LUCIANE GROCHOCKI RESENDE

VIABILIDADE DE *STREPTOCOCCUS MUTANS* APÓS TRATAMENTO COM CLAREADORES DENTAIS

CURITIBA

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Dissertação apresentada ao Programa de Pós-Graduação em Odontologia da Pontifícia Universidade Católica do Paraná, como parte dos requisitos para a obtenção do Título de Mestre em Odontologia – Área de Concentração em Dentística.

Orientador: Prof. Dr. Rodrigo Nunes Rached

Co-orientador: Prof. Dr. Edvaldo A. R. Rosa

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Resende, Luciane Grochocki
R433v 2007 Viabilidade de *streptococcus mutans* após tratamento com clareadores dentais / Luciane Grochocki Resende ; orientador, Rodrigo Nunes Rached ; co-orientador, Edvaldo A. R. Rosa. – 2007.
70 f. : il. ; 30 cm

Dissertação (mestrado) – Pontifícia Universidade Católica do Paraná, Curitiba, 2007
Inclui bibliografia

1. Dentes – Clareamento. 2. Streptococcus mutans. 3. Peróxidos. I. Rached, Rodrigo Nunes. II. Rosa, Edvaldo Antonio Ribeiro. III. Pontifícia Universidade Católica do Paraná. Programa de Pós-Graduação em Odontologia. IV. Título.

CDD 21. ed. – 617.63

"O homem que encontra o processo da educação
espiritualiza a vida,
entra triunfante para a eternidade."
Leocádio José Correia

DEDICO ESTE TRABALHO
A MINHA FAMÍLIA...

Ao meu marido **Sérgio**,

Por tudo que representa em minha vida.

Por todo amor, companheirismo e apoio, durante a realização deste curso.

Por todos os momentos que ficou me esperando, sem reclamar.

Por compreender meus momentos de afastamento, devido ao mestrado.

Ao meu filho **Gabriel**,

Por ser a razão da minha vida.

Por tentar entender a minha ausência neste momento de sua vida, por toda a força e incentivo.

Aos meus pais **Modesto e Joanina,**

Que me ensinaram através da dedicação, compreensão, dignidade e amor, a viver.

Pelo exemplo, amor, esforço e dedicação que sempre dispensaram comigo.

Aos meus irmãos **Luiz Daniel, Liliane e Rodrigo**,

Que me incentivaram, apoiaram. Sem o incentivo deles eu não estaria aqui.

Por terem compartilhado comigo todas as alegrias, tristezas, êxitos e tropeços durante toda minha vida, estimulando-me sempre a prosseguir.

"Ame-me quando eu menos merecer,
pois é quando eu mais preciso".
(Provérbio chinês)

AGRADECIMENTO ESPECIAL

Ao meu orientador professor **Dr. Rodrigo Nunes Rached**, meu profundo respeito e admiração pela sua competência e dedicação. Agradeço por todo o tempo que dispensou, sem nunca medir esforço, pelas cobranças e oportunidades. Por estar presente sempre quando necessário, dar os instrumentos elementais para que esta pesquisa se concretizasse. Sua determinação e apoio foram fundamentais para que as dificuldades fossem superadas. Tê-lo como orientador, além de privilégio, foi e será sempre uma honra. Obrigada por ser uma pessoa especial.

Ao meu co-orientador professor **Dr. Edvaldo A. R. Rosa**, pelo seu exemplo de competência profissional, dedicação e amor à ciência. Agradeço as oportunidades, os ensinamentos, à inspiração e o respeito por minha ignorância. Agradeço por toda a ajuda nos momentos que necessitei e por todo o tempo que disponibilizou sempre que precisei. Desculpe se em alguns momentos não atendi suas expectativas. Tê-lo como co-orientador foi um privilegio que muito contribuiu para meu crescimento. Muito obrigada.

A **Rosimeire T. Rosa**, pelo seu exemplo, competência, dedicação, paciência (muita paciência) para me ensinar que caminho trilhar. Agradeço a oportunidade de ter lhe conhecido e o privilégio de ter seus conhecimentos a minha disposição todos os dias. Por ter me apoiado, incentivado, aconselhado. Sem sua ajuda a concretização deste trabalho seria mais difícil. Obrigada de coração!

"Feliz daquele que transfere o que sabe e aprende o que ensina
(Provérbio chinês)

AGRADECIMENTOS

A **Pontifícia Universidade Católica do Paraná** pelo acolhimento e oportunidade de intensificar e aprimorar meus conhecimentos.

Ao professor **Dr. Sergio Vieira**, Diretor do programa Curso de Pós-graduação da PUCPR, pela dedicação, determinação, disciplina e empenho neste programa. Agradeço pelo seu acolhimento, por ter acreditado e me proporcionar esta oportunidade de crescimento formação profissional.

Aos professores **Danilo B. Menezes Caldas, Evelise Machado Souza, Janaína B. de Almeida, Rui Fernando Mazur, Sérgio A. Ignácio** agradeço a todos por todos os conhecimentos compartilhados, pela paciência, disponibilidade, carinho e amizade. em contribuir com seus conhecimentos práticos e teóricos.

Aos meus colegas de Mestrado **Andréa Freire, Marcos Kenzo Takahashi e Rafael Moura Jorge**, pelo convívio, amizade, ajuda nos momentos difíceis, conhecimento e comprometimento em todas as fases do nosso mestrado.

Agradecimento especial aos professores da minha banca de qualificação **Prof. Dr. Rodrigo Nunes Rached, Prof. Dr. Rui Fernando Mazur, Prof. Dr. Antonio Adilson Soares de Lima e Profª. Drª. Janaina B. de Almeida.**

Aos funcionários, **Silvana Casagrande Gabardo**, **Neide Borges dos Reis, Manolo Marino Gonzales, Cleomar Rodrigues Lemos, Aparecido Nunes Arquanjo, Marcos Vieira dos Santos e Diamir Desordi Polaquine** pela atenção, disponibilidade e incentivo.

.

A **Aline Mendes,** estagiária do Laboratório de Estomatologia, agradeço por ter compartilhado de todos os momentos difíceis, sempre me incentivando.

Aos funcionários do Laboratório de Bioquímica, **Cleide, Marcelo e Maurício,** por toda atenção dispensada, pela ajuda e pelos empréstimos, sem vocês meu trabalho não se concretizaria.

A todas as pessoas que de alguma forma colaboraram com minha formação profissional.

"Amigos são anjos que
nos ajudam a ficar em pé
quando nossas asas esquecem de voar".
(Autor desconhecido)

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1. ARTIGO EM PORTUGUÊS

1.1. PÁGINA TÍTULO

Toxicidade do peróxido de carbamida 16% e peróxido de hidrogênio 35% sobre S*treptococcus mutans*

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Palavras chave: clareamento dentário, S*treptococcus mutans*, peróxido de carbamida, peróxido de hidrogênio.

1.2. RESUMO

Objetivo: O objetivo deste estudo foi avaliar a toxicidade *in vitro* de agentes clareadores [peróxido de carbamida 16% (PC) e peróxido de hidrogênio 35% (PH)] sobre *Streptococcus mutans* crescido em fase planctônica e biofilme em dois tempos de exposição continuada. **Materiais e métodos:** O grupo experimental foi formado pela exposição das bactérias aos agentes clareadores PC e PH. Os grupos controles negativo foram formados por tratamentos com os excipientes (PCe, PHe) dos clareadores. O grupo controle positivo consistiu em espécimes que não foram expostos aos agentes clareadores ou seus excipientes. Os produtos PC, PH, PCe e PHe foram misturados com células crescidas em fase planctônica ou aplicados sobre biofilmes e imediatamente incubados por quatro períodos de tempo (2 e 24 horas) para PC e PCe, e (30 e 90 minutos) para PH e PHe. As misturas foram dispersas, diluídas seriadamente e plaqueadas, em triplicata. As espessuras dos biofilmes foram determinadas por meio de microscópio óptico. Os resultados foram submetidos aos testes Kruskal-Wallis e U de Mann-Whitney ($p<0,05$). **Resultados:** Os tratamentos PC, PH, PCe e PHe demonstraram efeito bactericida para as células em fase planctônicas, nos dois tempos investigados. Somente PC e PH apresentaram efeito bactericida em biofilme. PHe e CPe mostraram diferenças quando comparados com PH e PC, respectivamente, em todos os períodos do tempo. PCe24 apresentou redução bacteriana, mas não as eliminou totalmente. Os tratamentos com excipientes apresentaram redução tempo-dependente na viabilidade de células crescidas em biofilme para todos os períodos de tempo. **Conclusão:** Os agentes clareadores e seus excipientes apresentaram toxicidade para *Streptococcus mutans* crescido em fase planctônica e em biofilme, nos dois tempos investigados

1.3 INTRODUÇÃO

O peróxido de carbamida (PC) e peróxido de hidrogênio (PH) são produtos largamente utilizados para o clareamento dentário, ambos se decompondo, dentre outras substâncias, em oxigênio livre, o qual é responsável pelo efeito clareador [1]

As técnicas básicas de clareamento para dentes vitais são clareamento caseiro supervisionado e clareamento de consultório [2]. Para os clareamentos caseiros são usados géis de (PC) em concentrações de 10 a 16% e para o clareamento de consultório consiste no uso do peróxido de hidrogênio (PH) de 30 a 35%

Embora os agentes de clareamento dental venham sendo utilizados extensivamente, efeitos biológicos adversos sobre estruturas do organismo têm sido reportados, tais como alterações de sensibilidade, de tecidos dentais e de mucosa [3], mudanças morfológicas na superfície do esmalte como erosões, aumento de rugosidades e porosidades [4]. Efeitos adversos dos agentes clareadores em bactérias também têm sido observados. O PH é bacteriostático, mutagênico e são capazes de promover danos ao DNA bacteriano [5]. Ainda, o PH pode ter efeito genotóxico em bactérias [3].

