

Tese De Doutorado

**LIBERAÇÃO DE DOXICICLINA POR IMPLANTES DENTÁRIOS COM
SUPERFICIE NANO TRATADA, REVESTIDOS COM ÁCIDO POLI-
LÁCTICO-CO-GLICÓLICO (PLGA) PARA LIBERAÇÃO PROLONGADA
DE FÁRMACOS.**

Artur Breno Wanderley Alécio



Universidade Federal de Santa Catarina
Programa de Pós-graduação em Odontologia

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DE FÁRMACOS.**

Tese apresentada ao Programa de Pós-Graduação em Odontologia, do Centro de Ciências da Saúde, da Universidade Federal de Santa Catarina, como parte dos requisitos para obtenção do título de Doutor em Odontologia
Área de Concentração: Implantodontia.

Orientador: Prof. Dr. Ricardo de Souza Magini

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**LIBERAÇÃO DE DOXICICLINA POR IMPLANTES
DENTÁRIOS COM SUPERFÍCIE NANOTRATADA,
REVESTIDOS COM ÁCIDO POLI-LÁCTICO-CO-GLICÓLICO
(PLGA) PARA LIBERAÇÃO PROLONGADA DE FÁRMACOS**


Esta Tese foi julgada adequada para obtenção do Título de “Doutor em Odontologia” e aprovada em sua forma final pelo Programa de Pós graduação em Odontologia da Universidade Federal de Santa Catarina (PPGO/UFSC)

Florianópolis, 26 de março de 2018.

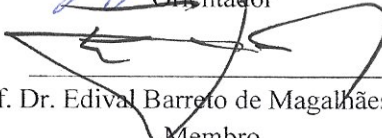


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Coordenadora do Curso de Pós-Graduação em Odontologia.

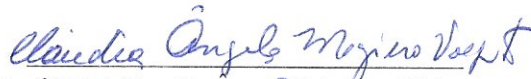
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"Todas as coisas são possíveis àquele que crê."

Marcos 9:23

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LISTA DE ABREVIATURAS E SÍMBOLOS

- < - Menor
- + - Mais
- - Menos
- % - Por cento
- cm**- Centímetro
- x**- Vezes
- g**- Grama
- h**- Hora
- Kv**- Quilovolts
- m**- Massa
- mL**- Microlitros
- mA**- Miliampère
- µg**- Microgramas
- mg**- Miligrama(s)
- mm**- Milímetro (s)
- nm**- nanometro
- p**- Valor de P
- pH**- Potencial hidrogênico
- PLGA**- Ácido polilático co-glicólico
- s** - Segundos
- v** - Volume
- °C**- Graus Celsius
- CC**- corrente contínua
- µm** -Micrometro(s)
- TiO₂**- dióxido de titânio
- IDSN**- Implante dental com superfície de nanotubos
- MTT**- Brometo de 3- [4,5-dimetiltiazol-2-il] -2,5-difenil-tetrazólio
- NH₄F**- Fluoreto de amônio
- DCM**- diclorometano
- TFA**- ácido trifluoroacético
- MEV**- Microscópico eletrônico de varredura

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CAPÍTULO I

RESUMO

Introdução: As superfícies dos implantes dentais comercialmente disponíveis podem ser facilmente contaminadas, resultando em rápida progressão da peri-mucosite e peri-implantite. **Objetivo:** Avaliar, in vitro, o padrão de liberação de doxiciclina por implantes dentais com superfície de nanotubos de titânio (IDSN) em diferentes pHs para examinar as novas técnicas de carregamento e revestimento químico. **Materiais e Métodos:** Nove IDSN foram carregados com doxiciclina e posteriormente revestidos com ácido poli-láctico-co-glicólico (PLGA). A cromatografia líquida de alto desempenho (HPLC) foi utilizada para medir as quantidades de doxiciclina liberada em um período de 30 dias. A citotoxicidade das IDSN foi avaliada por um ensaio utilizando brometo de 3- [4,5-dimetiltiazol-2-il] -2,5-difenil-tetrazólio (MTT). **Resultados:** Os IDSNs carregados com doxiciclina e revestidos com PLGA mostraram uma liberação média de fármaco durante o período experimental para os grupos de pH 7,4 (9,68 µg / mL), pH 6,4 (8,59 µg / mL); e pH 5,4 (13,30 µg / mL) A liberação de doxiciclina de IDSN foi mais rápida a pH 5,4 do que aquelas a pH 6,4 e 7,4 (P = 0,0031 e 0,0034, respectivamente). **Conclusão:** Este novo tratamento de superfície de implantes dentários com nanotubos de titânio e subsequente carregamento de drogas demonstrou biocompatibilidade e liberação sustentada de doxiciclina ao longo de um período de 30 dias. Estudos adicionais são necessários para adotar uma liberação de fármaco estável em um ambiente de pH neutro e justificar uma liberação constante de fármaco em um ambiente de pH ácido.

Palavras-chave: Implante dental, superfície nanotubular. Liberação de drogas, PLGA, Doxiciclina.

ABSTRACT

Introduction: Commercially available dental implant surfaces can be easily contaminated resulting in rapid progression of peri-mucositis and peri-implantitis. **Aim:** Evaluate, *in vitro*, the pattern of doxycycline release from by dental implants with titanium nanotube surface (DINS) at different pHs to examine novel drug loading and chemical coating techniques. **Materials & Methods:** Nine DINS were loaded with doxycycline and subsequently coated with poly lactic-co-glycolic acid (PLGA). High-performance liquid chromatography (HPLC) was used to measure the amounts of released doxycycline in a 30-day period. Cytotoxicity of the DINS was evaluated by an assay using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT). **Results:** The experimental DINS coated with doxycycline and PLGA showed a mean drug release during the experimental period for the groups pH 7.4 (9.68 μ g/mL), pH 6.4 (8.59 μ g/mL); and, pH 5.4 (13.30 μ l/mL) Doxycycline release from DINS was faster at pH 5.4 than those at pHs 6.4 and 7.4 (P= 0.0031 and 0.0034 respectively). **Conclusion:** This new surface treatment of dental implants with titanium nanotubes and subsequent drug loading demonstrated biocompatibility and sustained doxycycline release over a 30-day period. Additional studies are needed in order to adopt a stable drug release at neutral pH environment while warranting a constant drug release in an acidic pH environment.

Keywords: dental implants, nanotube surface, drug delivery, PLGA, doxycycline.

CAPÍTULO II

ARTIGO EM PORTUGUÊS

O Artigo abaixo foi formatado de acordo com as normas para elaboração de artigos do periódico *Jornal of Oral Implantology*.
(Anexo A)

TÍTULO: LIBERAÇÃO DE DOXICICLINA POR IMPLANTES DENTAIS COM SUPERFÍCIE NANOTRATADA, REVESTIDOS COM ÁCIDO POLILÁCTICO-CO-GLICÓLICO (PLGA) PARA LIBERAÇÃO PROLONGADA DE FÁRMACOS.

Título Abreviado: Implantes com superfície nanotratada para liberação de drogas

Categoria do Artigo: Artigo Original

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RESUMO

Introdução: As superfícies dos implantes dentais comercialmente disponíveis podem ser facilmente contaminadas, resultando em rápida progressão da perimucosite e peri-implantite. **Objetivo:** Avaliar, in vitro, o padrão de liberação de doxiciclina por implantes dentais com superfície de nanotubos de titânio (IDSN) em diferentes pHs para examinar as novas técnicas de carregamento e revestimento químico. **Materiais e Métodos:** Nove IDSN foram carregados com doxiciclina e posteriormente revestidos com ácido poli-láctico-co-glicólico (PLGA). A cromatografia líquida de alto desempenho (HPLC) foi utilizada para medir as quantidades de doxiciclina liberada em um período de 30 dias. A citotoxicidade dos IDSNs foi avaliada por um ensaio utilizando brometo de 3-[4,5-dimetiltiazol-2-il]-2,5-difeniltetrazólio (MTT). **Resultados:** Os IDSNs carregados com doxiciclina e revestidos com PLGA mostraram uma liberação média de fármaco durante o período experimental para os grupos de pH 7,4 (9,68 µg / mL), pH 6,4 (8,59 µg / mL); e pH 5,4 (13,30 µg / mL) A liberação de doxiciclina de IDSN foi mais rápida a pH 5,4 do que aqueles a pH 6,4 e 7,4 (P = 0,0031 e 0,0034, respectivamente). **Conclusão:** Este novo tratamento de superfície de implantes dentários com nanotubos de titânio e subsequente carregamento de drogas demonstrou biocompatibilidade e liberação sustentada de doxiciclina ao longo de um período de 30 dias. Estudos adicionais são necessários para adotar uma liberação de fármaco estável em um ambiente de pH neutro e justificar uma liberação constante de fármaco em um ambiente de pH ácido.

Palavras-chave: Implante dental, superfície nanotubular. Liberação de drogas, PLGA, Doxiciclina.

INTRODUÇÃO

Os implantes dentais estão associados a uma alta taxa de complicações; que podem ser de origem biológica e mecânica¹. As complicações biológicas podem ser: a mucosite peri-implantar, uma reação patológica reversível dos tecidos moles peri-implantares; e, peri-implantite, caracterizada pela destruição progressiva do osso ao redor do implante após a osseointegração². Essas complicações biológicas podem ser causadas por alterações inflamatórias induzidas por bactérias nos tecidos circundantes de um hospedeiro susceptível; portanto, as anormalidades no tecido ao redor do implante podem ser o principal motivo para falhas no implante³.

Nas reabilitações com implantes, geralmente a agregação bacteriana começa nos tecidos moles ao redor da coroa do implante. Se não for resolvida, a infecção pode atingir a interface implante-abutment.^{4, 5} A inflamação

pode progredir apicalmente e resultar em perda óssea vertical e horizontal. Este processo de infecção bacteriana peri-implante mostrou ser semelhante à progressão envolvida em doenças periodontais, apresentando características microbiológicas semelhantes.^{3, 5}

Estudos têm demonstrado que peri-implantitis ocorre em 28-56% dos implantes. As opções de tratamento podem ser cirúrgicas ou não cirúrgicas e envolvem processos de descontaminação química e física ⁶. Os métodos não cirúrgicos de tratamento de peri-implantite incluem instrumentação mecânica e o uso de uma variedade de agentes antibacterianos. ⁷ O uso de diferentes agentes antimicrobianos é possível, mas só é eficaz quando aplicado nos estágios iniciais da doença. ⁸ O uso de irrigantes desinfecionais subgingivais e o uso de antibióticos aplicados localmente, como as fibras de tetraciclina, foram empregadas, mas nenhum tratamento

proporcionou um efeito terapêutico conclusivo.^{9,10} A administração sistêmica de agentes antimicrobianos foi testada no tratamento de peri-implantitis,¹¹ porém os resultados foram limitados devido a cepas resistentes de bactérias e doses ineficazes de fármacos.

Os métodos mecânicos convencionais demonstraram ser ineficazes para o debridamento completo do defeito ósseo, bem como sobre a superfície do implante microestruturado contaminado.¹² Assim, a aplicação complementar de antibióticos e anti-sépticos sistêmicos ou locais tem sido geralmente recomendada.¹³ No entanto, devido à potencial resistência aos antibióticos e o efeito geralmente insuficiente dos agentes antimicrobianos para a erradicação bacteriana, bem como os fracos resultados da re-osseointegração após a sua aplicação adjuvante durante a terapia cirúrgica e não cirúrgica da peri-implantite,¹⁴ ainda

são necessárias novas abordagens para o tratamento das doenças peri-implantares.

Os sistemas de liberação local de medicamentos são utilizados na medicina para atingir o microorganismo específico. Uma vantagem da liberação local de antibióticos é que a quantidade total de fármaco utilizada é consideravelmente menor em comparação com a sua concentração após a administração sistêmica. Portanto, os efeitos colaterais, tais como; hipersensibilidade, desconforto gastrointestinal, náuseas, vômitos, colite pseudomembranosa e outros, são menos propensos a ocorrer quando os sistemas locais de liberação de medicamentos são usados. ^{15, 16}

A nanotecnologia foi introduzida na implantodontia, através da modificação de superfície dos implantes dentais por meio do tratamento eletroquímico em eletrólitos contendo flúor, o que resulta na formação de nanotubos de

dióxido de titânio (TiO₂) verticalmente a superfície do implante. Se essas estruturas ocorrem em dimensões apropriadas, elas podem permitir a aderência de células mesenquimais e apoiam o crescimento e a regeneração do tecido ósseo. Além disso, o aumento da superfície e do formato desses nanotubos, podem permitir o carregamento com agentes bioativos ou antimicrobianos; e portanto, servindo como sistemas *in situ* de administração de fármacos, que apresentam vantagens significativas quando comparados com um sistema de entrega sistêmico.¹⁷

A doxiciclina além dos efeitos anti-microbianos, mostrou melhorar a cicatrização de feridas, aumentar os mediadores osteogênicos^{18,19} e reduzir a atividade da collagenase.²⁰ Recentemente, os benefícios da doxiciclina como agente osteogênico foram observados em cirurgias per-radulares *in vivo*,²¹ no tratamento de defeitos infra-ósseos²² e na regulação de osteoclastogênese *in vitro*.^{23, 24}

O ácido poli-lático-co-glicólico (PLGA) tem sido utilizado como veículo de entrega para diversos tipos de antibióticos²⁵ devido ao seu perfil de degradação ajustável e produtos de degradação biocompatíveis. Vários estudos relataram a liberação de antibióticos incorporados ao PLGA ocorrendo durante o período de semanas a meses.²⁶ O PLGA carregado com antibióticos também tem sido utilizado na engenharia de tecidual, através de arcabouços, controlando a infecção, *in vitro*, ao longo de um período de oito semanas.²⁷

Para melhorar as propriedades físicas dos implantes e superar as desvantagens dos tratamentos atualmente disponíveis para as doenças peri-implantares, uma abordagem inovadora é a modificação da superfície de titânio com nanotubos para usar suas vantagens biocompatíveis e servir como reservatório de drogas para entrega local de fármacos. O objetivo deste estudo foi

avaliar, *in vitro*, o padrão de liberação de doxiciclina, em diferentes pHs, por implantes dentários de nanosuperfície, usando uma técnica de carga e revestimento de superfície proposta, bem como o seu comportamento de citotoxicidade.

