together should have similar dimensions and be labelled "a, b, c", etc.

- place magnification markers directly on the micrographs.

- authors should consider that the majority of figures will be reduced to the width of a single column (approximately 85 mm). Preferably figures should exactly match, or be no more than 1.5 times that width.

- authors can indicate if they feel a figure should be full page width.

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- unique, concise axis labels; do not repeat the Figure caption.

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- list together on a separate page.

- should be complete and understandable apart from the text.
- include key for symbols or abbreviations used in Figures.
- individual teeth should be identified using the FDI two-digit system.

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Correct Usage • use S.I. units (International System of Units). If non-SI units must be quoted, the SI equivalent must immediately follow in parentheses.

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- put leading zeros in all numbers less than 1.0
- write out number of ten or fewer (ten rats) except when indicating inanimate quantities (10 mL)
- always use digits for dates, dimensions, degrees, doses, time, percentages, ratios, statistical results, measurements, culture cells, and teeth.
- the complete names of individual teeth must be given in the text.

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