O aumento na aderência de bactérias cariogênicas sobre o esmalte *Streptococcus mutans* e *S. sobrinus* foi reportado após o uso de gel clareador (PC) durante 1 mês (8h diárias) em estudo *in vitro* [6] e após tratamento com PH *in vitro* [2]. As mudanças causadas na superfície do esmalte com conseqüente aumento da aderência bacteriana sugerem que ocorre um aumento de suscetibilidade à cárie após o clareamento. Entretanto, o PC 10% e o PH a 3% [1], equivalentes em potencial oxidativo, demonstraram elevado potencial anticariogênico durante o processo de clareamento, devido à elevação do pH da placa e da saliva [7], diminuindo o número de bactérias e reduzindo a quantidade de placa dental

[7,8]. Alkmin et al. [9] avaliaram a contagem de *S mutans* antes, durante e após o clareamento, e não observaram mudança significativa na contagem desta bactéria na cavidade oral. Contrariamente, Kraigher et al. [10] confirmaram em estudo com ratos o efeito bactericida dos clareadores em bactérias cariogênicas. Adicionalmente, foi demonstrado em estudo *in vitro* que géis clareadores não aumentam susceptibilidade à cárie em esmalte humano [11].

Diante da importância de se avaliar o efeito real dos agentes clareadores sobre bactérias envolvidas com a doença cárie, podendo-se desta forma predizer com maior segurança o impacto destas substâncias na experiência futura da cárie dos pacientes, o objetivo deste estudo foi avaliar a toxicidade *in vitro* dos agentes clareadores PC 16% e PH 35% sobre *S mutans* crescido em fase planctônica e biofilme em dois tempos de exposição continuada. A hipótese testada é que os tratamentos com géis clareadores provocam a morte de células de *S Mutans* em fase planctônica e em biofilme.

1.4. MATERIAIS E MÉTODOS

Neste estudo, foi empregada a linhagem CCT3440 de *Streptococcus mutans*, disponível no Laboratório de Estomatologia da PUCPR. A cepa foi reativada em infuso de cérebro e coração (BHI) (Biobrás Diagnósticos Ltda, Montes Claros, MG, Brasil) e incubada a 37°C em pCO_2 10%, por 24 horas. As células foram colhidas por centrifugação (BE 4004, Bio Eng Ind, Brasil) (5000 *g*) e lavadas com água destilada estéril. Após duas lavagens, os sedimentos foram ressuspendidos em água destilada estéril até uma turbidez próxima daquela do tubo #1 da escala de McFarland (aprox. 3×10^8 células/mL).

Os produtos testados foram um agente clareador "caseiro" a base de peróxido de carbamida ("PC", Whiteness® Perfect 16%, FGM, Joinville, Brasil) e um agente clareador "profissional" a base de peróxido de hidrogênio ("PH", Whiteness® HP Maxx 35%, FGM).

Os produtos avaliados e os grupos formados estão dispostos na Tabela 1. O grupo experimental foi formado pela exposição das bactérias aos agentes clareadores PC e PH. Os controles negativos foram formados pela exposição das bactérias aos excipientes dos géis clareadores (PCe, PHe). O controle positivo consistiu em espécimes que não foram expostos aos agentes clareadores ou seus excipientes, e submetidos às mesmas etapas dos grupos experimentais e controles negativos. Os tempos de exposição das bactérias ao PC e PCe foram de 2 e 24 horas. Já para PH e PHe os tempos foram de 30 e 90 minutos. Todos os grupos foram testados com bactérias crescidas em fase planctônica ou aplicadas sobre biofilmes. (Tabela 1).

Tabela 1- Produtos avaliados e grupos formados.

Material	Ingredientes	Tempos	Grupos
Whiteness® Perfect 16%	Peróxido de carbamida 16% (±0,5%), carbopol (934-P, 980-P), glicerina, água deionizada, nitrato de potássio, fluoreto de sódio 0,2%, estabilizantes, agentes neutralizantes	2 h	PC2
		24 h	PC24
Excipiente do Whiteness® Perfect 16%	Carbopol (934-P e 980-P), glicerina, água deionizada, nitrato de potássio, fluoreto de Sódio (0,2%), estabilizantes, agentes neutralizantes	2 h	PCe2
		24 h	PCe24
Whiteness® HP Maxx 35%	Peróxido de hidrogênio 33% (±2%), espessante, glicol, água deionizada, estabilizantes, agentes neutralizantes, carga inorgânica, corantes	30 min.	PH30
		90 min.	PH90
Excipiente do Whiteness® HP Maxx 35%	Espessante, glicol, água deionizada, estabilizantes, agentes neutralizantes, carga inorgânica, corantes	30 min.	PHe30
		90 min.	PHe90

Obs.: materiais e informações dos ingredientes fornecidos por FGM Ind., Joinville, Brasil.

Exposição das células planctônicas aos agentes clareadores

Alíquotas de 100 µL de suspensão bacteriana foram combinadas com os agentes clareadores (PC e PH) ou de seus excipientes (PCe e PHe). O produto PH foi preparado segundo as instruções do fabricante, misturando-se 1 gota de espessante para 3 gotas de PH. Para o excipiente de PH (PHe), utilizou-se 4 gotas do mesmo isoladamente. Similarmente em massa, utilizou-se aproximadamente 1g dos produtos PC e PCe. Em tubos de ensaio esterilizados de forma asséptica em cabine de segurança biológica (Classe II-A,VLFS 12, Veco do Brasil, São Paulo, SP, Brasil). Os tubos foram incubados em pCO_2 10% a 37°C. Réplicas dos tratamentos com o produto PC e PCe foram deixadas em incubação por 2 horas (tempo minimo de uso diário preconizado pelo fabricante). Nos tratamentos com o produto PH e PHe, o mesmo foi ativado utilizando-se o aparelho fotopolimerizador de luz halógena Optilight plus (Gnatus, Ribeirão Preto, SP, Brasil) com potência de 600 mW/cm², aferida com radiômetro (Gnatus, Ribeirão Preto, SP, Brasil), antes e ao término do experimento, (a aplicação da luz ocorreu em 3 etapas de 20 segundos), as misturas foram deixadas em incubação por tempos de 30 minutos. Decorridos os tempos de incubação, 9 mL de caldo Letheen (Becton Dickinson and Company, Sparks, MD, USA) foram acrescentados às misturas. Os conteúdos dos tubos foram homogeneizados em vórtex (AP56, Phoenix Ltda, São Paulo, SP, Brasil) e alíquotas de 100µL foram pipetadas e diluídas de forma seriada. De cada tubo da série de diluição alíquotas de 100µL foram aplicadas sobre BHI-ágar 1,6% (Biobrás, Montes Claros, MG, Brasil) e incubadas em PCO_2 10% e 37°C, por 24 horas. Para cada grupo, houve bactérias que não foram expostas a qualquer agente clareador ou seu respectivo

excipiente, formando desta forma o grupo controle, e submetidos às mesmas etapas dos grupos experimentais e controles negativos.

Exposição das células em biofilme aos agentes clareadores

Lâminas de vidro, para microscopia, previamente lavadas, desengorduradas, esterilizadas e tratadas com solução de L-lisina 0,1% foram imersas verticalmente em tubos plásticos cônicos de 50mL esterilizados contendo 30mL de caldo tríptico de soja (TSB) (Biobrás Diagnósticos Ltda, Montes Claros, MG, Brasil) enriquecido com 5% de glicose. O conjunto placa-lâmina foi pesado em balança analítica digital (Bel Mark U210A, Bel Engeneering, Piracicaba, SP, Brasil) (massa #2).

Aos tubos foram adicionadas alíquotas de 100μL de suspensão bacteriana e foram acondicionados em pCO_2 10% e 37°C. Decorrido 48 horas iniciais, a cada 24 horas, por cinco dias consecutivos, as lâminas foram transferidas para novos tubos com caldo TSB enriquecido com 5% de sacarose Após o período de formação de "biofilme cariogênico", as lâminas foram retiradas e rapidamente mergulhadas em água destilada estéril, para remoção de células não aderidas. Um dos lados de cada lâmina foi limpo com gaze esterilizada. Essa lâmina foi depositada em uma placa de Petri esterilizada, de tal forma que o biofilme formado ficou voltado para cima. O conjunto placa-lâmina-biofilme foi pesado em balança analítica digital. (massa #1).

Toda a área de biofilme formado foi recoberta com uma lâmina de vidro contendo cada agente clareador (PC e PH) ou de seus respectivos excipientes (PCe e PHe) de forma asséptica em cabine de segurança biológica. O produto PH foi preparado segundo as instruções do fabricante, misturando-se 1 gota de espessante para 3 gotas de peróxido de hidrogênio. O PHe foi usado apenas excipiente na medida de 4 gotas (equivalente a massa

de aproximadamente 1g). O produto PH, foi ativado utilizando um aparelho fotopolimerizador de luz halógena, conforme descrito anteriormente. Os tratamentos foram deixados em pCO_2 10% e 37°C. Réplicas dos tratamentos com o produto PC e PCe foram deixadas em incubação por tempos de 2 e 24 horas. Os tratamentos com o produto PH e PHe foram deixados em incubação por tempos de 30 e 90 minutos.

Decorridos os tempos de incubação, as lâminas de vidro foram transportadas para novos tubos plásticos cônicos de 50mL esterilizados contendo 30 mL de caldo Letheen. Os conteúdos dos tubos foram dispersos em banho de ultra-som (Thornton T7, Thornton Inpec Eletrônica Ltda, Ribeirão Preto, SP, Brasil) em potência máxima e posteriormente as lâminas foram retiradas. Os tubos foram homogeneizados em vórtex e alíquotas de 100µL foram tomadas e diluídas de forma seriada. De cada tubo da série de diluição, alíquotas de 100µL foram aplicadas sobre BHI-ágar 1,6% e incubadas em pCO_2 10% e 37°C, por 24 horas. Os resultados foram expressos em unidades formadoras de colônias de *S mutans* por mililitro (UFC/mL) e corrigidos para unidades formadoras de colônia *S mutans* por miligrama de biofilme (UFC/mg biofilme). Para cada grupo, houve bactérias que não foram expostas aos agentes clareadores ou seu respectivo excipiente, formando desta forma os grupos controles positivo, e submetidos às mesmas etapas dos grupos experimentais e controles negativos.