MATERIAIS E MÉTODOS

Tratamento Nanotubular na superfície dos Implantes dentais

Os implantes dentários pré-fabricados foram limpos por meio de sonicação em acetona (Fisher Scientific, Waltham, MA, EUA) durante 30 min, enxaguados em água deionizada e secos no ar. Os nanotubos foram incorporados perpendiculares às superfícies dos implantes dentários por meio de uma técnica de anodização eletroquímica. A anodização foi realizada em condições otimizadas, determinadas em estudos anteriores. O implante dental com superfície de nanotubos (IDSN) foi anexado a uma fonte de

tensão CC (Keithley 2400 SourceMeter) como o eletrodo de trabalho enquanto a malha de cobre era usada como o contra eletrodo. Ambos os eletrodos foram imersos em mistura de eletrólito de etileno glicol (Fisher Scientific), 0,3% em peso e Fluoreto de amônio (NH₄F) (Fisher Scientific) e 10% em volume de água deionizada. Os dados preliminares mostram que as melhores configurações para anodização das amostras são de 120 V durante 2 horas.¹⁷ As amostras anodizadas nesta configuração mostraram uma liberação de fármaco mais sustentada. Utilizou-se um agitador magnético para agitar o eletrólito à medida que a tensão CC constante de 120 V foi aplicada durante 2 h. A anodização foi realizada à temperatura ambiente.

Caracterização da superfície de Nanotube

As dimensões médias dos nanotubos foram verificadas utilizando uma Microscopia Electrónica de Escaneamento por Emissão (FESEM) (JEOL JSM-6320F). Para

determinar as dimensões dos nanotubos, os DINS foram colocados em uma fita de carbono condutor de dupla face e anexados a um talão de alumínio para imagens. Em seguida, o software ImageJ foi usado para medir as dimensões do nanotubo. Uma imagem microscópica eletrônica de varredura foi obtida a partir de uma amostra após ajuste das configurações de anodização e fabricação das amostras experimentais. A natureza cilíndrica e oca dos nanotubos TiO_2 conforme confirmada por meio de MEV (Figura 1) sugere a possibilidade de servir como transportadora para o uso de drogas e polímeros. Os nanotubos TiO_2 mostraram um diâmetro aproximado de 100nm e comprimento de 12 nm. Durante o estudo de liberação de fármaco (30 dias), a avaliação morfológica de MEV não mostrou alterações que poderiam ter ocorrido durante o experimento, quando comparadas às morfologias de carga pré e pós-fármaco. A estabilidade estrutural da modificação da superfície do nanotubo sugere que ela seja

uma superfície de implante dental promissora para o fornecimento prolongado de fármacos controlados pelo pH.

Desinfecção dos Implante Dentais com superfície nanotubular

Todas as amostras foram limpas em um banho ultrassônico utilizando tricloroetileno como detergente e lavadas duas vezes em etanol absoluto. Depois, a técnica de carga foi feita para os IDSN experimentais.

Carregamento de Doxiciclina e revestimento com PLGA

Nove IDSN foram carregados com doxiciclina. Os IDSN foram imersos em solução de doxiciclina em água desionizada a uma concentração de 50 mg / ml. Após o carregamento de doxiciclina na superfície nanotubular, os IDSN foram revestidos com uma solução de PLGA (60:40) em diclorometano (DCM) a uma concentração de 2% v/v.

Durante o revestimento PLGA, o citrato de trietilo a 5% v/v do peso de PLGA foi utilizado como plastificante.

Processo de carregamento de doxiciclina.

Os DINSs foram submersos em solução de doxiciclina preparada, sonicada (Bransonic 2800 banheira Ultra-som 40kHz, Danbury, Connecticut, EUA) durante 10 minutos, mantida sob vácuo durante 5 minutos e depois seca à temperatura ambiente por 10 minutos em uma capela de fluxo laminar. Todo o processo de carregamento foi repetido 3 vezes para maximizar o carregamento de doxiciclina no DINS.

Processo de revestimento polimérico.

Os IDSN carregados com doxiciclina foram submersos na solução PLGA. Em seguida, foram secos à temperatura ambiente durante 7 horas sob capela de fluxo laminar e, e posteriormente, secos a vácuo durante 9 horas. Todos os

procedimentos de revestimento de polímero foram repetidos 5 vezes para garantir o completo revestimento do IDSN carregado com doxíciclina com PLGA. Os IDSN revestidos foram caracterizados por meio de microscopia eletrônica de varredura (MEV, 15 kV, Cambridge 360) para composição atômica, espessura do revestimento e morfologia.

Análise em microscópio eletrônico de varredura (MEV)

Foram utilizados dois IDSN para análises morfológicas. As alterações da morfologia da superfície do implante foram identificadas por uma MEV (Carl Zeiss EVO 40, Peabody, Massachusetts, EUA) no UTHSC *College of Dentistry Laboratory of Bioscience Research*, seguindo as recomendações do fabricante. Os implantes foram fixados aos suportes para amostras e foram completamente inspecionados pelo MEV a 20,00 kV, com uma

magnificação de 50 x. A análise MEV foi realizada para verificar a homogeneidade da camada de revestimento.

Experimento de Liberação de drogas

As amostras de IDSN preparadas foram divididas em 3 grupos e distribuídas em meios de liberação com pH 5.4 6.4 e 7.4. A liberação *in vitro* de doxiciclina nas soluções tampão foi avaliada durante o período de 30 dias. Foram utilizadas soluções tampão de fosfato e bifosfotalato de pH 5,4, 6,4 e 7,4 como meio de liberação de fármaco. Durante o estudo de liberação de fármaco, as amostras para a análise de doxiciclina liberada foram tomadas em pontos de tempo designados.

Medição das Amostras

A cromatografia líquida de alto desempenho (HPLC, Waters Breeze System) foi utilizada para mensurar a liberação de fármacos. A análise cromatográfica foi

conduzida à temperatura ambiente (25 ° C) numa coluna Waters (150 x 4,6 mm, tamanho de partícula de 5,0 µm) com a fase móvel composta por uma mistura de água/ 0,1% de ácido trifluoroacético (TFA) e uma mistura de acetonitrilo / 0,1% de TFA com uma razão de volume de 60:40, que foi filtrada através de filtro com membrana de 0,2 µm. A taxa de fluxo foi de 1,0 mL / min. o comprimento de onda do detector foi ajustado em 360nm. O volume de injeção foi de 50 µl. As condições aplicadas na HPLC resultaram em um tempo de retenção de 2,0 min para a doxiciclina. Para a preparação da solução padrão foi realizada uma curva de calibração, um miligrama de doxiciclina foi pesado com precisão e diluído em 1 mL de água desionizada para fornecer a solução de reserva (1 mg / mL de solução de doxiciclina). A linearidade entre a concentração de doxiciclina e as áreas de pico integradas foi assegurada na faixa de concentração de fármaco de 1 µg/mL a 100 µg/mL. A curva de calibração obtida mostrou

linearidade dentro da faixa de concentração com um fator de correlação de 0.99917 (Gráfico 2). Antes da injeção, as soluções com as amostras para análise em HPLC foram diluídas 2 vezes com fase móvel e filtradas através de um filtro conduzido por seringa Fluoropore™ (PTFE) (0,45 µm).

Ensaio MTT

A citotoxicidade de IDSN contra os fibroblastos gengival foi avaliada por meio do ensaio de MTT (3-[4,5-dimetiltiazol-2-il] -2,5 difenil tetrazólio e de brometo de tetrazólio. Os fibroblastos gengivais normais foram cultivados e plaqueados (1×10^5 células) em um prato de 6 poços. Foram colocados três implantes de 2 experimentais (IDSN de 100 nm de diâmetro, IDSN de 180 nm) e um grupo de controle em poços individuais contendo fibroblastos anexados e incubados durante 3 dias. No final do período de incubação, os implantes foram removidos e a

viabilidade celular foi determinada pelo ensaio de proliferação de células MTT (Tabela 4).

Análise de dados

O significado das diferenças foi obtido por meio do sistema SAS (SAS Institute Inc (2010). O Sistema SAS, versão 9.3. SAS Institute Inc., Cary: NC). A média média, média, mínima, máxima acumulável e desvios-padrão foram calculados para o estudo de liberação. Um modelo de análise de variância linear generalizada foi ajustado para testar o efeito do pH e do tempo (dias) nas variáveis de resposta. A adequação do modelo foi avaliada através dos coeficientes de assimetria e curtose, o que permitiu a avaliação da adesão dos resíduos à distribuição gaussiana. Os efeitos significativos do pH foram complementados pelo teste pós-hoc de Tukey-Kramer e para o estudo do efeito significativo do tempo, os parâmetros ajustados desse efeito foram considerados como co-variável do modelo

(regressão linear simples). Em todos os testes, o nível de significância de 5% foi adotado.

RESULTADOS

Ensaio de HPLC

O Ensaio de HPLC mostrou uma liberação de doxiciclina durante um período de 30 dias, para os três grupos experimentais.

A doxiciclina exerce atividade anti-colagenase²⁰ no nível local de 1,2-8,1 $\mu\text{g}/\text{mL}$.²⁸ Os resultados mostraram que as IDSN inseridas em solução com pH 5.4 apresentaram uma explosão de liberação de fármaco de 112 $\mu\text{g}/\text{mL}$ nas primeiras 24 horas. Nos dois dias seguintes, a concentração média do fármaco liberado das IDSN reduziu para 45,45 $\mu\text{g}/\text{mL}$. Nos 8 dias seguintes, o valor médio do medicamento liberado foi de 13,0 $\mu\text{g}/\text{mL}$. Nos 17 dias seguintes, o valor médio da liberação do fármaco foi de

5,15 $\mu\text{g}/\text{mL}$. Os últimos 3 dias mostraram um valor médio de liberação de fármaco de 1,42 $\mu\text{g}/\text{mL}$. Todos os valores avaliados individualmente estavam acima da quantidade mínima de fármaco necessária para obter o efeito colagenase da doxiciclina quando emergiam os IDSN carregados em uma solução de pH 5.4 (Tabela 1).

Em relação aos IDSNs que foram inseridos em uma solução de pH 6,4, houve uma explosão de liberação de fármaco de 91,56 $\mu\text{g}/\text{mL}$ nas primeiras 24 horas. Nos dois dias seguintes, a concentração média do fármaco liberado das IDSN reduziu para 22,07 $\mu\text{g}/\text{mL}$. Nos 8 dias seguintes, o valor médio do medicamento liberado foi de 8,17 $\mu\text{g}/\text{mL}$. Nos 17 dias seguintes, o valor médio da liberação do fármaco foi de 3,11 $\mu\text{g}/\text{mL}$. Os últimos 2 dias mostraram um valor médio de liberação de fármaco de 1,26 $\mu\text{g}/\text{mL}$. Todos os valores avaliados individualmente estavam acima da quantidade mínima de fármaco necessária para obter o

efeito colagenase da doxiciclina quando emergiam os IDSN carregados em uma solução de pH 6,4 (Tabela 1).

Quando os IDSN s foram inseridos em uma solução de pH 7,4, houve uma explosão de liberação de fármaco de 96,55 $\mu\text{g/mL}$ nas primeiras 24 horas. Nos dois dias seguintes, a concentração média do fármaco liberado das IDSN reduziu para 26,86 $\mu\text{g/mL}$. Nos 8 dias seguintes, o valor médio do medicamento liberado foi de 4,49 $\mu\text{g/mL}$. Nos 17 dias seguintes, o valor médio de libertação do fármaco foi de 5,78 $\mu\text{g/mL}$. Os últimos 3 dias mostraram um valor médio de liberação de fármaco de 2,01 $\mu\text{g/mL}$. Todos os valores avaliados individualmente estavam acima da quantidade mínima de fármaco necessária para obter o efeito colagenase da doxiciclina quando emergiam os IDSN carregados em uma solução de pH 7,4 (Tabela 1).

Para todos os IDSN carregados e avaliados, a liberação do fármaco nos 3 pHs diferentes (7.4, 6.4 e 5.4) mostrou a

liberação do fármaco acima do intervalo mostrado para induzir a atividade anti-colagenase.

A concentração de fármaco liberado foi maior quando em meios ácidos. A concentração média de fármaco liberado em um período de 30 dias foi de 13.30 $\mu\text{g} / \text{mL}$, 8,59 $\mu\text{g} / \text{mL}$; e 9.68 $\mu\text{g} / \text{mL}$ para os grupos de pH 5,4, 6,4 e 7,4, respectivamente (Tabelas 1, 2 e 3).

Em todos os 3 grupos de pH, houve uma descarga de liberação do fármaco no primeiro dia. O grupo de pH 5,4 mostrou uma maior concentração do fármaco liberado pelo efeito do tempo no pH. Esta droga liberada foi estatisticamente significativa quando comparada com a concentração do fármaco liberado nos outros 2 grupos (Tabela 2 e Gráfico 3). Os grupos de pH 6,4 e 5,4 apresentaram maior liberação de fármaco no período experimental inicial quando comparado ao grupo 7.4; No entanto, esta não foi uma diferença estatisticamente

significante. O grupo de pH 7,4 mostrou maior liberação de fármaco cumulativo; No entanto, não houve diferença estatisticamente significativa entre os grupos (Gráfico 3).