Espessura do Biofilme

Cinco lâminas de vidro recobertas com biofilme foram usadas para estimativa de espessura do mesmo. As medidas foram feitas em 10 pontos eqüidistantes ao longo das lâminas. A determinação das espessuras dos biofilmes foi feita seguindo o protocolo

preconizado pelo Center for Biofilm Engineering da Montana State University (2007), em um microscópio óptico LABOPHOT-2 (Nikon Optics, Toquio, Japão).

Análise estatística

Os resultados obtidos para UFC/mg, não apresentaram valores de distribuição normal ($p<0,05$). O teste não-paramétrico de Kruskal-Wallis foi empregado. As comparações estatísticas dos dados obtidos para os tempos de tratamento para cada produto e seus respectivos excipientes foram feitas pelo teste U de Mann-Whitney. A análise estatística foi realizada usando o software SPSS 13.0. (SPSS Inc., Chicago, Il. EUA).

1.5 RESULTADOS

Os produtos comerciais Whiteness® Perfect 16%, Whiteness® HP Maxx 35% e seus excipientes mataram todas as bactérias crescidas em fase planctônica, nos dois tempos investigados.

Para as bactérias crescidas em biofilme, os grupos controle e os tratamentos PHe30, PHe90, PCe2, PCe24 tiveram crescimento bacteriano maior quando comparados com PH30, PH90, PC2, PC24 que não apresentaram crescimento bacteriano ($p<0,05$). PCe24 apresentou redução bacteriana estatisticamente significante, porem não as eliminou totalmente. (Tabela 2).

Para a variável tempo, comparando separadamente os tratamentos PHe30, PHe90, PCe2, PCe24, o tratamento PHe30 apresentou maior crescimento bacteriano quando comparado com PHe90 ($p=0,034$), ocorrendo o mesmo para PCe2 quando comparado com

PCe24 (p=0,000), mostrando que o produto excipiente promoveu redução de viabilidade tempo-dependente para as células em biofilme.

Tabela 2. Médias e desvios-padrão de UFC/mg de biofilme.

Tratamentos	n	Média	Desvios-padrão
Controle	10	2,60E+07 [a]	1,36E+07
PC2	10	0,00 [b]	0,00
PC24	10	0,00 [b]	0,00
PCe2	10	2,11E+07 [a]	8,53E+06
PCe24	10	4,04E+04 [a,b]	4,76E+04
PH30	10	0,00 [b]	0,00
PH90	10	0,00 [b]	0,00
PHe30	10	2,58E+07 [a]	9,45E+06
PHe90	10	1,63E+07 [a]	1,11E+07

Letras distintas indicam diferença estatisticamente significante (p<0,05).

Os biofilmes obtidos apresentaram espessura média de 397 (79,8) μm, com variação média da espessura de 207,1 μm (topo da lâmina) a 575,2 μm (base da lâmina).

1.6 DISCUSSÃO

Os tempos de 30 minutos e 90 minutos utilizados neste estudo para a técnica de clareamento em consultório com PH 35% teve como objetivo simular uma sessão clínica de 3 aplicações de 10 minutos e 3sessões desse procedimento, respectivamente. Os tempos de 2 horas e 24 horas utilizados para a técnica caseira supervisionada com gel de PC 16%,

teve como objetivo simular o tempo mínimo médio preconizado para uso diário (2 horas) e o uso de 2 horas diárias por 12 dias (24 horas). As mesmas justificativas se aplicam aos tempos realizados para os excipientes dos 2 peróxidos (grupo controle negativo).

Os estreptococos do grupo mutans são encontrados na cavidade bucal como células planctônicas e como células organizadas em biofilme. O biofilme é a modalidade mais comum de crescimento das bactérias na natureza, sendo que os organismos presentes nessa estrutura podem diferir basicamente das culturas planctônicas pelos seus padrões fisiológicos, expressão gênica e sensibilidade aos agentes antimicrobianos. Neste estudo, os géis clareadores e seus excipientes mataram as bactérias em fase planctônica para o tempo investigado. As células de microrganismos em estado planctônico são mais sensíveis que aquelas em biofilme [12], sendo que diferentes fatores contribuem para o fenômeno, tais como: a) limitação de nutrientes observada no interior do biofilme, a qual condiciona uma redução da atividade metabólica, diminue a de crescimento celular desses microrganismos e que também reduziria a efetividade de drogas capazes de interferir com a atividade metabólica bacteriana, b) na presença de matriz extracelular agindo como barreira física contra variações físico-químicas no meio circundante e c) pela formação de um fenótipo que modifica a suscetibilidade aos antimicrobianos [13].

O caráter oxidante, peculiar dos agentes clareadores, deriva do fato de que todos eles liberam formas ativas de oxigênio (radicais livres), altamente instáveis, as quais apresentam notável capacidade de reagir com outras substâncias orgânicas [14]. Apesar destes radicais serem responsáveis pela despigmentação da estrutura dental no clareamento, os mesmos interagem de forma prejudicial com diversos tipos celulares.Os tipos de oxigênio reativo são notadamente conhecidos como promotores de injúrias às

células vivas devido ao estresse oxidativo que promovem [14,15], podendo causar danos estruturais no DNA cromossômico, incorrendo em genotoxicidade, citotoxicidade ou mesmo apoptose celular [15,16].

S. mutans responde aos estresses ambientais tais como a oxidação, calor, acidez, salinidade elevada, pelo regulamento específico ou coordenado do nível de proteínas [17]. Essa bactéria é capaz de manter o seu metabolismo em ambientes ácidos como a placa dental submetida aos desafios cariogênicos, fator esse considerado como vantagem seletiva em condições ambientais que freqüentemente excedem os limites para o crescimento de outros organismos [18].

As diferentes formas de vida, sejam superiores ou inferiores, reagem ao estresse oxidativo de forma muito similar, tentando neutralizar os radicais livres por meio de enzimas com atividade catabólica. O sucesso deste processo de neutralização varia segundo o aporte genômico e o estado fisiológico do organismo. O *S. mutans* não possuem citocromos ou proteínas com porção heme, assim como não produz catalase , visto que a produção desse radical livre é uma característica inerente à espécie quando em ambiente aeróbio. Contudo, apresenta comportamento facultativo, graças aos genes *Nox-1* e *Nox-2* que codificam NADH oxidases [19] O ânion superóxido é neutralizado pela enzima superóxido dismutase, produzida pelo *S. Mutans* e por outros estreptococos bucais [20]. Entretanto, mesmo possuindo mecanismos de defesas complexos às injúrias aos quais os *S. mutans* estão expostos na cavidade bucal, estes mecanismos não foram efetivos para sua sobrevida frente ao estresse promovido pelos géis clareadores.

No presente estudo, os géis clareadores foram colocados em contato com o biofilme simulando as condições clínicas nas quais se preconiza que os dentes sejam isolados da

saliva, e o gel fique em contacto com a superfície dental. No caso do clareamento caseiro, onde inexiste profilaxia prévia, o biofilme provavelmente estará presente, havendo contato do gel com microrganismos presentes no mesmo**.** A espessura média (400 μm) dos biofilmes formados *in vitro* permitiu uma avaliação da penetrabilidade dos géis nos biofilmes. Valores de espessura próximos aos aqui reportados também foram obtidos por Zanin et al. [21] e refletem a espessura de biofilmes encontrados em áreas interproximais [22]. O uso da superfície de vidro para crescimento do biofilme é amplamente aceito [23] apresenta algumas vantagens como a fácil avaliação microscópica, equivalência com esmalte dental, possibilita trabalhar com uma superfície relativamente maior e que não necessita de polimentos, além do baixo custo de obtenção. Os biofilmes experimentais deste estudo certamente não refletem a realidade clínica, onde uma maior diversidade de organismos confere peculiaridades bioquímico-fisiológicas que um biofilme monoespecífico não alcança. Porém, como o objetivo desse estudo era avaliar a toxicidade dos géis clareadores sobre uma única espécie, acredita-se que o modelo fornece dados extrapoláveis.

Os genes bacterianos que codificam a formação de biofilmes estão intrinsecamente relacionados com genes que conferem resposta cruzada ou policistrônica aos diferentes tipos de estresse, incluindo o estresse oxidativo[19,24]; logo, era de se esperar que células crescidas em biofilme devam ser mais prontamente responsivas aos radicais livres formados. Os resultados obtidos, no entanto, mostraram que os géis clareadores conseguiram suplantar essa barreira e, hipoteticamente, provocar um estresse oxidativo de tamanha intensidade que reduz a viabilidade celular a níveis tão basais que não foi possível detectar sequer uma célula que gerasse colônias, após o desafio oxidativo.

Dentre os resultados obtidos, o fato dos excipientes dos clareadores provocarem a inviabilidade celular nas células em fase planctônica, podendo ser pela incorporação do glicol, provavelmente propilenoglicol, na formulação dos excipientes. Estudos anteriores mostraram que diferentes tipos de propilenoglicol apresentaram atividade antimicrobiana [25,26], provavelmente interferindo na "atividade de água" dos nos diferentes microrganismos. Propõe-se que, quando da mistura das suspensões bacterianas com os excipientes, o glicol passou a envolver todas as células, passando a exercer plenamente seu poder microbicida. O fato de que somente uma pequena porção das células em biofilme ($p>0.05$), muito provavelmente aquelas localizadas mais superficialmente, veio a morrer após 24 horas de exposição continuada reforça esse argumento, que é subsidiado pelos achados de Marcotte et al. [27] A possibilidade de que o Carbopol□ da formulação pudesse ter participado da erradicação do estreptococo foi afastada, pois os ácidos poliacrílicos são inócuos às bactérias [28].

A literatura traz dados conflitantes, oriundos das diferentes escolas, quanto o efeito dos agentes clareadores sobre a aderência bacteriana. Estudo realizado por Steinberg et al [29] mostra *in vitro* que o PC 10% diminuiu a aderência de *S. mutans, S. sobrinus* e *A. viscosus* na superfície de restaurações. Porém Gurgan et al [6] *in vitro* mostrarou que o PC 10% aumentou a aderência do *S. mutans* na superfície dental. A avaliação *in vitro d*o efeito de clareadores e encontrou aumento da rugosidade superficial do esmalte e o aumento da aderência pelo *S. Mutans* na superfície do esmalte [2]. Assim os clareadores podem afetar as estruturas dentais alterando o processo de formação do biofilme dental [29]. As mudanças causadas na superfície do esmalte e no aumento da aderência bacteriana sugerem que ocorre aumento de suscetibilidade à cárie após o clareamento, porém

Kraigher et al [10], realizado em ratos, confirmaram o efeito bactericida dos agentes clareadores nas bactérias cariogênicas. Adicionalmente, foi demonstrado em estudo *in vitro* que géis clareadores não aumentam a susceptibilidade à cárie em esmalte humano [11]. Os géis clareadores apresentaram efeito bacteriostático *in vitro* para o *S. mutans* e *Lactobacillus,* e diminuição na contagem de *Lactobacillus* na saliva *in vivo* [8]. Em oposição, Alkmin et al [9], analisaram o efeito de dois agentes clareadores *in vivo* contendo PC 10% e PH 7,5 % em *S mutans* através da contagem de bactérias na saliva, indicando que os agentes clareadores não mudaram o número de *S mutans* na cavidade bucal.

Estudos demonstram que ocorrem alterações superficiais e aumento de porosidades em esmalte e dentina depois do clareamento dental[4], estas alterações superficiais do esmalte provocadas pós-clareamento desapareceram em aproximadamente três meses [30]. Uma vez que *S mutans* tem maior afinidade para se aderir em superfícies retentivas, [2] os géis clareadores criaram superfícies que promovem a colonização bacteriana. De forma isolada, estas superfícies demoram um longo tempo para restabelecer-se e o paciente estaria susceptível a maior acúmulo de biofilme sobre a estrutura dental [2,6], sendo este formado por bactérias que sobrevivendo aos peróxidos. Esta hipótese é suportada por estudo que não demonstra diminuição do número de células de microrganismos na saliva pós-clareamento [9], tornando o esmalte rapidamente colonizável "de novo", havendo desta forma uma possibilidade de aumento do risco de cárie e doença periodontal.

Este estudo demonstrou que os peróxidos de hidrogênio e carbamida são bactericidas ao biofilme formado pelo *S mutans*, confirmando o estudo que indica o processo de clareamento possui elevado potencial anticariogênico [7], diminuindo o número de

bactérias, reduzindo a quantidade de placa e gengivite [7,8,10]. Este efeito bactericida pode ser favorecido pela permanência do PH na camada subsuperfícial do esmalte por até três semanas após o clareamento dental [31,32]. Diante destes achados, cabe ainda o questionamento se não ocorre seleção de cepas e se estas não tem sua virulência alteradas em um meio tão oxidativo.

Conclusão: Os agentes clareadores peróxido de carbamida 16% e peróxido de hidrogênio 35% e seus excipientes apresentaram toxicidade para *Streptococcus mutans* crescido em fase planctônica e em biofilme, nos tempos investigados. Os produtos excipientes promoveram redução de viabilidade tempo-dependente para as células em biofilme.

1.7. AGRADECIMENTOS

Os autores agradecem A FGM, produtos Odontológicos, Joinville, SC,Brasil, e Ganatus Equipamentos Médicos Odontológicos Ltda, Ribeirão Preto, SP, Brasil, que gentilmente nos cederam os produtos.

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2. ARTIGO EM INGLÊS

2.1 TITLE PAGE

Viability of *Streptococcus mutans* after dental bleaching treatments

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Key words: Dental bleaching, *Streptococcus mutans*, carbamide peroxide, hydrogen peroxide.

2.2. ABSTRACT

Objective: The aim of this study was to investigate the *in vitro* toxicity of bleaching agents (16% carbamide peroxide (CP) and 35% hydrogen peroxide (HP)) on *Streptococcus mutans* in biofilm and planktonic cells, after 2 and 24 h for CP or 30 and 90 min for HP. **Material and methods:** Experimental groups were formed by exposing the bacteria to the bleaching agents (CP and HP). Negative Control groups (CPe and HPe) consisted of specimens exposed to the excipients. The positive control group consisted of specimens neither exposed to bleaching agents nor to their excipients. The products were mixed with planktonic growth cells or applied on biofilms and immediately incubated for 4 periods of time (2 and 24h) for CP/CPe and (30 and 90 min) for HP/HPe. The mixtures were dispersed, serially diluted and plated in triplicate. The results were submitted to Kruskal-Wallis and U of Mann-Whitney Tests ($p<0.05$). **Results:** In all periods of time, CP, HP, CPe and HPe exhibited a bactericidal effect on planktonic cells, while only CP and HP exhibited a bactericidal effect in biofilm. HPe and CPe showed statistical differences when compared to HP and CP, respectively, in all periods of time. Although PCe24 reduced bacteria, it failed to eliminate it totally. The excipient products exhibited a time-dependent reduction on cell viability or biofilms for all periods of time. **Conclusions:** The commercial bleaching agents and their excipients were toxic to *Streptococcus mutans* in biofilm and planktonic environments, in the two investigated times.

2.3. INTRODUCTION

Carbamide peroxide (CP) and hydrogen peroxide (HP) are widely used for dental bleaching. These products are decomposed into free oxygen, among other substances, which is responsible for the bleaching effect [1]. The bleaching techniques for vital teeth are at-home bleaching and in-office bleaching [2]. At-home bleaching techniques uses 10% to 16% CP, while in-office bleaching techniques uses 30% to 35% HP.

With the extensive use of dental bleaching agents, adverse biological effects have been reported, such as sensibility, dental tissue and mucous alterations [3], morphologic changes on the enamel surface such as erosions, roughness and porosity [4]. Adverse effects of the bleaching agents in bacteria have also been observed. The hydrogen peroxide is known to be bacteriostatic, mutagenic and to promote damage to the bacterial DNA [5]. Genotoxic effect of bleaching agents on bacteria has also been suggested [3].

The increase of *Streptococcus mutans* and *S. sobrinus* cariogenic adherence was reported *in vitro* after exposure to CP and HP [6,2]. This finding suggests an increase in tooth decay susceptibility after bleaching. However, 10% CP and 3% HP, equivalent in oxidative potential [1], have shown a high anticariogenic potential due to an increase in plaque and saliva pH [7], decreasing the number of bacteria and reducing the dental plaque amount [7,8]. Alkmin et al [9] evaluated the *S Mutans* counting before, during and after the bleaching treatment and did not observe a significant change in the counting of this bacteria in the oral cavity. On the other hand, Kraigher et al [10] confirmed the bactericidal effect of the bleaching agents in cariogenic bacteria in a study with rats. Additionally, the vital tooth whitening does not increase caries susceptibility in human enamel [11].

The aim of this study was to investigate *in vitro* the viability of *S mutans* grown in biofilm and planktonic phase after treatments with 16% CP and 35% HP bleaching agents after different periods of continuous exposure. The tested hypothesis was that the treatment with the bleaching gels causes the death of the *S mutans* cells in biofilm and planktonic phase.

2.4. MATERIAL AND METHODS

In this study, the lineage CCT3440 of *Streptococcus mutans* was used. The strain was reactivated in brain and heart infusion (BHI) (Biobrás Diagnósticos Ltda, Montes Claros, MG, Brazil) and incubated at 37°C in 10% pCO_2 for 24 hours. The cells were obtained by centrifugation (Bio Eng Ind, BE 4004,Brazil) (5000 *g*) and washed with sterile distilled water. After being washed twice, the sediments were resuspended in sterile distilled water until turbidity similar to the one in tube # 1 of the McFarland scale (approximately 3×10^8 cells/mL) was achieved.

The tested products included a carbamide peroxide home-bleaching agent ("CP", Whiteness® Perfect 16%, FGM, Joinville, SC, Brazil) and a hydrogen peroxide professional bleaching agent ("HP", Whiteness® HP Maxx 35%, FGM, Joinville, SC, Brazil).

The products used and the groups formed are shown in Table 1. Experimental groups were formed by exposing the bacteria to the bleaching agents CP and HP. Negative control groups consisted of specimens exposed to the excipients (CPe and HPe). The positive control group consisted of specimens neither exposed to bleaching agents nor to their excipients, but submitted to the same protocol of the experimental groups and negative controls. The times of exposition of the bacteria were 2 and 24 h for CP and CPe, and 30

and 90 min. for HP and Hpe. All groups were tested with planktonic growth cells or applied on biofilms.

Table 1- Evaluated products and formed groups.

Product	Product description	Times	Groups
Whiteness® Perfect 16%	Carbamide peroxide16% (±0.5%), carbopol (934-P, 980-P), glycerin, deionizada water, potassium nitrate, sodium fluoride 0.2%, stabilizers, neutralizer agents	2 h	PC2
		24 h	PC24
excipients of Whiteness® Perfect 16%	Carbopol (934-P e 980-P), glycerin, deionizada water, potassium nitrate, sodium fluoride 0.2%,stabilizers, neutralizer agents	2 h	PCe2
		24 h	PCe24
Whiteness® HP Maxx 35%	Hidrogen peroxide 33% (±2%), thickening agent, glycol, deionizada water, stabilizers, neutralizer agents, inorganic fill, pigment	30 min.	PH30
		90 min.	PH90
excipients of Whiteness® HP Maxx 35%	Thickening agent, glycol, deionizada water, stabilizers, neutralizer agents, inorganic fill, pigment	30 min.	PHe30
		90 min.	PHe90

Obs.: materials and information of the ingredients supplied by FGM Ind., Joinville, Brazil.