Análise de Microscopia Electrónica de Varredura

A microscopia eletrônica de varredura (MEV) com ampliações de 25x (Figura 2), 50x (Figuras 3) e 500x (Figura 4) mostra a presença intacta do revestimento ácido poli-lático-co-glicólico (PLGA), mesmo após a lavagem inicial com água destilada.

Ensaio MTT

A citotoxicidade de IDSN contra os fibroblastos gengivais foi avaliada por meio do ensaio (3-[4,5-dimetiltiazol-2-il]-2,5 difenil tetrazólio e de brometo de tetrazólio). Os fibroblastos gengivais normais foram cultivados (1×10^5 células) em uma placa de 6 poços. Três

implantes foram colocados nos poços com fibroblastos e incubados durante 3 dias. No final do período de incubação, os implantes foram removidos e a viabilidade celular foi determinada pelo ensaio de proliferação de células MTT. O diâmetro de dois experimentos (100nm e 180nm) de implantes carregados com nanotubos não mostraram diferença estatística em relação ao crescimento celular quando comparados ao grupo controle (Tabela 4).

DISCUSSÃO

A doxiciclina exerce atividade anti-colagenase,²⁰ o que é um efeito desejável em pacientes com infecção periodontal e/ou peri-implantar. É demonstrado que uma dose mínima de 100 mg / dia de doxiciclina administrada de forma sistêmica, resulta no nível local de concentração de fármaco no sulco crevicular variando de 1,2-8,1 µg / ml.²⁸ As técnicas de revestimento de doxiciclina e PLGA propostas para as IDSN experimentais prolongaram a

liberação de fármaco até um período de estudo de 30 dias. Dosagens diárias, que neste estudo, estavam acima da dosagem mínima necessária para que o fármaco exercesse atividade anti-colagenase nos tecidos periodontais.

Os resultados do atual estudo mostraram que a doxiciclina liberada pelos IDSN ocorreu em maior concentração a um pH menor, o que pode ter sido devido à dissolução mais rápida da camada revestida com PLGA do IDSN em um ambiente ácido,^{29,30} liberando assim, uma maior concentração da doxiciclina. Além disso, houve uma maior liberação do fármaco a pH 6,4 no primeiro dia do período experimental. No presente estudo, os implantes imersos na solução de pH 5,4 mostraram uma maior concentração do medicamento liberado como o tempo decorrido. O objetivo desta técnica de carga/revestimento proposta é ter o medicamento disponível na presença de uma infecção, que é aqui simulada por imersão dos IDSN

na solução ácida de pH. Portanto, esse padrão de liberação em um ambiente ácido é aceitável (Gráfico 3). O PLGA proposto e os IDSNs revestidos parecem ser dispositivos promissores de liberação seletiva de fármacos. No entanto, em um ambiente saudável, o pH normal do plasma sanguíneo é 7.4.³¹ Idealmente, as IDSNs não deveriam liberar o medicamento a pH 7.4. No presente estudo, o fármaco foi liberado com um valor médio de 9,68 µl / mL a pH 7,4.

A saliva do paciente diagnosticado com periodontite generalizada crônica, apresenta pH em torno de $6,85 \pm 0,112$.³² A este pH, é esperado que o medicamento seja liberado pelo IDSN. No presente estudo, o valor médio do fármaco liberado pelos IDSNs a pH 6,4 foi de 8,59 µl / mL. Idealmente, os IDSNs devem liberar o fármaco a pH 6,4. No presente estudo, o fármaco foi liberado com um valor médio de 8,59 ug / mL a pH 6,4.

Nos tecidos inflamados, vários mediadores são recrutados para o fluido intersticial formando um exsudato inflamatório. As citocinas presentes no exudado recrutam leucócitos, que ativam o ácido láctico no exsudado, diminuindo o pH.³³ As altas concentrações de ions de hidrogênio do tecido inflamado mostraram diminuir o ph para 5.4. ³⁴ No presente estudo, o valor médio de O fármaco liberado das DINSs a pH 5,4 foi de 13,30 µl / mL. Durante todos os períodos de tempo avaliados neste estudo, os DINS liberam doxiciclina a pH 5,4 acima do intervalo mínimo indicado para exercer atividade anti-colagenase.²⁰

Os antibióticos, como a doxiciclina, são comumente utilizados para prevenir e tratar a peri-implantite. ⁷ A administração sistêmica de antibióticos pode resultar em doses inadequadas do fármaco no sulco crevicular.³⁵ Além da atividade anti-colagenase da doxiciclina, a mesma

mostrou-se exercer potencial regenerativo na regeneração óssea guiada ³⁶ e na osteointegração. ¹⁹

Uma concentração mínima de aproximadamente 1,4 µg/mL de doxiciclina localmente é necessária para ter seus efeitos osteogênicos benéficos.²⁴ O presente estudo mostrou que as dimensões propostas de nanotubos, técnicas de carregamento e revestimento de nanotubos dentários propostos foram capazes de manter uma concentração de fármaco suficiente ou acima do nível mínimo para promover atividades anti colagenase sustentadas por um período de 30 dias (Tabela 1). No ambiente ácido proposto, a concentração média do fármaco liberado foi de 13,30 µm / mL durante o período de 30 dias.

A biocompatibilidade dos IDSNs carregados e revestidos é um fator importante que precisa ser destacado. O PLGA aplicado na superfície do implante não alterou a resposta citotóxica do fibroblasto testado.

O presente estudo mostrou que a doxiciclina carregada/revestida proposta resultou em uma liberação lenta e prolongada de doxiciclina durante um período de 30 dias *in vitro*. Estudos futuros devem focar no controle da explosão inicial da liberação do medicamento para ampliar a vida do medicamento carregado. Além disso, a liberação do fármaco não deve ocorrer em pH neutro e somente ser liberado a um pH mais baixo, simulando um ambiente inflamatório, onde o fármaco seria, em última análise, necessário.³⁰ Assim, um sistema local de entrega de fármacos poderia ser a solução para atingir um efeito antibacteriano local suficiente, na busca de um implante dental que permita a redução ou controle de infecções peri-implantares no início do processo.

CONCLUSÃO

Este novo tratamento de superfície com nanotubos na superfície dos implantes dentais (protocolos de carga /

revestimento de drogas) mostrou biocompatibilidade e uma liberação de doxiciclina a longo de um período de 30 dias. Estudos futuros são necessários para estabilizar a liberação do fármaco pelos implantes, mantendo os implantes inertes a um pH neutro.

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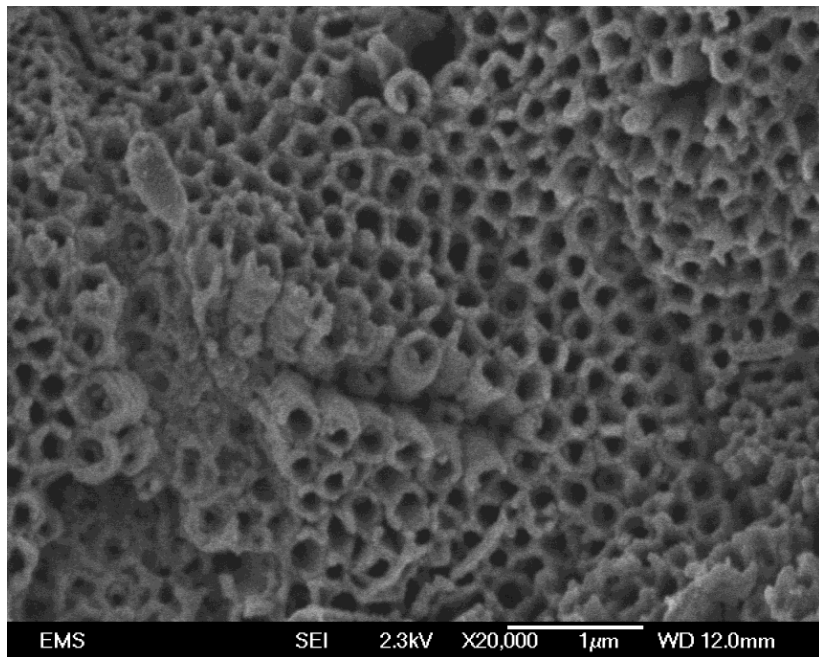
FIGURAS:

Figura 1) Microscopia eletrônica de varredura de um DINS com ampliação de 20 000 x.

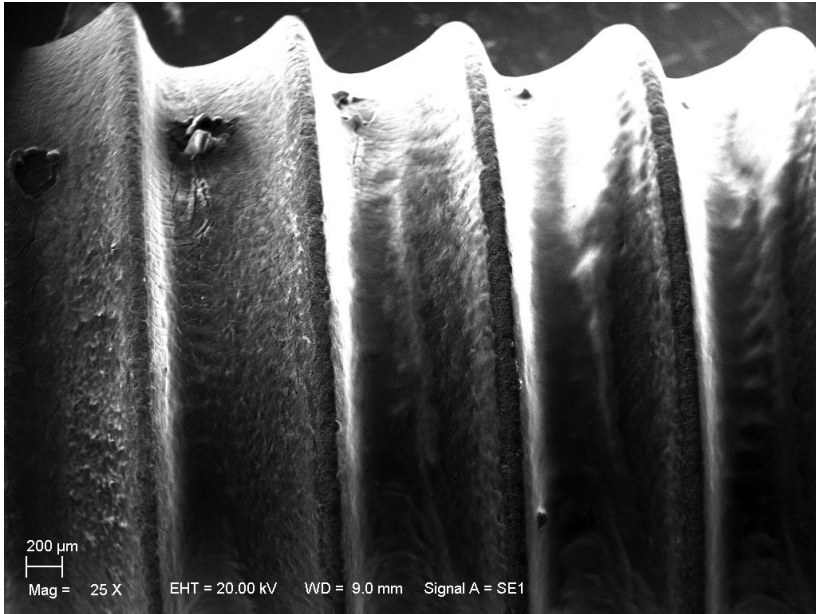


Figura 2) Microscopia eletrônica de varredura de um implante comercialmente disponível revestido com revestimento ácido poli-láctico-co-glicólico com aumento de 25x.

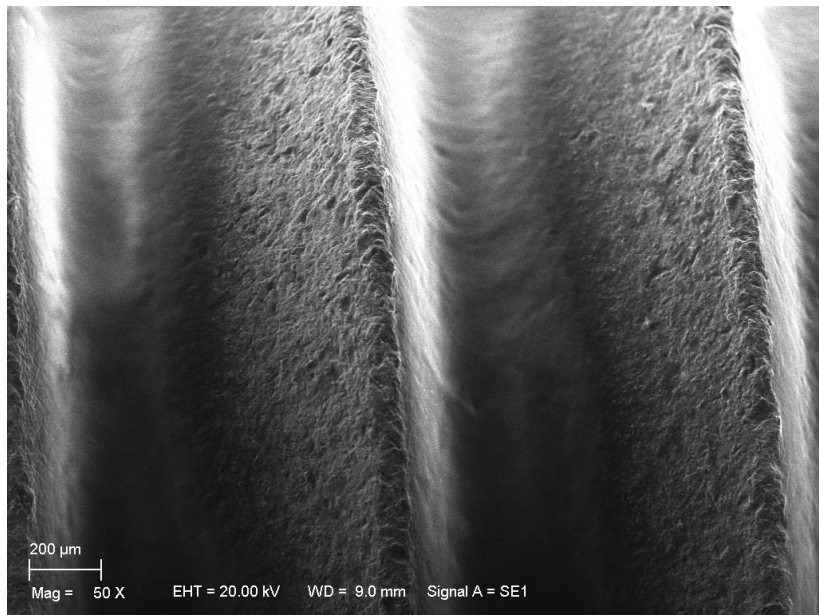


Figura 3) Microscopia eletrônica de varredura de um implante comercialmente disponível revestido com revestimento de poli-ácido láctico-co-glicólico com ampliação de 50x.

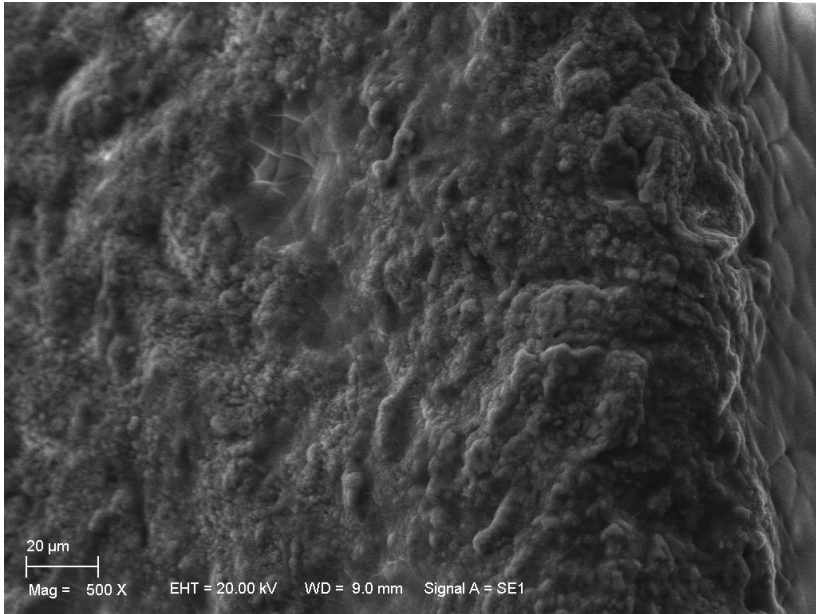


Figura 4) Microscopia eletrônica de varredura de um implante comercialmente disponível revestido com revestimento de ácido poli-láctico-co-glicólico com aumento de 500x.