Planktonic cells exposure the agents bleaching

Aliquots of 100 µL of bacterial suspension were combined with bleaching agents, CP and HP. The HP product was prepared according to the manufacturer's instructions, by mixing a drop of the thickening agent for 3 drops of HP. The HP excipient group (HPe) was formed by the thickening agent only, which was proportioned in the amount of 4 drops (equivalent to 1 g). 1 gram of the CP and CPe was used, respectively. In test tubes aseptically sterilized in a class II-A biological cabin safety (Veco do Brasil, Ind. Com. Equipam. Ltda, São Paulo, SP, Brazil). The treatments were left in 10% pCO_2 at 37°C. Replicas of the CP and CPe treatments were incubated for 2 hours (time minimo of diario use recommended by the manufacturer). In the treatments with HP and HPe, the products were irradiated with an Optilight plus (Gnatus, Ribeirão Preto, SP, Brazil) halogen light curing unit with irradiance of 600 mW/cm² surveyed with radiometer (Gnatus, Ribeirão

Preto, SP, Brazil), before and to the ending of the experiment, for 20 seconds at 3 different spots The mixtures were then incubated for 30 minutes.

After the incubation period, 9 mililiters of Letheen broth (Becton Dickinson and company, Sparks, MD, USA) were added to the mixtures. The tube content was homogenized in a vortex (AP56, Phoenix Ltda, São Paulo, SP, Brazil) and 100µL aliquots were taken and diluted serially. From each tube of the dilution series, 100µL aliquots were taken and applied over 1.6% BHI-agar and incubated in 10% pCO_2 at 37°C for 24 hours. There were bacteria in each group that were not exposed to any of the bleaching agents or to their excipients, forming thus, the control group, submitted to the same stages of the experimental groups and negative controls.

Biofilm exposure the agents bleaching

Glass plates used for microscopy were previously washed, ungreased, sterilized and treated with a 0.1% L-lisina solution. Then, the plates were vertically immersed in sterile 50 mL conic plastic tubes containing 30 mL of Triptic Soy Broth (TSB) (Biobrás, Montes Claros, MG, Brazil) enriched with 5% of glucose. The set plaque-plate was weighted in a digital analytical scale (Bel Mark U210A, Bel Engineering, Inc,Piracicaba, SP, Brazil) (mass #2).

Aliquots of 100 µL bacterial suspension were added to the tubes and then packed in 10% pCO_2 at 37°C. After the 48 initial hours, the plates were transferred to new tubes with the TSB enriched with 5% of saccharose every 24 hours for 5 consecutive days. After the formation period of the cariogenic biofilm, the plates were removed and then quickly immersed in sterile distilled water in order to remove the cells that did not adhere. One of

the sides of each plate was cleaned with sterile gauze. This plate was then deposited in a sterile Petri plate in such a way that the formed biofilm was facing upwards. The group plaque-plate-biofilm was weighted in a digital analytical scale (mass #1).

All the area with the formed biofilm was aseptically covered with a glass plate with each bleaching agent, CP and HP, or with their respective excipients, CPe and HPe, in a c biological cabin safety. The HP product was prepared according to the manufacturer's instruction, by mixing a drop of the thickening agent for 3 drops of HP. 4 drops (equivalent to 1 g) of the excipiente only was for the HPe group. HP and HPe groups were irradiated with an halogen light curing (as described previously). The treatments were left in 10% pCO_2 at 37°C. Replicas of the treatments with the CP and CPe product were incubated for 2 and 24 hours. The treatments with the HP and HPe product were incubated for 30 and 90 minutes. After the incubation period, the glass plates were transferred to new 50 mL sterile conic plastic tubes containing 30 mL of Letheen broth. The tube content was sparsed in an ultrasound bath (Thornton T7, Thornton Inpec Eletrônica Ltda, Ribeirão Preto, SP, Brazil) at maximum power and the plates were then removed. The tubes were homogenized in a vortex and 100µL aliquots were taken and diluted serially. From each tube of the dilution series, 100µL aliquots were taken and applied over 1.6% BHI-agar and incubated in 10% pCO_2 at 37°C for 24 hours. The results were shown in colony-forming units by of *S mutans* milliliteros (CFU/mL) and corrected to colony-forming units by microgram of biofilm (CFU /mg biofilm). The positive control group consisted of bacteria not exposed to any of the bleaching agents or to their excipient, but submitted to the same protocol of the experimental groups and negative controls.

Biofilm Thickness

Glass plates (5) covered with biofilm were used to estimate the thickness of the biofilm. The measurements were done at 10 equally distant spots along the plates. The measurement of the biofilm thickness was done according to the protocol recommended by the Center for Biofilm Engineering of Montana State University (2007), with a LABOPHOT-2 optical microscope (Nikon Optics, Toquio, Japan).

Statistical methods

The results of CFU/mg did not present normal distribution values ($p<0.05$), which suggested the use of the non-parametric Kruskal-Wallis test. The statistical comparisons of the data obtained for the treatment periods for each product and its respective excipients were done by U of Mann-Whitney test. The statistical analysis was done with the SPSS 13.0 software (SPSS Incorporation, Chicago, Il.).

2.5. RESULTS

The commercial products Whiteness® Perfect 16%, Whiteness® HP Maxx 35% and their excipients killed all the grown bacteria in the planktonic phase in both periods studied.

The groups control, PHe30, PHe90, PCe2, PCe24 treatments had a larger bacterial growth when compared to PH30, PH90, PC2, PC24, which did not present any bacterial growth in biofilm ($p<0.05$). Although PCe24 reduced bacteria, it failed to eliminate it totally. (Table 2)

Table 2. Averages and standard deviation of the biofilm CFU/mg.

Treatments	n	Average	Standard Deviation
Control	10	2.60E+07 [a]	1.36E+07
PC2	10	0.00 [b]	0.00
PC24	10	0.00 [b]	0.00
PCe2	10	2.11E+07 [a]	8.53E+06
PCe24	10	4.04E+04 [a,b]	4.76E+04
PH30	10	0.00 [b]	0.00
PH90	10	0.00 [b]	0.00
PHe30	10	2.58E+07 [a]	9.45E+06
PHe90	10	1.63E+07 [a]	1.11E+07

Distinct letters show statistically significant difference ($p<0.05$).

Comparing the PHe30, PHe90, PCe2, PCe24 treatments alone for the time variable, the PHe30 treatment presented a larger bacterial growth when compared to the PHe90 treatment ($p=0.034$). The same happened for PCe2 when compared to PCe24 ($p=0.000$), showing that the excipient products exhibited a time-dependent reduction on cell viability or biofilms for all periods of time.

The obtained biofilms presented an average thickness of 397 (79.8) μm, with an average variation of the thickness from 207.1 μm (top of the plate) to 575.2 μm (base of the plate).

2.6. DISCUSSION

The periods of 30 minutes and 90 minutes used in this study for the in-office bleaching with 35% HP aimed to simulate a one and three clinical sessions of three ten-minute applications each, respectively. The periods of 2 hours and 24 hours used in this study for the supervised home-bleaching technique with 16% CP gel aimed to simulate two-hour

daily sessions of 1 and 12 days, respectively. The same justifies the periods for the peroxide excipients groups.

Streptococcus mutans are found in the buccal cavity as planktonic cells and as cells organized in biofilm. The biofilm is the most common growth modality of the bacteria in nature, being thus that the organisms present in this structure may differ from the planktonic cultures basically by its physiologic types, genic expression and sensitivity to the antimicrobial agents. In this study, the CP and HP bleaching agents, as well as their excipients, killed the bacteria in the planktonic phase in all periods of exposure. The cells of microorganisms in the planktonic state are more sensitive than those in biofilm [12], considering that different factors contribute to the phenomenon, such as: 1) restriction of nutrients observed inside the biofilm, which determines a reduction of the metabolic activity that leads to a slower cellular growth speed of these microorganisms and that also reduces the efficacy of the drugs capable of interfering with bacterial metabolic activity; 2) extracellular matrix presence that acts as a physical barrier against physical and chemical variations in the surrounding medium or 3) a phenotype formation that modifies the susceptibility to the antimicrobials. [13].

The oxidant feature, specific of the bleaching agents, derives from the fact that all of them release active forms of highly unstable oxygen − free radicals − which have a remarkable capability of reacting with other organic substances [14]. Although these free radicals are responsible for the depigmentation of the bleached enamel, they are harmful to several types of live cells specially due to the oxidative stress that they induce [14,15], which cause structural damage to the cromossomic DNA, incurring in genotoxicity, citotoxicity or even cellular apoptosis [15,16].

S. mutans answers to the environmental stresses such as oxidation, heat, acidity, high salinity, by the specific and coordinated regulations of the protein levels [17]. This bacteria is capable of maintaining its metabolism in acidic environments such as the dental plaque submitted to the cariogenic challenges, and this factor is considered a selective advantage in environmental conditions that often exceed the limits for the growth of other organisms [18].

The different forms of life, superior or inferior, react to the oxidative stress in a very similar way, mainly by neutralizing the free radicals through enzymes with catabolic activity. This success of this approach varies according to the genomic contribution and the physiological state of the organism. The *S. mutans* does not have cytochromes or proteins with a hemi portion and does not produce catalase , which production is an inherent feature of the specie when in an aerobic environment. However, it presents a facultative behavior, thanks to the *Nox-1* and *Nox-2* genes that codify NADH oxidases [19]. The superoxide anion is neutralized by the superoxide dismutase, which is an enzyme produced by *S. mutans* and by other buccal streptococcus [20]. However, even with very complex defense mechanisms that protect *S. mutans* against injuries to which it is exposed to in the oral cavity, these mechanisms were not effective for its survival under the stress promoted by the bleaching.

In this study, the bleaching agents were kept in contact to the biofilm, similarly as in the clinical condition, where the teeth must be isolated from saliva and the gel must contact the dental surface. In at-home bleaching, the absence of a previous prophylaxis will preserve the present biofilm, thus promoting a contact of the gel to their microorganisms. The average thickness (± 400μm) of the biofilms may, *a priori*, seem high . Similar values

to the one reported here were also obtained by Zanin et al. [21] for biofilm in interproximal areas [22]. The use of a glass surface for the growth of the biofilm is widely accepted [23], it presents some advantages such as easy microscopic evaluation, similarity to the tooth enamel, it enables to work with a relatively larger surface that does not require polishing, besides the low acquisition cost. The experimental biofilms used in this study certainly do not reflect the clinical reality, where a larger diversity of microorganisms grants biochemical and physiological peculiarities that a monospecific biofilm does not have. Nevertheless, as the aim of this study was to evaluate the toxicity of the bleaching gels on just one bacterial species, the model provides data that can be extrapolated.