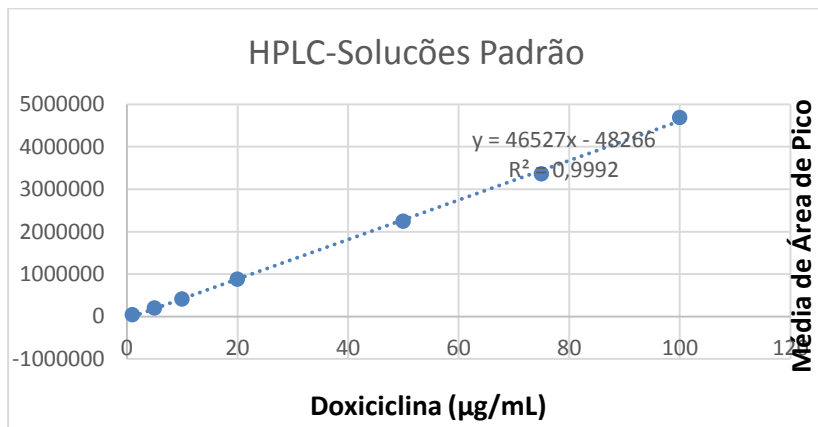
GRÁFICOS:

Gráfico 1) Curva de calibração das soluções padrão utilizadas para análises de HPLC. As áreas integradas de picos de HPLC foram representadas graficamente em função da concentração de doxiciclina em soluções padrão (eixo X).

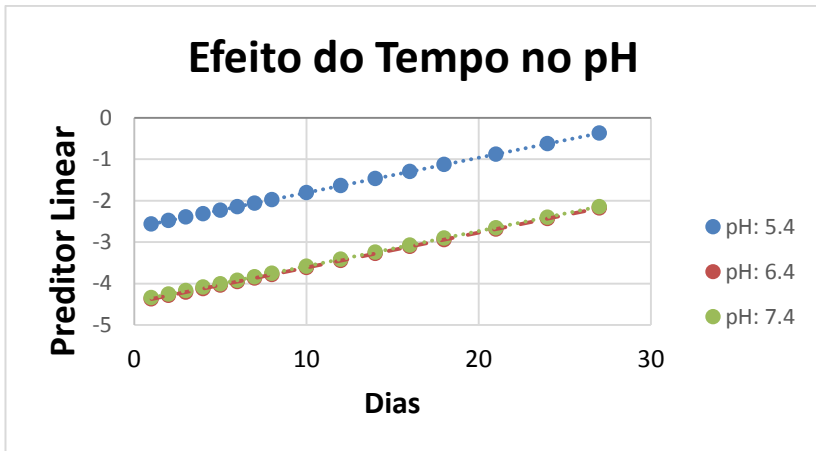


Gráfico 2) Efeito da liberação de doxiciclina ao longo do tempo. Observe, diferença estatisticamente significativa a pH 5,4 quando comparada aos pH 6,4 e 7,4.

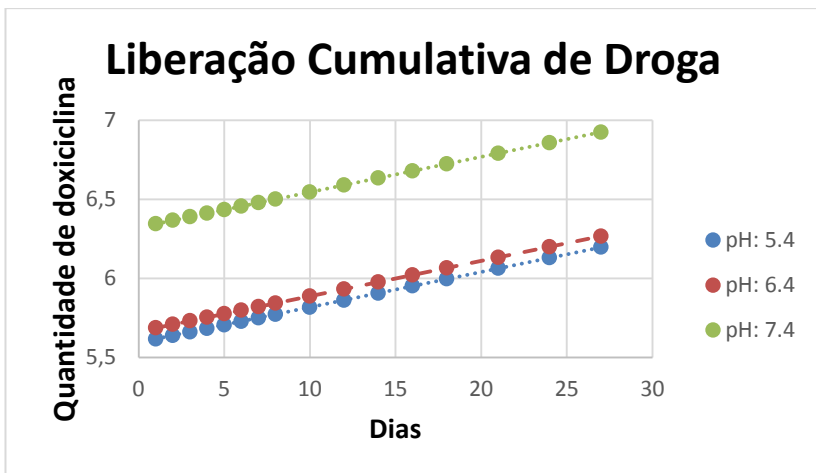


Gráfico 3) Quantidade média acumulada de trinta dias (μg) de doxiciclina liberada a pH 5,4, 6,4 e 7,4.

TABELAS

pH	7.4	6.4	5.4
DIA	Quantidade(μg)	Quantidade (μg)	Quantidade (μg)
1	96,55	91,56	112,44
2	28,21	25,91	59,22
3	25,51	18,23	31,67
4	8,62	9,10	33,16
5	6,90	18,06	19,32
6	6,35	13,18	15,98
7	5,33	7,20	9,68
8	4,43	8,16	11,38
10	4,30	9,65	14,49
12	4,90	11,61	17,56
14	14,62	11,20	21,83
16	21,33	9,82	16,87
18	23,43	8,86	11,90
21	12,94	3,88	6,93
24	11,14	3,78	6,39
27	9,97	3,73	6,12
30	6,03	3,79	4,26

Tabela 1) Quantidade média diária (μg) de doxiciclina liberada pelos DINS de 100 nm de diâmetro sob os pHs 5.4, 6.4 e 7.4.

Análise das Variáveis			
pH	N Obs	Média	DP
5.4	51	13.30	33.42
6.4	51	8.59	24.08
7.4	51	9.68	26.21

Tabela 2) Média, desvio padrão (DP) e número de observações (N Obs) de doxiciclina liberadas sob pHs 5.4, 6.4 e 7.4 em um período de 30 dias.

Diferenças dos pHs			
Múltiplas comparações: <i>Tukey-Kramer</i>			
pH	pH	DP	Valor de P
5.4	6.4	0.3185	0.0031
5.4	7.4	0.3185	0.0034
6.4	7.4	0.3185	0.9946

Tabela 3) O teste estatístico de Tukey-Kramer ajustado para comparações múltiplas foi aplicado para diferenças de pH pH 5,4, 6,4 e 7,4 grupos mínimo de meios quadrados. Valores para a média, erro padrão (DP) e valor de P.

IDSN*	Viabilidade Celular
100 nm diameter IDSN	2.9×10^4
180 nm diameter IDSN	2.24×10^4
Controle**	2.45×10^4

* IDSN: implantes dentários tratados com superfície de nanotubos

** Implante comercialmente disponível sem tratamento superficial de nanotubos (Biohorizons Co, Birmingham, AL, EUA)

Tabela 4) Resultado do teste MTT mostra ausência de citotoxicidade em ambos, teste IDSN e implantes de controle.

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CAPÍTULO III

ARTIGO EM INGLÊS

O Artigo abaixo foi formatado de acordo com as normas para elaboração de artigos do periódico *Jornal of Oral Implantology*. (Anexo A)

TITLE: DOXYCYCLINE RELEASE OF DENTAL IMPLANTS WITH NANOTUBE SURFACE, COATED WITH POLY LACTIC-CO-GLYCOLIC ACID (PLGA) FOR EXTENDED PH CONTROLLED DRUG DELIVERY.

Running Head: Nanotube treated implants for drug delivery

Article Category: Original Article

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ABSTRACT

Introduction: Commercially available dental implant surfaces can be easily contaminated resulting in rapid progression of peri-mucositis and peri-implantitis. **Aim:** Evaluate, *in vitro*, the pattern of doxycycline release from by dental implants with titanium nanotube surface (DINS) at different pHs to examine novel drug loading and chemical coating techniques. **Materials & Methods:** Nine DINS were loaded with doxycycline and subsequently coated with poly lactic-co-glycolic acid (PLGA). High-performance liquid chromatography (HPLC) was used to measure the amounts of released doxycycline in a 30-day period. Cytotoxicity of the DINS was evaluated by an assay using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT). **Results:** The experimental DINS coated with doxycycline and PLGA showed a mean drug release during the experimental period for the groups pH 7.4 (9.68 μ g/mL), pH 6.4 (8.59 μ g/mL); and, pH 5.4 (13.30 μ l/mL) Doxycycline release from DINS was faster at pH 5.4 than those at pHs 6.4 and 7.4 (P= 0.0031 and 0.0034 respectively). **Conclusion:** This new surface treatment of dental implants with titanium nanotubes and subsequent drug loading demonstrated biocompatibility and sustained doxycycline release over a 30-day period. Additional studies are needed in order to adopt a stable drug release at neutral pH environment while warranting a constant drug release in an acidic pH environment.

Keywords: dental implants, nanotube surface, drug delivery, PLGA, doxycycline.

INTRODUCTION

Dental implants are associated with a high complication rate; which vary from biological and mechanical¹. Biological complications can be peri-implant mucositis, a reversible pathological reaction of the peri-implant soft tissues; and, peri-implantitis, characterized by progressive destruction of bone around the implant after osseointegration.² These biological complications may be caused by bacteria-induced inflammatory changes in the surrounding tissues of a susceptible host; therefore, abnormalities in the tissue around the implant may be the main reason for implant failures.³

In cement retained implant crowns, usually bacterial aggregation begins in the soft tissues around the implant crown. If not resolved, the infection may reach the implant–abutment interface.^{4,5} The inflammation may progress apically and result in vertical and horizontal bone loss. This peri-implant bacterial infection process has shown to be similar to

the progression involved in periodontal diseases, presenting similar microbiological characteristics.^{3,5}

Studies have shown that peri-implantitis occurs in 28-56% of the implants. Treatment options may be surgical or non-surgical and involves chemical and physical decontamination processes.⁶ The Non-surgical methods of treating peri-implantitis includes mechanical instrumentation and the use of a variety of antibacterial agents.⁷ The use of different antimicrobial agents is possible but is only effective when applied during the early stages of the disease.⁸ The use of subgingival disinfecting irrigants, and the use of locally applied antibiotics, such as tetracycline fibres, were employed, but neither treatment provided a conclusive therapeutic effect.^{9,10} The systemic administration of antimicrobial agents was tested in the treatment of peri-implantitis¹¹ however, the results were limited due to resistant strains of bacteria and ineffective drug dosages.

Conventional mechanical methods have shown to be ineffective for complete debridement of the bony defect as well as of the contaminated microstructured implant surface.¹² Thus, adjunctive application of systemic or local antibiotics and antiseptics has been generally recommended.¹³ However, due to potential antibiotic resistance and the generally insufficient effect of the antimicrobial agents for bacterial eradication as well as the poor results of re-osseointegration following their adjunctive application during peri-implantitis non-surgical and surgical therapy,¹⁴ novel approaches are still necessary in the treatment of peri-implant diseases.

Drug delivery systems have been used in medicine to target specific microorganism. An advantage of the local delivery of antibiotics is that the total amount of drug used is considerably lower compared to its concentration after systemic administration. Therefore, side-effects, such as; hypersensitivity, gastrointestinal discomfort, nausea, vomiting,

pseudomembranous colitis, and others, are less likely to occur when the local drug release systems are used.^{15, 16}

Nanotechnology has introduced a surface modification to dental implants by electrochemical treatment in fluoride containing electrolytes, which results in the formation of arrayed vertical titanium dioxide (TiO₂) nanotubes. If these structures occur in appropriate dimensions, they may enable the adherence of mesenchymal stem cells and support growth and regeneration of bone tissue. Furthermore, the increased surface area and format of these nano modified surfaces may allow loading with bioactive agents or antimicrobials; hence, serving as *in situ* drug delivery systems, which have significant advantages when compared with a systemic delivery system.¹⁷

Doxycycline has shown to improve wound healing, to increase osteogenic mediators,^{18, 19} and to reduce collagenase activity.²⁰ Recently, the benefits of doxycycline as an osteogenic agent were observed in *in vivo* peri-radicular surgeries,²¹ in the

treatment of infra-bony defects²² and in the downregulation of osteoclastogenesis *in vitro*.^{23, 24}

poly lactic-co-glycolic acid (PLGA) has been used as a delivery vehicle for almost all types of antibiotics²⁵ due to its tunable degradation profile and biocompatible degradation products. Several studies have reported the release of antibiotics from PLGA to occur over the span of weeks to months²⁶. Antibiotic-loaded PLGA has also been incorporated into tissue engineering scaffolds for the purpose of mitigating infection over eight-weeks *in vitro*.²⁷

To improve the physical properties of implants and overcome the disadvantages of the treatments currently available for peri-implantitis, an innovative approach is the modification of the titanium surface with nanotubes in order to use its biocompatible advantages and to serve as a drug reservoir for local drug delivery. The aim of the study was to evaluate *in vitro* the pattern of doxycycline release, at different pHs, by a

nanosurface dental implants, using a proposed loading and surface coating technique, as well as the cytotoxicity behavior.

MATERIALS & METHODS

Nanotube Treatment of The Dental Implants

Prefabricated dental implants were cleaned by means of sonication in acetone (Fisher Scientific, Waltham, MA, USA) for 30 min, rinsed in deionized water and dried in air. Nanotubes were incorporated perpendicular to the dental implant surfaces by means of an electrochemical anodizing technique. Anodization was performed under optimized condition, determined in previous studies. The dental implant with nanotube surface (DINS) were attached to a DC voltage source (Keithley 2400 SourceMeter) as the working electrode while copper mesh was used as the counter-electrode. Both electrodes were immersed in electrolyte mixture of ethylene

glycol (Fisher Scientific), 0.3 wt% NH₄F (Fisher Scientific) and 10 vol.% deionized water. Preliminary data shows the best settings for anodization of samples is 120V for 2 hrs.¹⁷ The samples anodized in this setting showed a more sustained drug release. A magnetic stirrer was used to agitate the electrolyte as constant DC voltage of 120 V was applied for 2h. Anodization was performed at room temperature.