The bacterial genes that codify the biofilm formation are intrinsically related with the genes that grant a cross or polycistronic response to the different types of stress, including the oxidative stress [19,24] thus, it was expected that the cells grown in biofilm are more readily responsive to the formed free radicals. However, the results of the present study showed that the bleaching gels are able to overcome this barrier and, hypothetically, cause a high oxidative stress that reduces the cellular feasibility to levels so low that no colonies could were detected.

Another result of this study was the fact that the bleaching excipients also caused cellular inviability for the cells in the planktonic phase. This might be due to the fact that the manufacturer adds glycol, very likely a propilenoglycol, to the excipients formulation. Former studies have shown that different types of propilenoglycol presented antimicrobial activity [25,26] that probably interfere in the “water activity” of the different microorganisms. The fact that just a small part of the cells in biofilm died after twenty four hours of continuous exposition are very likely due to the death of cells located more

superficially, as supported by Marcotte et al [27]. The possibility that the Carbopol might have participated in the streptococcus eradication was discarded because the polyacrylic acids are innocuous to the bacteria [28].

The literature presents conflicting data, proceeding from different schools, regarding the effect of the bleaching agents about the bacterial adherence. Studies show, *in vitro,* that the 10% CP decreases the adherence of the *S. mutans, S. sobrinus* and *A. viscosus* on the surface of the restorations [29]. *In vitro* studies show that the 10% CP increases the adherence of the *S. mutans* on the dental surface [6]. Another study evaluated, *in vitro,* the effect of the bleaching agents and found an increase of the superficial roughness in the enamel and an increase of the adherence of the *S. mutans* on the dental surface [2]. Therefore, the bleaching agents can affect the dental structures, altering the formation of the dental biofilm [29]. The changes caused on the enamel surface and in the increase of the bacterial adherence suggest an increase in the tooth decay susceptibility after the bleaching, although the study of Kraigher et al[10] in rats confirmed the bactericide effect of the bleaching agents in cariogenic bacteria. Additionally, the vital tooth whitening does not produce caries susceptibility in human enamel [11].The bleaching gels presented a bactericide effect *in vitro* for the *S. mutans* and *Lactobacillus* and, as well as a decrease in the *Lactobacillus* counting in saliva *in vivo* [8]. In contradiction, Alkmin et al [9], analyzed *in vivo* the effect of two bleaching agents containing 10% CP and 7.5% hydrogen peroxide in *S mutans*, by counting the bacteria in saliva, which indicated that the bleaching agents do not change the number of *S mutans* in the buccal cavity.

Studies show that there were superficial alterations and a porosity increase in the enamel and dentine after the dental bleaching [4]. These superficial alterations of the

enamel that happen after the bleaching disappear in approximately three months [30]. Once the *S mutans* have more affinity to adhere to retentive surfaces [2], the bleaching gels might create surfaces that promote bacterial colonization. In an isolated way, while these surfaces take a long time to recover, the patient may be susceptible to a greater accumulation of biofilm on the dental structure [2,6], with this biofilm being formed by bacteria that have survived the peroxide. This hypothesis is supported by a study that did not show any decrease in the microorganisms' cells number in saliva after bleaching [9], which makes the enamel quickly colonizable "de novo".

This study demonstrated that the HP and the CP are bactericide to the biofilm formed by the *S mutans*, which confirms the study that indicate that the bleaching process have a high anticariogenic potential [7,9,10]. This bactericide effect may be intensified by the fact that the PH may stay in the sub-superficial layer of the enamel for up to 3 weeks after the dental bleaching [31,32], thereby affecting at an unknown extension the bacteria in the mouth. According to these propositions, it is inquiring that a bacterial strain selection or virulence factors change are feasible to occur in such an oxidative medium.

Conclusion: The viability of *Streptococcus mutans grown i*n biofilm and planktonic phase was totally compromised by the commercial bleaching. The excipients caused a time-dependent reduction of biofilm cell viability for all periods of exposure.

2.7. ACKNOWLEDGEMENTS

We are thankful to the FGM and to the Gnatus Equip. Med. Odon. Ltda for dontion the products.

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APÊNDICE

APÊNDICE 1- TABELAS

Tabela 1 – Descrição de grupos, materiais clareadores para planctônicas

PLANCTÔNICAS			
GRUPO	**GEL CLAREADOR**	**TEMPO**	**n**
CONTROLE	Sem tratamento	Todos os tempos	**20**
PH30	Peróxido de hidrogênio*	30 minutos	**10**
PHe30	Excipiente do Peróxido de hidrogênio*	30 minutos	**10**
PC2	Peróxido de carbamida ¤	2 horas	**10**
PCe2	Excipiente do Peróxido de carbamida ¤	2 horas	**10**

* Whiteness® HP Maxx 35% (FGM); ¤ Whiteness® Perfect 16% (FGM);

Tabela 2 – Descrição de grupos, materiais clareadores para Biofilme

BIOFILME			
GRUPO	**GEL CLAREADOR**	**TEMPO**	**n**
CONTROLE	Sem tratamento	Todos os tempos	**40**
PH30	Peróxido de hidrogênio*	30 minutos	**10**
PHe30	Excipiente do Peróxido de hidrogênio*	30 minutos	**10**
PH90	Peróxido de hidrogênio*	90 minutos	**10**
PHe90	Excipiente do Peróxido de hidrogênio*	90 minutos	**10**
PC2	Peróxido de carbamida¤	2 horas	**10**
PCe2	Excipiente do Peróxido de carbamida¤	2 horas	**10**
PC24	Peróxido de carbamida¤	24 horas	**10**
PCe24	Excipiente do Peróxido de carbamida¤	24 horas	**10**

* Whiteness® HP Maxx 35% (FGM); ¤ Whiteness® Perfect 16% (FGM);

Tabela 3- Componentes dos Géis clareadores fornecidas pelo fabricante

Peróxido de Carbamida Whiteness® Perfect 16% (FGM) Indicação: Clareamento caseiro (moldeira individual)	**Ingrediente Ativo**	Peróxido de Carbamida a 16% +/- 0,5%
	Ingredientes Inativos (excipiente)	Carbopol (934-P e 980-P) Glicerina, Água Deionizada, Nitrato de Potássio, Fluoreto de Sódio (0,2%), Estabilizantes, Agentes Neutralizantes
Peróxido de Hidrogênio Whiteness® HP Maxx 35% (FGM) Indicação: Clareamento em consultório	**Ingrediente Ativo**	Peróxido de Hidrogênio a 33% +/- 2%
	Ingredientes Inativos (excipiente)	Espessante, Glicol, Água Deionizada, Estabilizantes, Agentes Neutralizantes, Carga Inorgânica, Corante FGM01 Corantes para cor verde (mistura)

Tabela 4– Descrição de grupos conforme tratamento e tempo

Controle	1
PHe30	2
PHe90	3
PH30	4
PH90	5
PCe2	6
PCe24	7
PC2	8
PC24	9

Tabela 5- Comparações múltiplas não paramétricas de Kruskal-Wallis

Tratamento	Tratamento	Diferença observada	Diferença mínima (p=0,05)	Significativa (p=0,01)
1	2	1,80	36,23	41,95
1	3	10,20	36,23	41,95
1	4	52,80	36,23	41,95
1	5	52,80	36,23	41,95
1	6	2,80	36,23	41,95
1	7	27,80	36,23	41,95
1	8	52,80	36,23	41,95
1	9	52,80	36,23	41,95
2	3	12,00	36,23	41,95
2	4	54,60	36,23	41,95
2	5	54,60	36,23	41,95
2	6	4,60	36,23	41,95
2	7	29,60	36,23	41,95
2	8	54,60	36,23	41,95
2	9	54,60	36,23	41,95
3	4	42,60	36,23	41,95
3	5	42,60	36,23	41,95
3	6	7,40	36,23	41,95
3	7	17,60	36,23	41,95
3	8	42,60	36,23	41,95
3	9	42,60	36,23	41,95
4	5	0,00	36,23	41,95
4	6	50,00	36,23	41,95
4	7	25,00	36,23	41,95
4	8	0,00	36,23	41,95
4	9	0,00	36,23	41,95
5	6	50,00	36,23	41,95
5	7	25,00	36,23	41,95
5	8	0,00	36,23	41,95
5	9	0,00	36,23	41,95
6	7	25,00	36,23	41,95
6	8	50,00	36,23	41,95
6	9	50,00	36,23	41,95
7	8	25,00	36,23	41,95
7	9	25,00	36,23	41,95
8	9	0,00	36,23	41,95

Valores em vermelho indica diferença estatisticamente significante (p<0,05) entre os tratamentos.

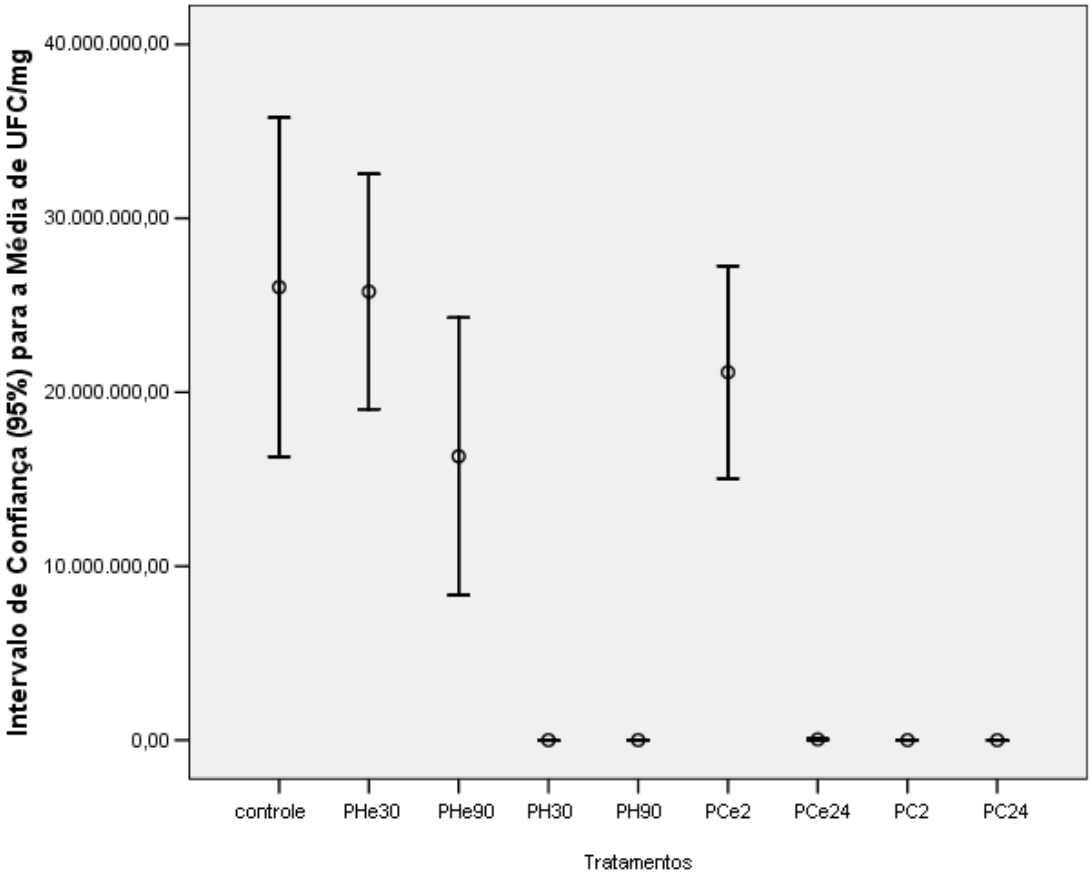
Tabela 6- Média e desvio padrão para Biofilme

	Tratamentos	n	Média	Desvio-padrão
1	**Controle**	22	2,60E+07[a]	1,36E+07
2	**PHe30**	10	2,58E+07[a]	9,45E+06
3	**PHe90**	10	1,63E+07[a]	1,11E+07
4	**PH30**	10	0,00E+00[b]	0,00E+00
5	**PH90**	10	0,00E+00 [b]	0,00E+00
6	**PCb2**	10	2,11E+07[a]	8,53E+06
7	**PCb24**	10	4,04E+04[a,b]	4,76E+04
8	**PC2**	10	0,00E+00 [b]	0,00E+00
9	**PC24**	10	0,00E+00 [b]	0,00E+00

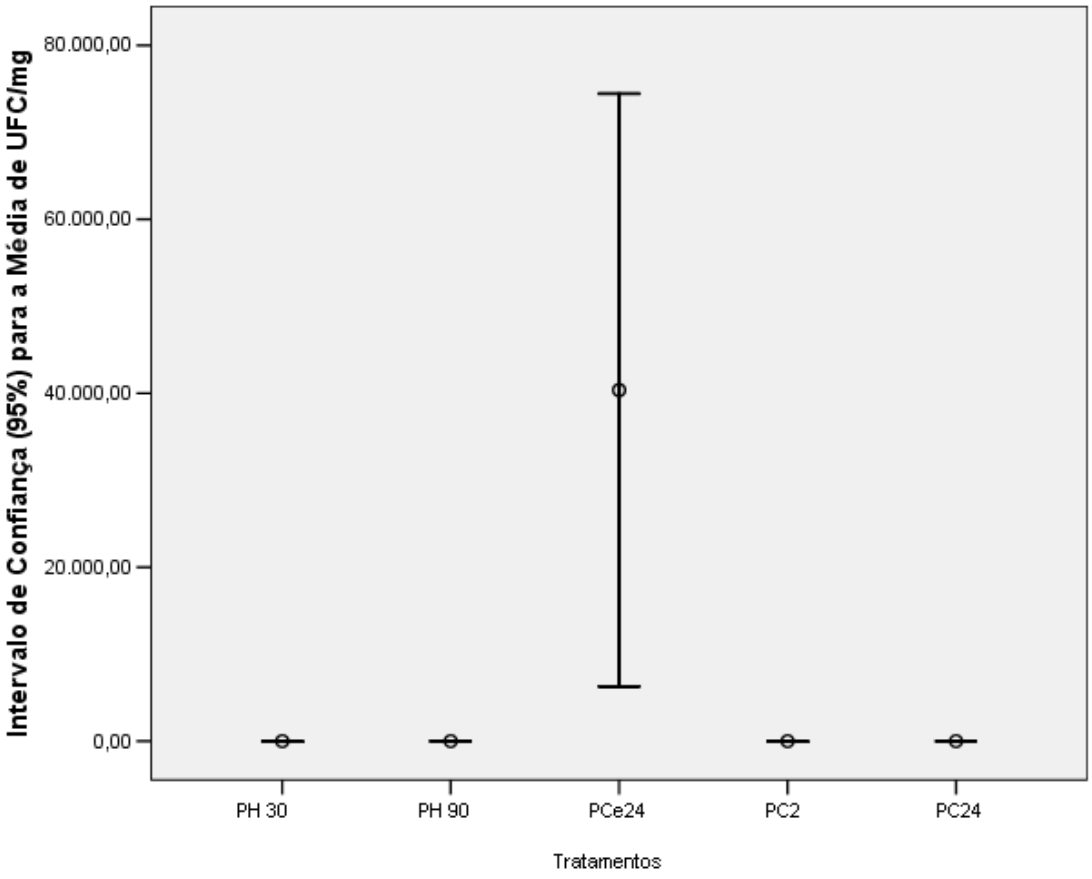
NOTA: Letras diferentes indicam diferença estatisticamente significante ($p < 0,05$).

APÊNDICE 2- GRÁFICOS

Intervalo de confiança (95%) para a média de UFC/mg



Intervalo de confiança (95%) para a média de UFC/mg- para o grupo 7-



ANEXOS

ANEXO 1- NORMAS DA REVISTA

Acta Odontologica Scandinavica's

Instructions for Authors

The scope of the journal covers all aspects of dentistry, both basic and clinical science. In general, analytical studies are preferred to descriptive studies. Articles reporting novel research showing cause and effect relationships for experimental studies and explanatory / associative relationships for those of an observational nature are favored. Hypothesis driven research are encouraged since simple descriptive reports tend to have relatively low scientific priority for publication.

Original research papers, review articles, short communications, and letters to the Editor will be considered for publication. Review articles may be invited by the Editor-in-Chief, but will be subjected to peer review. Proposals for review articles should be discussed with the Editor prior to submission. Short communications should not be longer than two printed pages, and should contain new and important information. Short communications should follow the usual division into Material and methods etc. and have a short abstract.

For more information on most aspects of scientific writing, consult *Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Writing and Editing for Biomedical Publication*, published by the International Committee of Medical Journal Editors and freely available at: www.icmje.org.

Manuscript submission

All submissions should be made in final, fully corrected form online at ***Acta Odontologica Scandinavica's*** **Manuscript Central site**. New users should first create an account. Once a user is logged onto the site submissions should be made via the Corresponding Author Centre.

Receipt of the manuscript in the editor's office will be acknowledged via email. The manuscript will then be distributed to two or more external peer reviewers allotted a time period of four weeks for the review. The reviewers will be anonymous, but the authors' names will be known to the reviewers. If reviewers' opinions conflict, the manuscript may be sent to an extra reviewer. Efforts are made to avoid all types of conflict of interest when the reviewers are selected. The authors must always suggest two reviewers, and also have the option to name unsuitable reviewers.

Cover letter

It must be stated in the cover letter, that the manuscript has not been published, simultaneously submitted, or already accepted for publication elsewhere, and that all authors have read and approved the manuscript. If there is more than one author, the contribution of each author should be stated. Gift authorship is not acceptable. State any conflict of interest related to individual authors' commitments and any project support.

Give reference (registration number or the like) to research ethical permission, if applicable, as locally regulated for research on humans or animals.

Make a full statement about all submissions and previous reports that could be regarded as redundant or as duplicate publication of the same or similar work, and alert the editor if the manuscript includes subjects about which the authors have published a previous report or have submitted a related report to another journal. Refer to and reference any such report in the new paper. Upload copies of such material as supplementary files.

Authors are responsible for obtaining permission from everyone acknowledged by name in Acknowledgment section, because readers may infer their endorsement of the data and conclusions. Therefore, state explicitly that acknowledged persons have seen the text and given their permission to be named.

Manuscript preparation

Editors and reviewers spend many hours reading manuscripts, and therefore they appreciate receiving material that has been carefully prepared in accordance with these Instructions to Authors.

Authors are advised to consult a recent issue of the Journal to be familiar with its style and format. The whole manuscript should be submitted in correct English. Authors whose native language is not English are strongly recommended to obtain assistance from someone proficient in scientific English. Manuscripts not submitted in the proper format or in poor English may be returned without review.

The parts of a manuscript should be as follows: Title page, abstract page, introduction, material and methods, results, discussion, acknowledgments, references, figure legends, tables, and figures, arranged in that order. This division is also appropriate for short communications. For review papers and qualitative studies, other headings may be used as appropriate. Use the built-in system of headings of your word processor to divide up and clarify the text; however, use not more than three levels of division. All text files (including abstract, keywords and figure legends) should be uploaded onto Manuscript central as one MS Word file and in the format described below.

To facilitate the editing of your manuscript, and lessen the time to publication, please adhere to the following simple general guidelines and advices:

1 . Use double spacing throughout and left and margin of at least 3 and 5 cm, respectively leaving space for the reviewer to add notes.

2 . Begin each of the following sections on a separate sheet: title page; abstract and key words; body of the text starting with introduction; acknowledgements; references; figure legends and tables (each on a separate sheet).

3 Use font Times New Roman 12 point. If you use a non-English word processor, program it for English. Be especially careful to use full stop as decimal point, not comma. As spell checker, use American English. Check that diacritic signs are found in names only.