Nanotube Surface Characterization

The average nanotube dimensions were verified using a Field Emission Scanning Electron Microscopy (FESEM) (JEOL JSM-6320F). In order to determine dimensions of nanotubes, the DINS were placed on a double-sided conductive carbon tape and attached to an aluminum stub for imaging. Next, *ImageJ* software was used to measure the nanotube dimensions. A scanning electron microscopic image was obtained from a sample after adjustment of the anodization

settings and fabrication of the experimental samples. The cylindrical and hollow nature of the TiO₂ nanotubes as confirmed by the SEM (Figure 1) suggests the possibility of serving as carrier for drug and polymer loading. The TiO₂ nanotubes showed an approximate diameter of 100nm and length of 12 nm. During the 30-day drug release study, SEM morphological evaluation showed no changes that could have incurred during the experiment, when compared to the pre- and post-drug loading morphologies. The structural stability of the nanotube surface modification suggests it as being a promising dental implant surface for long-term pH controlled drug delivery.

Sterilization of the Dental Implant Nanotube Surface (DINS)

All samples were cleaned in an ultrasonic bath using trichlorethylene as detergent and rinsed two times in absolute

ethanol. Afterwards loading technique was made to the experimental DINSs.

DINS Doxycycline Loading and PLGA Coating

Nine DINS were loaded with doxycycline by dipping DINS in doxycycline solution in deionized water at a concentration of 50mg/ml. After loading doxycycline in nanotubes on surface, DINS were further coated using a PLGA solution in dichloromethane (DCM) at a concentration of 2% w/v . During the PLGA coating, triethyl citrate at 5% w/w of the weight of PLGA was used as a plasticizer.

Doxycycline Loading Process.

The DINSs were submerged in prepared doxycycline solution sonicated (Bransonic 2800 Ultrasonic bath 40kHz, Danbury, Connecticut, USA) for 10 minutes, maintained under vacuum

for 5 minutes, and afterwards dried at room temperature for 10 minutes in a fume hood. The whole loading process was repeated 3 times in order to maximize doxycycline loading on the DINS.

Polymer Coating Process.

Doxycycline-loaded DINS were submerged in PLGA solution. Next, wet DINS were dried at room temperature for 7 hours under the hood and, then, dried under vacuum for 9 hours. The whole polymer coating procedures were repeated 5 times in order to warrant complete covering doxycycline-loaded DINS with PLGA. The coated DINS were characterized by scanning electron microscopy (SEM, 15 kV, Cambridge 360) for atomic composition, coating thickness and morphology.

Scanning Electron Microscope (SEM) Analysis

Two DINS were used for morphological analysis. Implant surface morphology alterations were identified by a SEM (Carl Zeiss EVO 40, Peabody, Massachusetts, USA) at the UTHSC College of Dentistry Laboratory of Bioscience Research following manufacturer's recommendations. Implants were secured to the STEM sample holders, and they were fully inspected by the SEM at 20.00 kV, at 50 x magnification for damage. SEM analysis was conducted to verify thickness of coating layer.

***In vitro* Drug Release**

Prepared DINS samples were divided in 3 groups and assigned for release media of pHs 5.4 6.4 and 7.4. In vitro doxycycline release in those buffer solutions was lasted for 30 days. Biphthalate and phosphate buffer solutions of pHs 5.4, 6.4 and 7,4 were used as drug release media. During the drug release study, samples for the analysis of released doxycycline were taken at designated time points.

Samples Measurement

High-performance liquid chromatography (HPLC, Waters Breeze System) was used for drug release study. The chromatographic analysis was conducted at ambient temperature (25°C) on a Waters column (150 x 4.6 mm, 5.0 µm particle size) with the mobile phase composed of a mixture of water and 0.1% of trifluoroacetic acid (TFA) and a mixture of acetonitrile/0.1% TFA at a volume ratio of 60:40, which was filtered through 0.2µm membrane filter. The flow rate was 1.0 mL/min. the detector wavelength was set at 360nm. Injection volume was 50 µL. The HPLC condition resulted in the retention time of 2.0 min for doxycycline. For preparation of standard solution for a calibration curve, one milligram of doxycycline was accurately weighed and diluted in 1 mL of deionized water to provide the stock solution (1mg/mL doxycycline solution). Linearity between doxycycline concentration and integrated peak areas was assured in the drug concentration range of 1 µg/mL to 100 µg/mL. Obtained

calibration curve showed linearity within the concentration range with a correlation factor of 0.99917 (Graph 2). The sample solutions for HPLC analysis were diluted 2 times with mobile phase and filtered through a Fluoropore™ (PTFE) syringe driven filter (0.45µm) before injection.

MTT Assay

Cytotoxicity of DINS against the gingival fibroblasts was evaluated by means of the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. Normal gingival fibroblasts were cultured and plated (1x10⁵ cells) in a 6 well dish. Three implants from 2 experimental (100 nm diameter DINS, 180nm diameter DINS) and one control group were placed in individual wells containing attached fibroblasts and incubated for 3 days. At the end of the incubation period, the implants were removed and the cell viability was determined by MTT cell proliferation assay (Table 4).

Data Analysis

The significance of differences was obtained by means of sistema SAS (SAS Institute Inc (2010). The SAS System, release 9.3. SAS Institute Inc., Cary:NC). The mean, median, minimum, maximum, cumulative percentage average and standard deviations were calculated for the release study. A generalized linear mixed model of variance analysis was fitted to test the effect of pH and time (days) on the response variables. The adequacy of the model was evaluated through the coefficients of asymmetry and kurtosis, which allowed the evaluation of the adherence of the residues to the Gaussian distribution. Significant pH effects were complemented by the Tukey-Kramer post-hoc test and for the study of the significant effect of time the adjusted parameters of this effect were considered as co-variable of the model (simple linear regression). In all tests the significance level of 5% was adopted.

RESULTS

HPLC Assay

HPLC Assay showed a release of doxycycline during a 30-day period, for all three experimental groups.

Doxycycline exerts anti-collagenase activity²⁰ at the local level of 1.2-8.1 µg/mL.²⁸ The results showed that DINSs inserted in solution with pH 5.4 showed a burst of drug release of 112 µg/mL in the first 24 hrs. In the following 2 days, the mean concentration of the drug released from the DINSs reduced to 45,45 µg/mL. In the following 8 days, the mean value of the released drug was 13,0 µg/mL. In the following 17 days, the mean drug release value was 5,15 µg/mL. The last 3 days showed a mean value of drug release of 1,42 µg/mL. All the values evaluated individually were above the range of drug necessary to obtain the collagenase effect of the doxycycline when emerging the loaded DINS in a 5.4 pH solution (Table 1).

When the DINSs were inserted into a 6.4 pH solution, there was a burst of drug release of 91.56 $\mu\text{g/mL}$ in the first 24 hrs. In the following 2 days, the mean concentration of the drug released from the DINSs reduced to 22.07 $\mu\text{g/mL}$. In the following 8 days, the mean value of the released drug was 8,17 $\mu\text{g/mL}$. In the following 17 days, the mean drug release value was 3,11 $\mu\text{g/mL}$. The last 3 days showed a mean value of drug release of 1,26 $\mu\text{g/mL}$. All the values evaluated individually were above the range of drug necessary to obtain the collagenase effect of the doxycycline when emerging the loaded DINS in a 6.4 pH solution (Table 1).

When the DINSs were inserted into a 7.4 pH solution, there was a burst of drug release of 96.55 $\mu\text{g/mL}$ in the first 24 hrs. In the following 2 days, the mean concentration of the drug released from the DINSs reduced to 26,86 $\mu\text{g/mL}$. In the following 8 days, the mean value of the released drug was 4,49 $\mu\text{g/mL}$. In the following 17 days, the mean drug release value

was 5,78 $\mu\text{g}/\text{mL}$. The last 3 days showed a mean value of drug release of 2.01 $\mu\text{g}/\text{mL}$. All the values evaluated individually were above the range of drug necessary to obtain the collagenase effect of the doxycycline when emerging the loaded DINS in a 7.4 pH solution (Table 1).

For all loaded DINS evaluated, the drug release at the 3 different pHs (7.4, 6.4 and 5.4) showed drug release above the range shown to induce anti-collagenase activity.

The released drug concentration was higher when in acid media. The mean released drug concentration in a 30-day period was 13,30 $\mu\text{g}/\text{mL}$, 8,59 $\mu\text{g}/\text{mL}$; and, 9,68 $\mu\text{g}/\text{mL}$ for the pH groups 5.4, 6.4 and 7.4, respectively (Tables 1, 2, 3 and 4).

In all 3 pH groups, there was an increased discharge of the drug after the first day. The pH 5.4 group showed a higher concentration of the drug released by effect of time on pH. This drug released was statistically significant when comparing the concentration of the drug released in the other 2 groups (Table

2 and Graphic 3). The pH groups 6.4 and 5.4 showed higher drug release in the initial experimental period when compared to group 7.4; however, this was not a statistically significant difference. The pH 7.4 group showed higher cumulative drug released; however, there was no statistically significant difference between the groups (Graph 3).

Scanning Electron Microscopy Analysis

Scanning electron microscopy (SEM) shows presence of and intact the poly lactic-co-glycolic Acid (PLGA) coating, even after the initial wash with distilled water at 25x (Figure 2), 50x (Figures 3), and 500x (Figure 4) magnifications.

MTT Assay

Cytotoxicity of DINS against the gingival fibroblasts was evaluated by means of the MTT ((3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay. Normal gingival fibroblasts were cultured and plated (1×10^5 cells) in a 6-well dish. Three implants were placed in the wells with fibroblasts

and incubated for 3 days. At the end of the incubation period, implants were removed and the cell viability was determined by MTT cell proliferation assay. The two-experimental diameter (100nm, and 180nm) nanotube loaded implants did not show statistical difference in cell growth when comparing to the control group (Table 4).

DISCUSSION

Doxycycline exerts anti-collagenase activity²⁰, which is a desirable effect in patients presenting periodontal and/or peri-implant infections. It has been shown that the minimum dose necessary taken systemically of 100mg/day of doxycycline, results in the local level of drug concentration in the crevicular sulcus that ranges from 1.2-8.1 μ g/mL.²⁸ The proposed doxycycline loading and PLGA coating techniques for the experimental DINS a prolonged drug release up to a 30-day study period. Daily dosages that were above the minimum dosage necessary for the drug to exert anti-collagenase activity

in the periodontal tissues.¹⁸

The results of the current study showed that the doxycycline released from the DINSs was at a higher concentration at a lower pH, which could have been due to the faster dissolution of the DINS's PLGA coated layer in an acidic environment,^{29,}³⁰ releasing a greater concentration of the doxycycline to the embedding solution. In addition, there was a higher release of the drug at pH 6.4 in the first day of the experimental period. In the present study, the implants immersed in the pH 5.4 solution showed a higher concentration of the released drug as time elapsed. The objective of this proposed loading/coating technique would be to have the drug available in the presence of an infection, which is here simulated by immersing the DINSs in the acidic pH solution. Therefore, this release pattern in an acidic environment is acceptable (Graph 3). The proposed PLGA loaded and coated DINS seem to be promising selective drug releasing devices. However, in a healthy environment, the

normal pH of blood plasma is 7.4.³¹ Ideally, the DINSs should not release the drug at pH 7.4. In the present study, the drug was released at a mean value of 9,68 $\mu\text{L}/\text{mL}$ at pH 7.4.

Saliva of patient presenting chronic generalized periodontitis, has shown to be chronic generalized periodontitis was 6.85 ± 0.11 .³² At this pH, it is expected for the drug to be released from the DINS. In the present study, the mean value of the released drug from the DINSs at pH 6.4 was 8,59 $\mu\text{L}/\text{mL}$, Ideally, the DINSs should release the drug at pH 6.4. In the present study, the drug was released at a mean value of 8,59 $\mu\text{g}/\text{mL}$ at pH 6.4.

In the inflamed tissues, several mediators are recruited into the interstitial fluid forming an inflammatory exudate. Cytokines present in the exudate recruit leukocytes, which actively pump lactic acid into the exudate lowering the pH.³³ The high hydrogen ion concentrations of the inflamed tissue, has shown to go down to pH 5.4.³⁴ In the present study, the mean value of

the released drug from the DINs at pH 5.4 was 13,30 µl/mL. During all time periods evaluated in this study, the DINs release doxycycline at pH 5.4 above the minimum range indicated for it to exert anti-collagenase activity.²⁰

Antibiotics, such as doxycycline (Doxy), are commonly used to prevent and treat peri-implantitis.⁷ The systemic administration of antibiotics may result in inadequate dosage of the drug at the crevicular sulcus.³⁵ In addition to doxycycline's anti-collagenase activity,²⁰ it has shown to exert regenerative potential in guided bone regeneration³⁶ and osseointegration.¹⁹

A minimum concentration of approximately 1.4µg/mL of doxycycline is necessary locally to have its beneficial osteogenic effects.²⁴ The present study showed that the proposed dental implant nanotube dimensions, loading and coating techniques were able to sustain a sufficient drug concentration to or above the minimum level to promote anti-

collagenase activities sustained for a 30-day period (Table 1). At the proposed acidic environment, the mean concentration of the drug released was 13,30 $\mu\text{m}/\text{mL}$ for the period of 30 days.

The doxycycline loaded and coated DINS's biocompatibility is an important factor that needs to be highlighted. The PLGA applied to the surface of the implant did not alter the tested fibroblast's cytotoxic response (table 4).

The present study showed that the proposed loaded/coated doxycycline resulted in a slow and prolonged release of doxycycline for a 30-day period *in vitro*. Future studies are in controlling the initial burst of the drug release in order to extend the life of the loaded drug. In addition, the drug release should not occur in neutral pH and only be released at lower pH, simulating an inflammatory environment, where the drug would ultimately be required.³⁰ Thus, a local drug delivery system could be the solution to achieve a sufficient local antibacterial effect, in the search for a dental implant that

would allow reduction or control of peri-implantitis infections at the initiation of the process.

CONCLUSION

This novel dental implant nanotube surface treatment and drug loading/coating protocols showed biocompatibility and a long-term doxycycline release for a 30-day period. Future studies are necessary to stabilize the unloading of the drug from the implants in a controlled manner, maintaining the implants unloaded at neutral pH.

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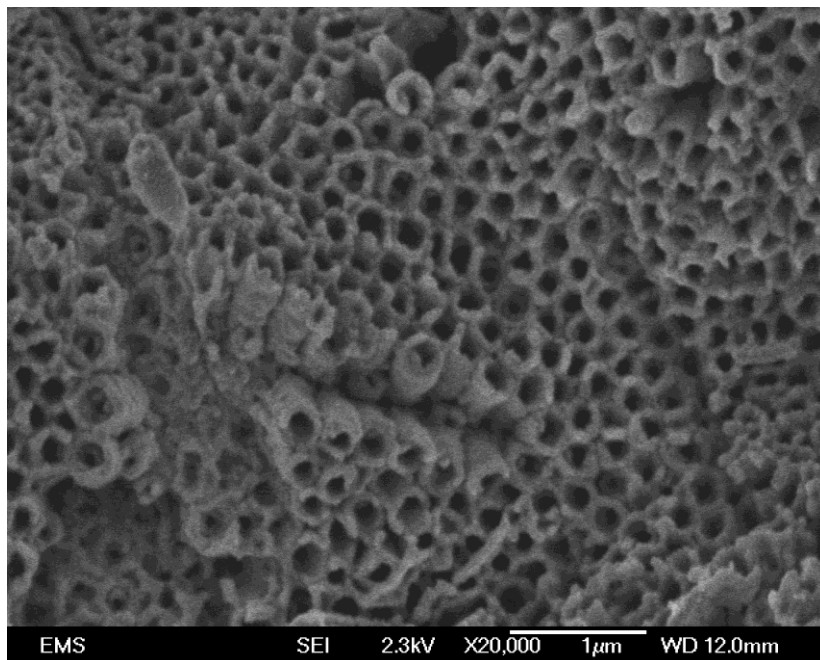
FIGURES

Figure 1) Scanning electron microscopy of a DINS at 20,000x magnification.

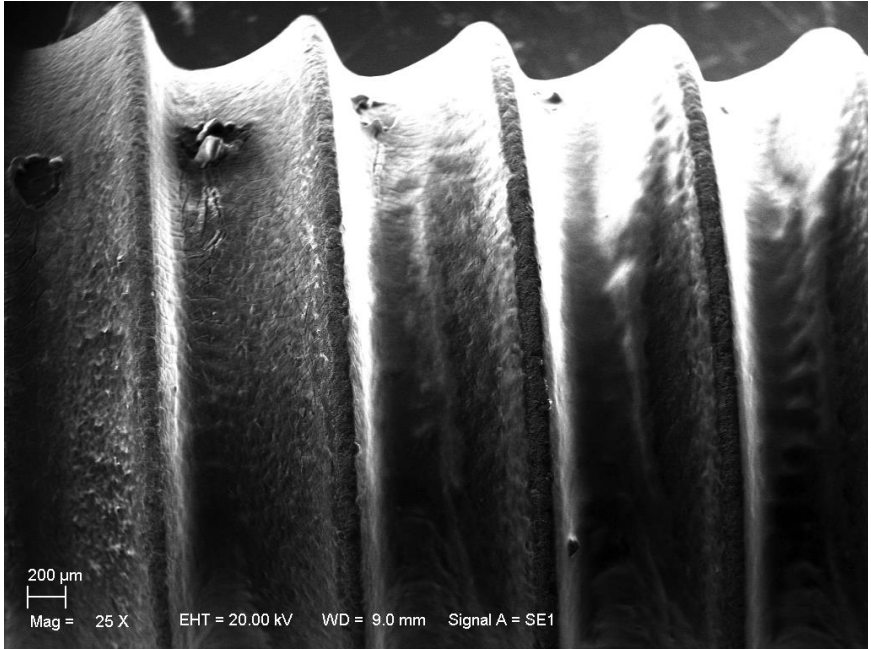


Figure 2) Scanning electron microscopy of a commercially available implant coated with poly lactic-co-glycolic Acid coating at 25x magnification.

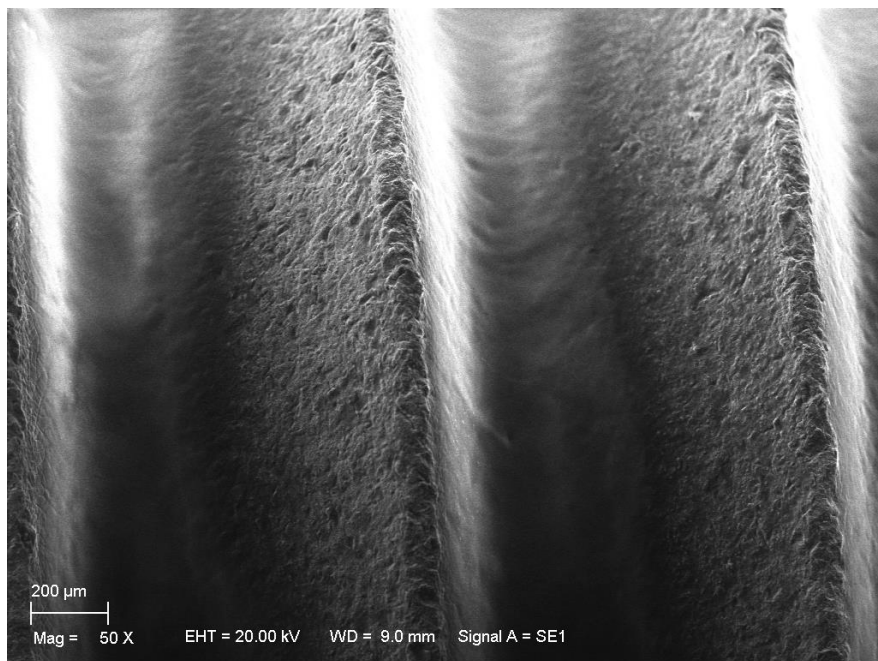


Figure 3) Scanning electron microscopy of a commercially available implant coated with Poly Lactic-co-Glycolic Acid coating at 50x magnification.

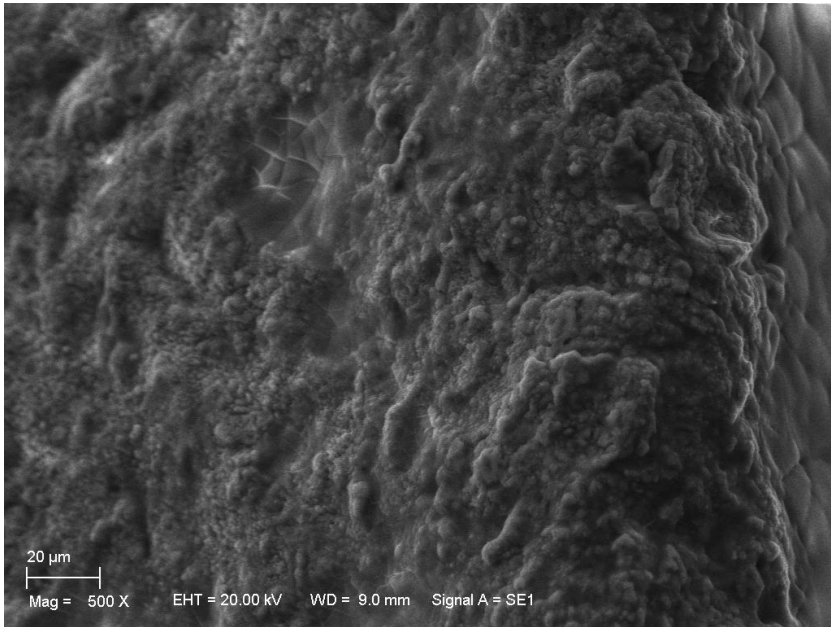
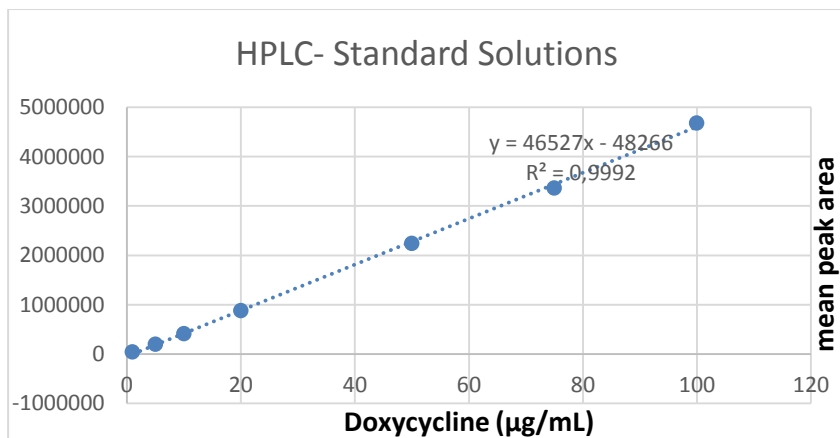
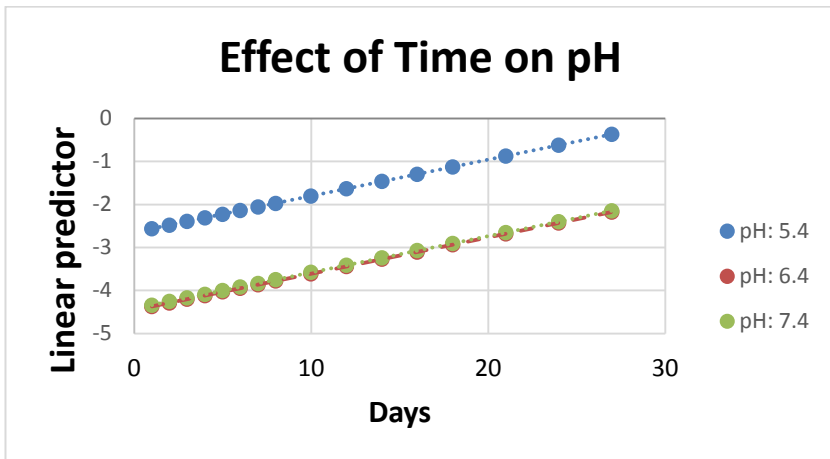


Figure 4) Scanning electron microscopy of a commercially available implant coated with poly lactic-co-glycolic Acid coating at 500x magnification.

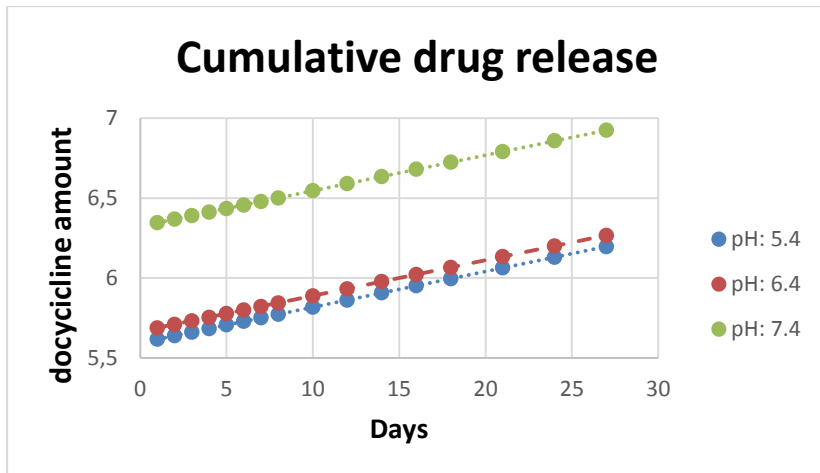
GRAPHS



Graph 1) Calibration curve of the standard solutions used for HPLC analysis. Integrated areas of HPLC peaks were graphed as a function of doxycycline concentration in standard solutions (X-axis).



Graph 2) Effect of doxycycline release over time. Note, statistically significant difference at pH 5.4 when compared to pHs 6.4, and 7.4.



Graph 3) Thirty-day cumulative average quantity (μg) of doxycycline released under pH 5.4, 6.4, and 7.4.

TABLES

pH	7.4	6.4	5.4
Day	Quantity (μg)	Quantity (μg)	Quantity (μg)
1	96,55	91,56	112,44
2	28,21	25,91	59,22
3	25,51	18,23	31,67
4	8,62	9,10	33,16
5	6,90	18,06	19,32
6	6,35	13,18	15,98
7	5,33	7,20	9,68
8	4,43	8,16	11,38
10	4,30	9,65	14,49
12	4,90	11,61	17,56
14	14,62	11,20	21,83
16	21,33	9,82	16,87
18	23,43	8,86	11,90
21	12,94	3,88	6,93
24	11,14	3,78	6,39
27	9,97	3,73	6,12
30	6,03	3,79	4,26

Table 1) Daily average quantity (μg) of doxycycline released from 100 nm diameter DINS under pH 5.4, 6.4, and 7.4.

Variable Analysis			
<i>pH</i>	<i>N Obs</i>	<i>Mean</i>	<i>STD</i>
5.4	51	13.30	33.42
6.4	51	8.59	24.08
7.4	51	9.68	26.21

Table 2) Shows; mean, standard deviation (STD) and number of observations (N Obs) of doxycycline released under 5.4 , 6.4 and 7.4 in a 30-day period.