4 . Number the pages consecutively beginning with the title page. Do not number lines.
5 . Use the left alignment feature for a paragraph.
6 . Avoid end-of-line hyphens.
7 The beginning of paragraphs should be properly marked with an indent.
8 . Use a single hyphen to hyphenate compound words and a double hyphen (–) to indicate a dash in the text.
9 . Enter only one space after the full stop at the end of a sentence.
10 . Be consistent: use the same form of units, etc., and key these elements in exactly the same way throughout the manuscript. Prut a space between the digits and the unit, e.g. 5.2 mm.
11 . When emphasizing words (seldom necessary), use the italics feature of your word processor software rather than the underline feature.
12 Do not use the lowercase l for 1 (one) or the uppercase O for 0 (zero), use the proper numerals instead.
13 . Use the space bar only as a word separator, not as a tabulator.
14 . Format tables using the table functions of your word processor.

Title page

The title page has to contain the following information:
1 . A concise but fully informative title (a subtitle may be used in addition, but must be short). Avoid unnecessary words such as "Study on", "An investigation of" etc, and also affirmative wordings. The title should include species used (if appropriate) and any non-standard acronyms or abbreviations should be avoided. Include all information in the title necessary to make electronic retrieval of the article both sensitive and specific. Do not capitalize the title: only the first word and proper nouns have capital initials
2. The full name of each author. Use capital letters. Do not include academic degrees.
3. The departments and institutions to which the work should be attributed, including the city and country, for each author. Using numbering in superscript, key each author to the relevant institution.
4. A short title not exceeding 40 letters and spaces for use as a running head.
5. Give the name and current address of the author to whom correspondence, proofs, and reprints are to be sent. Include telephone and telefax numbers as well as e-mail address. Observe that these data will be published with the paper.
6. The number of figures and tables.

Abstract and key words

Present the abstract limited to 250 words on a separate page. The abstract should briefly state the objective of the investigation, basic procedures, main findings, and principal conclusions. Use only standard abbreviations, and include no references. Structure the abstract using the headings ***Objective, Material and methods, Results***, and ***Conclusions*** in one paragraph.

Give not more than five key words in alphabetical order after the abstract, and, wherever possible, use terms from the Medical Subject Headings list of *Index Medicus*. Do not repeat words from the article title.
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Introduction

Provide a context or background for the study (i.e. the nature of the problem and its significance). Give only strictly pertinent references and do not include data or conclusions from the work being reported. In the last paragraph of the section, state the aim of the study concisely, and, where applicable, give the research hypothesis (but not the null hypothesis). When drawing comparisons for experimental or interventional studies, the latter must always be expressed explicitly.

Material and methods

In this section, describe all methods, materials and subjects so that researchers can readily repeat the study. Use appropriate subheadings for the different sections to obtain clarity. Define the material and equipment used in as detailed manner as necessary by, for example, name, product number and batch, and identify the manufacturer by product, city, and country in parentheses. For common methods, a brief description and a reference may be enough; however, if you deviate from the common method, give a full description. Quantitative estimates of the validity and reliability of the methods are desirable. Report length, height, weight, and volume in metric units (meters, kilograms, or liters), or their decimal multiples. Give temperatures in degrees Celsius and use of the International System of Units (SI) is recommended. Correct unit abbreviations should be used (e.g. "yr", "wk", "d", "h", "min", "s" and "μm"). For many details the Biochemical Journal web site http://www.biochemj.org/bj/bji2a.htm#NOMENCLATURE can be a valuable resource. Scientific names of bacteria, binomials in italics, must be given in full when first mentioned. Subsequent mention may abbreviate genus, taking care that this abbreviation is unambiguous (Staph. or Strep. instead of S.).

Describe subjects participating in the study in detail so that a similar group of subjects can be identified readily. Include eligibility and exclusion criteria and a description of the source population. If applicable, describe ethical aspects here. Indicate that informed consent has been obtained. However, submit such details as the diary number in the covering letter.

When submitting review manuscripts, particularly Systematic Reviews, include a section describing the methods used in locating, selecting, extracting, and synthesizing data. Summarize these methods in the abstract.

Statistics

Conclude the Material and methods section with a paragraph dealing with statistics, if applicable. Name and specify all non-descriptive statistical methods if applicable. The praxis of naming statistical terms and methods is very variable. Therefore, define statistical terms, abbreviations, and most symbols. However, the following abbreviations

may be used without definition: ANOVA (analysis of variance), CI (Confidence interval), r (coefficient of correlation, sample), r^2 (coefficient of determination, sample), R (coefficient of multiple correlation), R^2 (coefficient of multiple determination), CV (coefficient of variation), df (degrees of freedom), n (number of observations), NS (non significant), P (probability (level of significance)), SD (standard deviation), SEM (standard error of the mean), t (statistical datum derived in Student's t test), F (variance ratio). Use mean(SD) for mean and standard deviation, for example "The mean(SD) was 19.2(2.3)". Median, range etc. are written out in text and tables. Specify the computer software used. Authors are advised to consult a statistician or a person with in-depth statistical knowledge.

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Present your results in logical sequence giving the main or most important findings first, usually in past tense, without subjective comments and reference to previous literature. For clarity, the results section may have subheadings. The Result section is not the place for interpretation of the data, and must not include any references to other articles.

Do not repeat in the text, data easily found in the tables or illustrations (double documentation is not acceptable). Restrict tables and figures to those needed to explain the argument of the paper and to assess its support. Use graphs as an alternative to tables with many entries; do not duplicate data in graphs and tables. Avoid non-technical uses of technical terms in statistics, such as "random", "normal," "significant," "correlations," and "sample."

Tables

Present each table on a separate sheet. Do not submit tables as graphics but use the table facility of most word processors. Format the table as you expect it to appear in print and therefore hide internal vertical and horizontal lines. Number the tables consecutively in Roman numerals and give each a short descriptive heading. Give each column a short or abbreviated head. Place explanatory matter in footnotes to the table, not in the heading. Explain all non-standard abbreviations in footnotes to the table.

If data from another published or unpublished source are used, obtain permission and acknowledge fully. As far as possible, tables have to be self-explanatory and understandable without reference to the text of the article.

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Upload figures (illustrations) in electronic form in JPG or TIFF file format only. Optimize the size of the file for printing with 800 DPI for line graphics and 300 DPI for halftone figures, but depending on the character of the article and the quality of those electronic files, the author(s) may be asked to supply files of higher quality. Consult the editor if you have special figures to submit such as camera-ready originals or transparencies. Authors will be charged for the extra cost of reproducing illustrations in color.
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Discussion

The Discussion section should present the interpretation of the findings. This section is the only proper section for subjective comments. Authors are strongly urged to avoid undue repetition of what has already been reported in the results section, or introduced in the introduction.

The last paragraph should be dedicated to the conclusions of the study. There ought to be a correspondence between the aims and hypotheses in the end of the introduction and conclusions.

Acknowledgements

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References

References in languages not understood by all scientists must be avoided. Therefore, only articles written in English should be used as references. This applies also to law texts, other official texts and internet sites. Furthermore, avoid references difficult to retrieve, e.g. old textbooks, journals not indexed in Medline, etc. Avoid references to websites, since these are often changed or removed.

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References in the text

The number of references should not normally exceed 40, and 20–30 references are frequently adequate. However, for review articles there is no upper limit. Number each reference consecutively in the order in which they are first mentioned in the text. Identify references in the text by Arabic numerals in square brackets. If more than one reference, separate them by a comma: [2,4-6,8]. Avoid putting references in tables and figure legends. Give cross-references by the name of the author followed by the appropriate number in parentheses, e.g. "Lagerlöf [1] has reported ...", "Oliveby & Lagerlöf [2] have reported ...", or simply by giving the appropriate number in parentheses, e.g. "As has recently been reported [1] ...". When there are three or more authors, give only the name of the first author, followed by 'et al.': "Oliveby et al. [1] have reported…". Ensure that all listed references are cited in the text.

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At the end of the paper references should be listed in numerical order, in the style shown in the following examples, preceded by the number. For reference list entries, follow the style set out in the examples below. Abbreviate the names of journals in accordance with *MedLine*. List the names of the first six authors in reference-list entries before adding 'et al.'

Here are some examples to follow:

Journals

Standard journal article

[1] Flink H, Tegelberg Å, Thörn M, Lagerlöf F. Effect of oral iron supplementation on unstimulated salivary flow rate: A randomized, double-blind, placebo-controlled trial. J Oral Pathol Med 2006;35:540-7.

[2] Twetman S, Axelsson S, Dahlgren H, Holm AK, Källestål C, Lagerlöf F, et al. Caries-preventive effect of fluoride toothpaste: A systematic review. Acta Odontol Scand 2003;61:347-55.

Article in supplement or special issue

[3] Fleischer W, Reimer K. Povidone iodine antisepsis. State of the art. Dermatology 1997;195 Suppl 2:3-9.

Corporate (collective) author

[4] American Academy of Periodontology. Sonic and ultrasonic scalers in periodontics. J Periodontol 2000;71:1792-801.

Unpublished article

[5] Garoushi S, Lassila LV, Tezvergil A, Vallittu PK. Static and fatigue compression test for particulate filler composite resin with fiber-reinforced composite substructure. Dent Mater 2006. In press.

Books and other monographs

Personal author(s)

[6] Hosmer D, Lemeshow S. Applied logistic regression, 2nd edn. New York: Wiley-Interscience; 2000.

Chapter in book

[7] Nauntofte B, Tenovuo J, Lagerlöf F. Secretion and composition of saliva. In: Fejerskov O, Kidd EAM, editors. Dental caries: The disease and its clinical management. Oxford: Blackwell Munksgaard; 2003. p. 7-27.

No author given

[8] World Health Organization. Oral health surveys - basic methods, 4th edn. Geneva: World Health Organization; 1997.

More information about other reference types is available at www.nlm.nih.gov/bsd/uniform_requirements.html, but observe some minor deviations (no full stop after journal title, no issue or date after volume, etc).

Abbreviations

Use only standard abbreviations. Consult *Scientific Style and Format. The CBE Manual for Authors, Editors, and Publishers*, 7th ed. ISBN 0-9779665-0-X, 2006. Explain any non-standard abbreviations (to be avoided if possible) in the text at first mention. Avoid abbreviations in the title of the article. Give tooth designations in accordance with the two digit system (ISO 3950-1977).

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