Differences of pH Least Squares Means			
Multiple Comparisons: <i>Tukey-Kramer</i>			
pH	pH	STE	P value
5.4	6.4	0.3185	0.0031
5.4	7.4	0.3185	0.0034
6.4	7.4	0.3185	0.9946

Table 3) Tukey-Kramer statistical test adjusted for multiple comparisons was applied for pH 5.4, 6.4 and 7.4 groups for differences of pH least square means. Values for mean, standard error (STE) and P value.

DINS*	viable cells/well
100 nm diameter DINS	2.9×10^4
180 nm diameter DINS	2.24×10^4
Control**	2.45×10^4

*DINS: Dental implants treated with nanotube surface

** Commercially available implant without nanotube surface treatment (Biohorizons Co, Birmingham, AL, USA)

Table 4) MTT assay result showing absence of cytotoxicity in both, test DINS and control implants.

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CAPÍTULO V

ANEXO

Normas do periódico *Journal of Oral Implantology* para publicação.

JOURNAL OF ORAL IMPLANTOLOGY

INFORMATION FOR CONTRIBUTORS

The *Journal of Oral Implantology (JOI)* seeks to bring information of interest to scientists, clinicians, laboratory owners and technicians, manufacturers, and educators. This information includes, but is not limited to, scientific articles, basic and clinical research, research reviews, case letters and research letters, and book and article reviews. None of these necessarily represent the opinions or views of the American Academy of Implant Dentistry (AAID), the Editors or members of the Board, or the Institution with which the author(s) are affiliated. Articles are welcomed from all clinicians and scientists. Membership in the AAID is not a prerequisite for submission. This journal does not necessarily offer approval of products advertised within it.

MANUSCRIPT SUBMISSION

See below section entitled **MANUSCRIPT SUBMISSION INSTRUCTIONS** for how to submit manuscripts through the online peer review system. Submitted articles should be for exclusive publication in the *JOI*, with the understanding that they have not been published elsewhere in any form and will not be submitted elsewhere unless rejected. Authors should always retain a complete copy of their manuscripts.

General Publication Policies (applies to all manuscript types)

Manuscripts will be considered for publication only if they

- __are focused;
- __are based on a sound hypothesis and an adequate investigation method analyzing a statistically relevant series, leading to relevant results that back the conclusion;
- __are well written in simple, scientific English grammar and style;
- __are presented with a clear message and containing new information that is relevant for the readership of the journal;

- _add new information to the existing body of knowledge or present new points of view on known treatments, pathologies, or implant related issues; and
- _have contributions by all authors on the paper.

Manuscript Types

Editorials

To be commissioned by the Editor-in-Chief.

Clinical Research Papers & Dental Implant Science Research Papers

Clinical and Dental Implant Science Research papers should be formatted as follows:

- _Title page
- _Abstract
- _Key Words
- _Text
 - Introduction
 - Methods
 - ∅ Subjects (human, animal, in vitro)
 - ∅ Instrumentation/Measurement
 - ∅ Materials
 - ∅ Procedures
 - Results
 - Discussion
 - Conclusion
- _References
- _Tables
- _Captions to figures
- _Figures: charts, illustrations, photos

Please see the Appendix to these instructions for a list of criteria that the reviewers will use to evaluate your paper. This list should guide you when writing your paper for review with JOI.

Clinical Case Letters & Research Letters

Clinical Case Letters and Research Letters are intended to inform, entertain, and inspire the readers. These letters normally contain three parts: 1) Introduction, 2) a description of the case or method and its outcome or result, and 3) discussion. Like all letters, they do not have an abstract. Explanatory and graphic

pictures (up to a maximum of 15) are highly recommended in this format.

- **Clinical Case Letters** should present in the introduction a diagnostic conundrum or a practical clinical problem, and introduce the authors' therapeutic logic. The description of the case should contain the history, examination, investigations, management, and outcome of the case. The discussion should educate the reader and open the debate on the many therapeutic options, and the logic of their choices considering the risks and potential outcomes. Clinical Case Letters should enlighten readers about an interesting clinical situation or therapeutic option. They can also serve as the introduction of a new technique, new material or therapeutic approach, or as the first step before a clinical research protocol. Rarity and overspecialization are not necessary, but originality is highly recommended.

- **Research Letters** should present in the introduction an interesting basic science problem or concept to be examined and discussed, followed by a description of methods of investigation and results, and discussion of the data. This format is limited to simple protocols, which do not require a full research article. This kind of article must be particularly reader-friendly and didactic, even if it refers to a dense basic science topic. This format has to be considered as a pedagogic tool for research communication, and not as a format for the publication of large amounts of data. Research Letters can follow the classical 3-part format (introduction, method, discussion) or use a more open format for the purpose of illustrating a concept. The open format can be used as a discussion on a hot research topic or as an introduction to new research perspectives.

Review Papers

Review papers in *JOI* are normally submitted by invitation, but we do consider unsolicited submissions. The purpose of a Review is to bring the reader up-to-date with research in a particular aspect of implant dentistry, highlighting areas of special interest and progress. Because the readership of *JOI* is wide-ranging it is essential that the Review is easily comprehensible to a nonspecialist in the discipline. However, the article should also

aim to provide an authoritative in-depth discussion of current progress and problems and should not consist of a laborious report that includes every paper in the area.

The author should not be concerned with providing a comprehensive list of references; references of importance and particular interest are all that are required. The author should identify areas in the field where further developments are impending or of urgent need, and any areas (such as techniques) that may be of consequence to implant dentistry. Please note that Reviews in *JOI* should not contain any original research.

Mini-Review Papers

Mini-reviews are highlights or summaries of research in an evolving area in implant dentistry from the previous 2–3 years. Mini-reviews are not intended to be comprehensive overviews; rather, they are meant to highlight recent and important developments in a specific subject area.

Mini-reviews should not include unpublished original research and should set the topic in the context of the relevant literature. A small amount of speculation of possible upcoming developments is appropriate in the Conclusions section of the paper.

Letters to the Editor

JOI welcomes Letters to the Editor. To keep the letter timely and relevant the editorial staff will expedite submission of Letters to the Editor. Only letters of the highest quality will be published, and the following guidelines must be adhered to:

- _Letters are meant to be focus pieces and, therefore, are limited to no more than **600 words**. One reference should include a reference to the *JOI* article being addressed.
- _It is recommended that you limit your letter to one or two important and critical points to which you wish to provide a clear and precise discussion regarding the previously published article.
- _One should support all assertions by peer reviewed literature, which should be primary research or large clinical studies rather than a case report.
- _Please include any financial disclosures at the end of the letter. This would include any potential conflicts of interest not just related to the specific content of your letter, but also the content of the *JOI* article and other related areas.
- _Please recognize that letters that are essentially in agreement with the author's findings and offer no additional insights or

provide little new information for publication. Likewise, letters that highlight the writer's own research or are otherwise self-promotional will receive a low publication priority.

- _There may be a need for additional editing. Should editing be required, the letter will be sent back to the author for final approval of the edited version.
- _It is important to use civil and professional discourse. It is not advisable that one adopts a tone that may be misconstrued to be in any way insulting.
- _Letters that are anecdotal are not acceptable for publication. While personal experiences can have great value in patient care, it is generally not strong evidence to be placed in a Letter to the Editor.

Book Review

A review of a book should be no more than **400 words**.

Article Review

A review of a journal article should be no more than **400 words**.

Manuscript Preparation

General Comments

JOI style is based on the *AMA Manual of Style*, 10th edition.

Some specifics are noted below. Papers should be submitted in this style. Failure to do so will result in the paper being immediately returned to the author, and may lead to significant delays in publication. Spelling is that of American usage. Papers should be typed in Times New Roman, 12-point font, with text double-spaced and with a margin of at least 1 in. (3 cm) all round. Using these formatting specifications, the number of printed (published) pages may be estimated using the following equation:

No. of manuscript pages ÷ 2.5 = No. of printed pages

Headings

Headings appropriate to the nature of the paper enhance readability. They should be kept to a minimum and may be removed by the Editors. Only two categories of headings should be used: 1st level headings should be typed in all capital letters; 2nd level headings should be typed in lower case with an initial capital letter.

Quantitative Analysis

If any statistical methods are used, the text should state the test or other analytical method applied, basic descriptive statistics,

critical value obtained, degrees of freedom, and significance level (eg, ANOVA, $F = 2.58$; $df = 4.58$; $P < .001$). If a computer data analysis was involved, the software package and manufacturer should be mentioned. Descriptive statistics may be presented in the form of a table or included in the text.

Abbreviations, Symbols, and Nomenclature

Only standardized or generally accepted terms should be used. Abbreviations must be defined when initially used in the text. For further details concerning abbreviations, see Baron DN, ed. *Units, Symbols, and Abbreviations: A Guide for Biological and Medical Editors and Authors*. London: Royal Society of Medicine, 1988. The minus sign should be -. If a special designation for teeth is used, a note should explain the symbols. Scientific names of organisms should be binomials, the generic name only with a capital, and should be in italicized font. Microorganisms should be named according to *Manual of Clinical Microbiology*. 10th ed. Versalovic J, Carroll KC, Funke G, Jorgensen JH, Landry ML, Warnock DW, eds. Washington DC: American Society of Microbiology; 2011.

Drugs

Use only generic (nonproprietary) names in the text. Suppliers of drugs used may be named in the Acknowledgments section.

Gender References

Do not use “he”, “his”, “she”, or “her” when the sex of the person is unknown; use the term “the patient” or “patient” etc. Avoid alternatives such as “he/she”. Patients should not be automatically designated as “she”, and doctors as “he”.

Tooth Numbers

When authors wish to list tooth numbers, edentulous sites, or implant locations, *JOI* requires the use of the ADA’s Current Dental Terminology, 2011-2012. This system assigns #1 to the maxillary right 3rd molar and moves around the upper arch to #16, the maxillary left 3rd molar, then continues with the mandibular left 3rd molar as #17, and ends with the lower right 3rd molar as #32. See <http://www.ada.org> for more information.

Manuscript Files to Include in Submission

Cover Letter

The cover letter should contain the following information in the form of a letter addressed to the Editor-in-Chief:

- _Why the paper is being submitted

- What each of the authors contributed to the paper
- The complete contact information for the corresponding author

Title Page

JOI conducts double-blind reviews of all submitted articles. Each submission should include a document, separate from the manuscript, that contains the following information:

- Full article title
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- All author names (in the exact same order as the names are entered into the submission form in the peer review system)
- Earned degrees (PhD, DDS, etc) for all authors
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- Complete contact information for the corresponding author (address, telephone/fax numbers, e-mail address)
- Acknowledgments
- Conflict of interest statement

Please note that the qualifications and professional titles of the authors will not be included in the published paper. The name of the institution where the research was performed also will not be included in the published paper.

Manuscript

Assemble the manuscript in the following order, with each item beginning a new page. Please turn on line numbering in the Word file.

- **Abstract & Key Words (not applicable for Case Letters and Research Letters)**
 - 250 words or fewer
 - Do not use subheadings or abbreviations
 - Should be one continuous paragraph
 - Must contain all relevant information, including results and conclusion
 - Should contain 6 or fewer key words

- **Body Text.** Please ensure that the body text of your paper conforms to the following structure: Introduction, Materials and Methods, Results, Discussion, and Conclusion.
 - *Introduction*
 - ∩ Present the type and extent of the problem

studied.

- ✎ Review briefly the relevant literature.
 - ✎ State the rationale for the study.
 - ✎ Explain the purpose in writing the paper.
 - ✎ State the method of investigation and the reasons for the choice of a particular method.
 - ✎ Write in the present tense.
- *Materials and Methods* ▪ ✎ Give the full details but limit references.
- ✎ Write in the past tense.
 - ✎ Include exact technical specifications, quantities, and generic names.
 - ✎ Limit the number of subheadings, and use the same subheadings in the results section.
 - ✎ State the statistical analysis used.
 - ✎ Indicate that the methodology was reviewed by an independent statistician (for Clinical Research and Dental Implant Science Research papers only)
- ✎ Do not mention the investigators' qualifications or the institution where the work was performed.
- *Results* ▪ ✎ Do not describe methods.
- ✎ Present results in the past tense.
 - ✎ Present representative data rather than endlessly repetitive data.
 - ✎ Use tables where appropriate, and do not repeat information that can be found in the text.
- *Discussion* ▪ ✎ Discuss—do not reiterate—the data found in the results section.
- ✎ Point out exceptions and lack of correlations in the data. Do not try to disguise or “spin” data.
 - ✎ Show how results concur and/or contrast with previous work.
 - ✎ Discuss the implications of the study's findings.
- *Conclusion* ▪ ✎ State your conclusions clearly.

- ∩ Conclusion must be supported by and limited to the results of the study.

- *Abbreviations* ▪ ∩ Include a list of all abbreviations used in the paper with definitions for each abbreviation.

- **References.** Do not use endnotes; instead, type in all references as text. References strictly follow *AMA Manual of Style*, 10th edition. In-text citations to references should be indicated using superscripted numbers in numerical order. The references should then be listed at the end of the article in the order they are mentioned in the text. Unpublished observations, personal communications, submitted papers not yet accepted, and abstracts may not appear in the reference section. Refer to written, not oral, communications parenthetically in the text. Also refer to web sites parenthetically in the text. Include among the references papers accepted but not yet published, and label them as “in press.” Sample references are below:

- *Article from a journal*

Davarpanah M, Martinez H, Tecucianu JF, Hage G, Lazzara R. The modified osteotome technique. *Int J Periodontics Restor Dent*. 2001;21:599–607.

- *Chapter from a book*

Jensen OT. Guided bone graft augmentation. In: Buser D, Dahlin C, Schenk RK, eds. *Guided Bone Regeneration in Implant Dentistry*. 1st ed. Chicago, Ill: Quintessence Publishing Co Ltd; 1994: 234–264.

- *Book*

Misch CE. *Contemporary Implant Dentistry*. St Louis, Mo: Mosby Year Book; 1993.

- *Paper*

Ho E, Marcolongo M. The effect of coupling agents on hydroxyapatite/polymethylmetacrylate composite. Paper presented at: Drexel University Research Day, April 22, 2003;

Philadelphia, Pa.

- *Web*

Freiberg RJ, Boutossov D, Cozean C. Role of water irrigation during laser ablation of hard dental tissue. Available at: http://www.laserdentistry.org/praf/edu_overview.cfm. Accessed February 15, 2004.

- **_Tables.** Tables should be numbered consecutively and titled. Use the table function within Microsoft Word to create tables. Table columns should have explanatory headings. Each table should appear on a separate page in the manuscript file following the references. Tables must provide information that cannot be adequately dealt with in the text and should not duplicate (or be a rewording of) information presented in the text. Tables will be formatted and paged in *JOI* style by the Publisher.

- **_Captions to figures.** Please supply complete captions for all figures on a separate page at the end of the manuscript. Authors should not use symbols in figure captions; instead, a key should be included as part of the figure. Submit each part of a multi-part figure in separate files. Use letters in the caption for the corresponding figure.

Figures

For electronic figures, the Publisher will accept .eps, .tif, .pdf, and .jpg formats. Images must be at least 4.0 in. (10.2 cm) in width with a resolution of at least 200 dpi. Figure quality may be checked using the complimentary Allen Press VeriFig service available at <http://verifig.allenpress.com/login>. Do not embed figures within the Microsoft Word document containing the manuscript. It is the author's responsibility to obtain written permission to use figures that have appeared in another publication. Proof of permission to use previously published figures must be presented at the time of submission, and credit to the original source must be given in the figure caption.

Rebuttal Letter/Response to Reviewers (for Revised Submissions Only)

When submitting a revised paper that previously received a Major Revision or Minor Revision decision, please include a letter that provides a response for each point raised by the reviewers. The

letter should also describe all the changes made to the paper.

Reprints

Authors will receive a complimentary PDF reprint of their article 3–4 weeks after publication. Paper reprints are available for purchase at the time of publication. The corresponding author will be sent an informational email when it is time to place orders for paper reprints.

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Upon passing through the initial quality check, your paper will be assigned to the Editor-in-Chief who will make an initial evaluation of the suitability of the paper for peer review. If the Editor-in-Chief determines that the paper is not suitable for peer review, for any reason, the paper will receive an immediate Editor Rejection. Should it be determined that the paper can be reviewed, reviewers will be assigned. *JOI* requires two reviews for each paper; however, the Editor-in-Chief may decide to send the paper to additional reviewers if necessary. Once the reviews have been submitted, a decision will be made. Each paper will receive one of the following four decisions:

- **_Accept.** Congratulations! Your paper has been accepted for publication and will be published in the next available issue.
- **_Major Revision.** For this decision type, the Editor-in-Chief has significant changes that you will need to make before your paper can be reconsidered for publication. In most cases, revisions of papers with Major Revision decisions will be sent to the original reviewers for re-review.
- **_Minor Revision.** For this decision type, the Editor-in-Chief has minor changes that you will need to make before your paper will be accepted for publication. In some cases, revisions of papers with Minor Revision decisions will be sent to the original reviewers for re-review.
- **_Reject.** A reject decision indicates that the paper is unsuitable for publication in *JOI*.

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Login To register yourself in the peer review system, go to <https://www.editorialmanager.com/aaaid-joi/>. Click on Register Now and follow the instructions. You will receive an email notifying you of your registration, Login ID, and temporary password. You may be asked to create a new password the first

time you login. **Main Menu** Once you have registered and signed in, you will be directed to the Main Menu. In your Main Menu will see three boxes: *New Submissions*, *Revisions*, and *Completed*.

From these boxes you can perform the following tasks: *New Submissions*

- Submit manuscripts
- Check status of submissions

Revisions

- Submit revised manuscripts
- Check status of revisions

Completed

- Check for decisions

Submitting a Manuscript

As a submitting Author, your role in the review process begins when you submit a manuscript. Either click on the link in the email you received when you registered, or navigate to the system URL and login manually, and select Submit New Manuscript from the “New Submissions” box. This will take you to your *New Submission* page.

Article Type

Select an article type from the dropdown menu.

Click the “Next” button.

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On the *Enter Title* page, a Full Title and a Short Title (being mindful of word limits) in the boxes provided.

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Authors

On the *Add/Enter/Remove Authors* page, you will automatically be listed as the corresponding author. You may add more author names by clicking the “+Add Another Author” button (please note that a first and last name, academic degrees, and an email address are required for each author).

If you wish to reorder the list of author names, click and hold the vertical blue bar to the left of the name. You may then drag the author name to a different position in the list.

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On the *Submit Abstract* you can either type or copy and paste the abstract (be mindful of word limit) of your manuscript. Please note that even though Clinical Case Letters and Research Letters are not published with an abstract, the system requires that an abstract is submitted. It is only necessary to type the following into the box: “An abstract is not required.”

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Classifications are used to aid in the selection of reviewers with the appropriate specialties for a submission. On the *Select Classifications* page, you may choose classifications for your manuscript by clicking “Select Classifications.”

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On the *Additional Information* page, there are several questions that must be answered:

- Conflict of interest. Please note that the information entered into this box must also be included in the conflict of interest statement that is included on the title page.
- Dual publication
- Acknowledgment that copyright transfer form has been signed. Please note that only the corresponding author must fill out and sign the copyright transfer form.
- AID funding acknowledgment
- Open access
- Page charges

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On the *Enter Comments* page, enter any comments you wish to make to the editorial office regarding your submission. Comments entered on this page will NOT appear in your manuscript or be shown to reviewers.

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On the *Suggest Reviewers* page, you may enter the names and contact information for potential reviewers. Fill out the information and click “Add Reviewer” at the bottom of the page. Please note that suggesting reviewer is not required.

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Attach Files

You are now ready to attach the files for your manuscript. Select the file type of each attachment from the drop-down menu at the top of the screen. Your options are

- _Title Page
- _Article File
- _Appendices
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- _Cover Letter
- _Figure
- _Supplemental Material
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Type a name for each file in the “Description” window (default will be the item selected). You may either drag the file over to the submission form and drop it, or use the “Browse” button to locate and select the file. You may change the order of the files before you proceed by numbering them sequentially and clicking on “Update File Order.”

Once you have added all your files and placed them in the correct order, click “Next” to build your PDF. Make sure all of your files are accounted for in the table and click the “Build PDF for my Approval” button.

Please note that you are not yet finished with the submission process.

Approve Submission

After you click the “Build PDF for my Approval” button, you will be taken to a page with instructions regarding approval of the PDF of your submission.

Click on the [Submissions Waiting for Author’s Approval](#) link.

You will be taken to the *Submissions Waiting for Approval by Author* page. Until Action Links appears you cannot check and approve your submission. If the Action column is blank, please wait until Action Links appears. This may take several minutes depending on the size of the files you uploaded.

You may view, edit, approve and/or remove your submission using the Action Links drop-down menu. You must first view the submission. Click the View Submission link to download your PDF file, view it for accuracy, and ensure it appears as intended. If you wish to make changes, click the Edit Submission to return to the New Submission screen. Click the tab on the left side of the screen that corresponds to the portion of your submission that you want to edit.

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Submitting Revisions for Major or Minor Revision Decisions

The decision letter you receive from the Editor-in-Chief will indicate whether your manuscript needs revisions before further consideration for publication. Either minor or major revisions will need to be made and the manuscript resubmitted to start the review process over.

Access the manuscript to make revisions either by using the link in the email or clicking Submissions Needing Revision in the “Revisions” box on your Main Menu.

Select Revise Submission from the Action Links. This will bring up a confirmation window. Click “OK” if you are ready to proceed. You will be directed to a *Revised Submission* screen where you will resubmit your manuscript with revisions. Tracking information and identification (such as the manuscript number) will be carried over from the initial submission.

During the process you will be required to upload a file containing your response to the reviewers’ comments. In addition, the new manuscript file should have the track changes function in Word activated when the text is revised so that any changes made are readily visible to the Editorial Staff and Reviewers.

Original files can be included or excluded by using the check boxes at the bottom of the screen. New files are added in the same way as in the original submission, on the *Attach Files* screen. You may move from your original submission any files that are still applicable in your revision (eg, any figure files that were not changed when you revised your paper).

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Check Status & Revisions

Once you have submitted your manuscript with revisions, it will

appear in the *Revisions* box on your Main Menu. Click the Revisions Being Processed link to view the current status of the revision.

APPENDIX

Reviewer Form

Clinical Research Papers and Dental Implant Science

Research Papers

Positive declarative statements are used by the reviewer to rate the submitted manuscript. The reviewer rates each statement using a scale from 1 to 4, as follows:

1 = Strongly Disagree

2 = Disagree

3 = Agree

4 = Strongly Agree

NA/NC = Not Applicable or No Comment by the reviewer.

I. Title and Abstract

A. Title clearly identified target(s) population and variables under study.

B. Abstract was well written and clearly described the purpose, methods {subjects, instrument(s), design, procedures}, important findings, implications, theoretical support, limitations, and recommendations for future research.

II. Introduction

A. A clear statement of the problem was provided.

B. The rationale for the study was very logical and convincing.

C. A review of literature was current, thorough, accurate, and clearly related to the statement of the problem.

D. Terms were clearly defined.

E. The specific purpose of the study, research questions, or hypotheses was a logical extension of the problem, rationale, and literature review.

F. Theoretical foundation for the study was provided and supported.

G. A clearly written research question(s) was provided.

H. The introduction was well organized and well written.

III. Methods

A. Methodology was reviewed by an independent statistician.

B. Human or Animal Selection and Protections

1. Protection of humans or animals was clearly described and complied with national and international protection guidelines.

- 2. Human or animal selection and exclusion criteria were clearly described.
- 3. Random selection of humans or animals and random assignment to groups was clearly described.
- 4. Differential selection of humans or animals and threats to internal validity were clearly controlled.
- 5. Humans or animals were not selected on basis of extreme scores.
- 6. Interaction of human or animal selection and threats to external validity were clearly controlled.
- 7. The selection and protection text was well organized and well written.

C. Instrumentation/Measurement

- 1. The instrumentation was appropriate.
- 2. The instruments were calibrated for the population sampled.
- 3. The instrumentation measurements were reliable and valid.
- 4. Experimenter bias was controlled.
- 5. Test environment was controlled.
- 6. Instructions to humans were controlled.
- 7. Adequate selection and measurements of independent variable(s) were described.
- 8. Adequate selection and measurements of dependent variable(s) were described.
- 9. The instrumentation text was well organized and well written.

D. Materials

- 1. Materials were clearly described.
- 2. Materials were referenced to connect readers with vendors.
- 3. Materials were well organized and well written.

E. Procedures

- 1. The research design was appropriate for the study.
- 2. The procedures controlled threats to internal validity (confidence that independent and dependent variables were experimentally related).
 - a. Extraneous variables in the study were controlled.
 - b. Potential confounding variables were controlled.
 - c. Variable relationships (e.g. convincing antecedence conditions) were controlled.
 - d. Causality was clearly described.
 - e. The procedures controlled threats to external validity were well controlled (e.g. history, maturation, etc.).

3. The procedures supported external validity.

- a. Population validity (research sample like the population being generalized) was controlled.
- b. Ecological validity (research procedures generalized across settings) was controlled.
- c. Threats to external validity were controlled (e.g. interaction effects of testing, etc.)
- d. Procedures were well organized and well written.

IV. Results (Quantitative)

A. Results were reviewed by an independent statistician.

B. Data organization and tabulation procedures were clear.

C. A clear and measurable question(s) from the introduction was analyzed.

D. Informal Analysis were effectively used and presented (e.g. boxplots, scatterplots, etc.) to informally answer the research question(s). Tables and figures from this informal analysis were labeled, used within text, self-explanatory, and efficiently used.

E. Summary Descriptive Statistics were effectively used, tabled, and integrated into text. Tables and figures from descriptive statistics were labeled, used within text, self-explanatory, and efficiently used.

F. Formal Statistical Analysis, when used, effectively: controlled for mathematical assumptions and alpha levels, answered the research question(s), and provided a clear summary table(s) and figure(s) of the software output results. Tables and figures from the formal statistical analysis were labeled, used within text, self-explanatory, and efficiently used.

G. The name and version of the statistical software used was provided in text and cited in references.

H. The name of the statistical tool(s) used is exactly the same as used by the statistical software publisher, and this name is consistently used throughout the manuscript.

I. The results were clearly related to research question(s) asked without overgeneralizing the results.

J. Tables and figures from a formal statistical analysis were: clearly and accurately labeled, were used with text, were self-explanatory, and efficiently communicated useful information related to the research question(s).

K. Results were well organized and well written.

V. Discussion, Summary, and Conclusions

A. The discussion, summary, and conclusions were clearly related to the research problem and the research question (questions) investigated.

B. Limitations of the study were clearly discussed.

C. Conclusions were drawn directly and accurately from results.

D. Reasonable explanations were given for unusual, atypical, or discrepant results.

E. The results were clearly related to one or more theoretical explanations.

F. The results were empirically argued as externally valid to the population from which the sample was taken.

G. Implications for application of findings were empirically discussed and not overgeneralized beyond the scope of the study.

H. Suggestions for future research were empirically discussed and limited to the results of the study.

I. The discussion, summary, and conclusions were limited to the empirical findings of the study, well organized, and well written.

VI. References

A. References (not a bibliography) were cited in text and all text citations were listed in references.

B. References followed the Journal of Oral Implantology requirements.

C. References were well organized and well written.

VII. The Manuscript

A. The manuscript was well written and clearly presented.

B. The manuscript was accurate and efficiently presented as a convincing empirically persuasive argument.

Comments: