UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

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CIRURGIÃO-DENTISTA

ANÁLISE DA FARMACOCINÉTICA E DOS ÍNDICES PK/PD DA DOXICICLINA NO PLASMA, FLUIDO GENGIVAL E SALIVA E AVALIAÇÃO DE SEU EFEITO SOBRE A OSTEOCLASTOGÊNESE MEDIADA POR RANKL

Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas, para obtenção do título de Doutor em Odontologia - Área de Farmacologia, Anestesiologia e Terapêutica do Programa de Pós-Graduação em Odontologia.

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"Eu preferirei sempre aqueles que sonham... embora se enganem; aqueles que esperam... embora, às vezes, suas esperanças fracassem; aqueles que apostam na utopia... embora, em seguida, fiquem no meio do caminho. Aposto nos que confiam em que o mundo pode e deve mudar; naqueles que acreditam que a felicidade virá. Só daqueles que esperam será o reino da felicidade"

José Luis Martin Descalzo

RESUMO

Doxiciclina (Dox) é um antimicrobiano pertencente à família das tetraciclinas com um amplo espectro de ação contra bactérias Gram-positivas e Gram-negativas. Além de suas propriedades antimicrobianas, Dox é atualmente empregada na periodontia como um modulador da resposta do hospedeiro (MRH), ao inibir a atividade da enzima metaloproteinase de matriz (MMP), a qual está relacionada ao processo de destruição tecidual. Neste contexto, este trabalho teve os seguintes objetivos: 1-determinar os parâmetros farmacocinéticos e integrar os índices PK/PD da Dox para o plasma, fluido gengival (FG) e saliva; 2-analisar os efeitos *in vitro* e *in vivo* da Dox sobre a osteoclastogênese com a finalidade de elucidar possíveis propriedades biológicas adicionais deste fármaco como MRH. Para a análise farmacocinética, 12 voluntários receberam dose oral única de 100 mg de Dox. Sangue, FG e saliva foram coletados em tempos pré-determinados e a concentração da Dox nestes fluidos foi determinada por bioensaio. A análise dos principais índices PK/PD da Dox foi realizada considerando o CIM para *P. gingivalis*. Para o segundo objetivo, o efeito da Dox sobre os processos de diferenciação e ativação osteoclástica foi verificado, respectivamente, pela contagem de células $TRAP^+$ multinucleadas geradas a partir de células precursoras estimuladas com sRANKL na presença ou ausência de Dox e pela análise das lacunas de reabsorção formadas por estas células, quando cultivadas sobre discos de dentina. *In vivo,* o efeito da Dox sobre a osteoclastogênese foi determinado através da indução deste processo em calvária de camundongo. Solução de sRANKL/LPS foi injetada na região da calvária e os animais receberam, por gavagem, Dox ou placebo diariamente. Após 10 dias, a calvária foi removida para análise histoquímica. Em acréscimo, a atividade da Dox sobre a expressão de genes responsáveis pelos processos de diferenciação e ativação osteoclástica foi analisada por RT-PCR. Durante os experimentos *in vitro* e *in vivo*, a produção e atividade da MMP foram verificadas através de Western-blot e Zimograma respectivamente. Os resultados demonstraram que as maiores concentrações de Dox foram observadas no plasma, seguido pelo FG e saliva. A análise dos índices PK/PD da Dox indicou que a dose de 100 mg foi insuficiente para se obter os valores ideais antimicrobianos preconizados na

literatura para os parâmetros ASC/CIM e C_{max}/CIM. Os experimentos *in vitro* e *in vivo* sobre o efeito da Dox como MRH demonstraram que este fármaco inibiu os processos de diferenciação e ativação dos osteoclastos. Dox também modulou a expressão de proteínas diretamente relacionadas a osteoclastogênese, incluindo TRAP, Catepsina K e c-Myc. Finalmente, embora a síntese da MMP não tenha sido afetada, a atividade da MMP foi reduzida na presença de Dox. Portanto, os resultados do presente estudo sugerem que uma dose inicial maior do que 100 mg é necessária para alcançar o valor preconizado para ASC/CIM e Cmax/CIM, com a finalidade de se obter os melhores resultados clínicos antimicrobianos. A análise da Dox como MRH indicou que este fármaco pode atuar neste processo não somente pela sua capacidade de inativar a MMP, e sim, por apresentar a propriedade de inibir a diferenciação e ativação osteoclástica, incluindo a modulação de sua expressão gênica.

Palavras-chave: Doxiciclina; farmacocinética; osteoclastos; RANKL.

ABSTRACT

Doxycycline (Dox), a member of the tetracycline family, is an antimicrobial agent with a broad-spectrum of activity against Gram-positive and Gram-negative bacteria. In addition to its antimicrobial properties, Dox is used in the treatment of periodontal diseases as a host response modulator by inhibiting the activity of an important enzyme, matrix metalloproteinase (MMP), which is related to the process of tissue destruction. In this context, this study had the following aims: 1-to determine the pharmacokinetic parameters of Dox and to integrate the PK/PD indices for plasma, gingival crevicular fluid (GCF) and saliva; 2-to analyze the effects *in vitro* and *in vivo* of Dox on the osteoclastogenesis and on the osteoclast activation in order to elucidate additional biological properties of Dox on the host response modulation (HRM). Twelve volunteers received single oral administration of Dox (100 mg). Blood, GCF and saliva were collected and the concentrations were measured by bioassay technique. The PK/PD analyses were carried out using the MIC for *P. gingivalis*. For the second objective, the effect of Dox on the osteoclast differentiation and activation processes was determined, respectively, by the counting of $TRAP^+$ multinuclear cells derived from osteoclast precursory cells sRANKL-stimulated in the presence or absence of Dox and by the analysis of the resorption areas formed by these cells when cultured on dentin discs. *In vivo,* Dox's effect on the osteoclastogenesis was verified using the model of osteoclastogenesis induction in mouse calvaria. sRANKL/LPS was injected in the supra-calvaria area and the animals received Dox or placebo daily by gavage. After the experimental period of 10 days, the calvariae were removed for histochemistry analyses. In addition, the effect of Dox on the expression of genes related to the osteoclast differentiation and activation processes was carried out using RT-PCR technique. MMP production and activity were ensured during *in vitro* and *in vivo* experiments by Western-blot and Zymography, respectively. The results demonstrated that Dox achieved the highest concentration in the plasma, following by GCF and saliva. PK/PD analyses showed that the dose of 100 mg was insufficient to get the antimicrobial levels indicated in the literature for AUC/MIC and C_{max}/MIC indices. *In vitro* and *in vivo* studies of Dox's effects on the HRM demonstrated that this drug could inhibit the

osteoclast differentiation and activation process. Dox also showed an important property of down-regulation in the expression of proteins directly related to osteoclastogenesis, including TRAP, Cathepsin K and c-Myc. Finally, although Dox did not affect the expression of MMP protein, MMP activity was remarkably decreased by Dox. Therefore, the present study suggests that higher doses than 100 mg would be necessary to obtain effective antimicrobial levels and the effect of DOX on the HRM can be due to not only by MMP inhibition but also by the direct effect on RANKL-mediated osteoclast differentiation and activation, including its gene regulation.

Key words: Doxycycline; pharmacokinetics; osteoclasts; RANKL.

LISTA DE ABREVIATURAS E SIGLAS

ALP	Fosfatase alcalina
CIM	Concentração inibitória mínima
DOX	Doxiciclina
ERK	Proteína cinase ativada por sinais extracelulares
FG	Fluido gengival
JNK	Proteína cinase c-Jun N-terminal
LPS	Lipopolissacarídeo
MAPK	Proteína cinase ativada por mitógenos
M-CSF	Fator estimulante de colônia de macrófagos
MMP	Metaloproteinase de matriz
MRH	Modulador da resposta do hospedeiro
NFATc	Fator nuclear de célula T ativada
NFκB	Fator nuclear κB
PK/PD	Farmacocinética/Farmacodinâmica
RANK	Receptor ativador do fator nuclear κB
RANKL	Receptor ativador do fator nuclear κB ligante
RT-PCR	Reação em cadeia da polimerase com transcrição reversa
TRAF	Fator associado ao receptor do fator de necrose tumoral
TRAP	Fosfatase ácido tartarato resistente

SUMÁRIO

1. INTRODUÇÃO

As doenças periodontais (DP) caracterizam-se como um processo inflamatório crônico que acomete os tecidos periodontais, levando a perda progressiva de inserção, com reabsorção óssea e migração apical do epitélio juncional, constituindo-se hoje como uma das principais causas de perda do elemento dentário (Dumitrescu *et al.*, 2004). O tratamento mecânico (raspagem e aplainamento radicular) representa ainda hoje a forma mais eficaz para o controle desta patologia, porém, o reconhecimento de que as DP estão relacionadas com um grupo determinado de microrganismos provocou um aumento no interesse do uso de antimicrobianos como coadjuvante no tratamento destas doenças (Freeman *et al.*, 1992).

A terapia antimicrobiana visa reforçar o tratamento periodontal mecânico e dar suporte às defesas do hospedeiro, inibindo os microrganismos que permanecem viáveis após a terapia convencional. Porém, para que um fármaco seja efetivo no tratamento das DP, há a necessidade de níveis terapêuticos adequados no sítio da infecção. Atualmente, apenas alguns antibióticos possuem esta propriedade de se concentrar no fluido gengival (FG) e na saliva a partir de sua administração sistêmica, possibilitando assim, a sua indicação na periodontite (Pascale *et al.*, 1986; Ramberg *et al.*, 2001).

A doxiciclina ($C_{22}H_{24}N_2O_8.H_2O$) é um antibiótico pertencente ao grupo das tetraciclinas, produzida a partir de uma cultura mutante de *Streptomyces aureofaciens* (Amato Neto, 1994). Este fármaco tornou-se disponível comercialmente em 1962, sendo considerado uma tetraciclina de segunda geração ou de ação lenta por apresentar meia-vida mais prolongada, penetração tecidual mais intensa e maior atividade antimicrobiana (Cunha *et al.*, 2000).

A doxiciclina (Dox) é um antibiótico de amplo espectro, com atividade contra microrganismos Gram-positivos e Gram-negativos (Zhanel *et al.*, 2004). Este antimicrobiano apresenta vantagens sobre os outros membros de seu grupo que incluem: baixa interferência com os alimentos durante o processo de absorção, índice reduzido de efeitos colaterais gastrointestinais e posologia conveniente, a qual favorece a aceitação do tratamento pelo paciente (Barza *et al.*, 1975).

O mecanismo de ação da Dox assemelha-se a outras tetraciclinas. Em concentrações adequadas, este antibiótico exerce atividade bacteriostática por inibir a síntese protéica ao impedir a ligação do t-RNA à subunidade menor do ribossomo bacteriano - 30S. Em acréscimo, a Dox induz danos na membrana citoplasmática, promovendo assim, a perda de nucleotídeos e outros componentes celulares (Joshi & Miller, 1997).

Na atualidade, diferentes métodos vêm sendo utilizados na tentativa de determinar a melhor posologia (dose/freqüência) antimicrobiana a ser empregada na prática clínica. De acordo com Van Bambeke & Tulkens (2001), o desenvolvimento de um esquema posológico efetivo inicia-se com o conhecimento das características químicas da molécula e culmina com a avaliação de sua atividade microbiológica e entendimento de suas propriedades farmacocinéticas (informações referentes aos processos de absorção, distribuição, metabolização e excreção) e farmacodinâmicas (dados sobre os prováveis mecanismos de ação e conhecimento da concentração inibitória mínima – CIM dos patógenos envolvidos).

Neste contexto, diferentes autores preconizam a utilização de índices que correlacionam às propriedades farmacocinéticas (PK) e farmacodinâmicas (PD) com a finalidade de aperfeiçoar a posologia destes fármacos, obtendo assim, as melhores taxas de erradicação bacteriana com os menores níveis de resistência (Jacobs, 2001; Frimodt-Moller, 2002).

Dentre os principais índices que relacionam PK/PD dos agentes antimicrobianos, destacam-se a relação entre a área sob a curva e a CIM (ASC/CIM), e a concentração máxima e a CIM (C_{max}/CIM) para os fármacos concentração-dependentes. Para os fármacos tempo-dependentes, o tempo em que a concentração excede o valor de CIM (T>CIM) constitui-se como o principal parâmetro a ser estudado (Barger *et al.*, 2003).

Além do aspecto microbiano, estudos atuais na área de Periodontia têm-se dirigido à elucidação do exato mecanismo da reabsorção óssea que ocorre na evolução das DP. Isto se deve ao fato de que embora a placa bacteriana seja considerada como o agente etiológico necessário para o início da DP e subseqüente degradação dos tecidos periodontais, a direta exposição aos componentes bacterianos isoladamente é responsável

apenas por uma proporção relativamente pequena da destruição periodontal (aproximadamente 20%). O maior componente da degradação das estruturas periodontais é o resultado da ativação da resposta imuno-inflamatória local do próprio hospedeiro (Salvi and Lang, 2005).

O tecido ósseo é formado por células (osteoblastos e osteoclastos), minerais (cálcio e fósforo) e matriz orgânica (proteínas colágenas e não-colágenas). Os osteoblastos sintetizam e mineralizam a matriz protéica com cristais de hidroxiapatita, enquanto os osteoclastos promovem a reabsorção óssea, mantendo assim uma constante remodelação tecidual (Wittrant *et al.*, 2003). Porém, diante de certos quadros patológicos, há um desequilíbrio em favor da reabsorção óssea, ocasionando assim a perda de sua matriz (Gravallese *et al.*, 2001).

Recentemente, uma nova proteína da família do Fator de Necrose Tumoral (TNF) denominada Ligante do Receptor Ativador do Fator NF-Kappa B (RANKL) tem sido relacionada com o processo de reabsorção óssea (Wittrant *et al.*, 2003). RANKL é uma proteína de 317 aminoácidos, expressada por osteoblastos e células do estroma (Feng, 2005). A interação de RANKL ao Receptor Ativador do Fator NF-Kappa B (RANK), presente nos osteoclastos e em seus precursores celulares, promovem a ativação de sinalizadores intracelulares, tais como, o Fator Nuclear de célula T ativada (NFAT), Ca^{2+}/Calcineurin, NF-Kappa B e proteínas cinases ativadas por mitógenos (MAPK), induzindo assim, os processos de diferenciação e ativação dos osteoclastos (Jimi & Glosh, 2005).

De acordo com Teitelbaum *et al.* (2000), os osteoclastos são células multinucleadas, formadas pela fusão de progenitores mononucleares da família dos monócitos/macrófagos, sendo que a sua atividade pode ser avaliada por marcadores bioquímicos específicos. Um marcador clássico é representado pela Fosfatase Ácida Tartarato-Resistente (TRAP). TRAP é uma enzima lisossômica, que quando sintetizada pelos osteoclastos reflete a sua atividade, sendo estes denominados TRAP-Positivos ($TRAP^{+}$) (Feng *et al.* 2005).

Além deste papel de marcador biológico, a TRAP possui também uma importante função no processo de reabsorção óssea, devido a principal proteína da matriz

óssea, a osteonectina, constituir-se como um substrato para TRAP, e portanto, promovendo a degradação catabólica da matriz óssea (Oddie *et al.*, 2000). Ek-Rylander *et al.* (1994) acrescentam que a TRAP também exerce uma atividade reguladora da adesão dos osteoclastos à superfície óssea.

Outras importantes proteínas relacionadas com o processo de reabsorção óssea são representadas pela metaloproteinase de matriz (MMP) e pela Catepsina K. Esta última é responsável pela degradação dos cristais de hidroxiapatita, resultando na exposição do colágeno da matriz óssea. MMP compreende uma família de endopeptidases com atividade proteolítica, a qual pode liderar a destruição do tecido periodontal através da degradação do colágeno presente no tecido ósseo e gengival (Kadoglou & Liapis, 2004). Dentre as diferentes MMPs, a MMP-9 destaca-se por constituir-se como uma das mais abundantes no fluido gengival e por desempenhar um importante papel na atividade invasiva dos osteoclastos em pacientes com periodontite (Preshaw *et al.*, 2005).

Com estes conhecimentos, uma nova alternativa terapêutica vem sendo proposta na tentativa de controlar a progressão da DP, representada pelo processo de reabsorção óssea. Este "novo" grupo de fármacos foi denominado de moduladores da resposta do hospedeiro (Salvi & Lang, 2005). Neste contexto, Golub *et al.* (2001) destacam o uso da Dox em doses sub-antimicrobianas no controle da perda óssea alveolar, ao inibir a atividade da MMP e, consequentemente, a degradação do colágeno.

Portanto, em decorrência da atividade da Dox sobre a microbiota periodontal, mas, principalmente de sua propriedade na modulação da perda óssea alveolar através da inibição da MMP, este fármaco vem se destacando atualmente como coadjuvante no tratamento periodontal, especialmente na periodontite crônica (Emingil *et al.*, 2004; Lee *et al.*, 2004; Novak *et al.*, 2002).

Sendo assim, para um correto emprego da Dox na prática periodontal, torna-se necessário um conhecimento prévio das propriedades farmacocinéticas e farmacodinâmicas. Porém, a literatura indexada atual é escassa em trabalhos que correlacionam estes dois parâmetros (PK/PD) para este fármaco. Em acréscimo, embora o efeito da Dox na modulação da perda óssea seja atribuído principalmente a sua atividade de inibição da MMP, o exato mecanismo de ação da Dox sobre os processos de diferenciação

e ativação dos osteoclastos permanece ainda desconhecido. Portanto, este trabalho teve os seguintes objetivos: 1. Determinar os parâmetros farmacocinéticos e integrar os índices PK/PD da doxiciclina para o plasma, fluido gengival e saliva após administração oral de 100 mg em 12 voluntários portadores de doença periodontal, otimizando assim, a sua posologia no tratamento das doenças periodontais; 2. Elucidar o efeito da Dox sobre os processos de diferenciação e ativação de osteoclastos mediada por RANKL, objetivando elucidar possíveis propriedades biológicas deste fármaco na modulação da resposta do hospedeiro em processos de destruição óssea.

2. CAPÍTULOS

Esta tese está baseada na Informação CCPG/002/06 (Anexo 1) e na aprovação pela Congregação da Faculdade de Odontologia de Piracicaba em sua 105ª Reunião Ordinária em 17/12/2003, que regulamenta o formato alternativo para tese de Doutorado e permite a inserção de artigos científicos de autoria do candidato.

Assim sendo, esta tese é composta de dois capítulos contendo artigos que se encontram em fase de submissão ou submetidos para publicação em revistas científicas, conforme descrito a seguir:

Capítulo 1

Artigo *"Pharmacokinetics and PK/PD of doxycycline in plasma, gingival crevicular fluid and saliva after single oral dose"*

Este artigo está em fase de submissão ao periódico: *Journal Clinical of Periodontology.*

Capítulo 2

Artigo *"Suppression of RANKL-mediated bone resorption by doxycycline"*

Este artigo está submetido ao periódico: *Bone (Anexo 4).*

2.1 CAPÍTULO 1

Pharmacokinetics and PK/PD of doxycycline in plasma, gingival crevicular fluid and saliva after single oral dose.

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Abstract

The aim of this study was to investigate the pharmacokinetics (PK) and pharmacodynamics (PD) indices of doxycycline (Dox) in plasma, gingival crevicular fluid (GCF) and saliva in patients with periodontal disease. A single 100 mg oral dose of Dox was administered to 12 volunteers of both genders (6 males and 6 females). Plasma, GCF and saliva were collected from pre-dose to 24 h. Dox concentration was quantified by a bioassay technique and PK parameters (C_{max}; T_{max}; $T_{1/2}$; AUC_{0-24}; $AUC_{0-\infty}$; Vd and Cl) were determined. PK/PD indices (T>MIC; AUC/MIC and C_{max}/MIC) were estimated for *P. gingivalis*. The highest Dox concentration (C_{max}) in plasma, GCF and saliva were 1.8 μg/mL at 2 h, 1.3 μg/mL at 2 h and 0.4 μg/mL at 3 h, respectively. Dox also showed high volume of distribution (VD) indicating that Dox was able to penetrate into biological membranes and body tissues. Elimination half-life ($T_{1/2}$) was 15.5 h in plasma, 21.2 h in GCF and 20.6 h in saliva. The C_{max}/MIC and AUC/MIC ratios for *P. gingivalis* (considering a 0.25 μg/mL MIC) were 7.2 and 86.8 h for plasma, 5.2 and 70.8 h for GCF, and 1.6 and 20 h for saliva, respectively. These results suggest that it could be necessary higher doses than 100 mg to obtain effective antimicrobial levels considering a 12 or 24 h dose interval.

Keywords: Doxycyline; pharmacokinetic; pharmacodynamic; periodontal disease.

Introduction

A successful periodontitis therapy requires a reduction in the number of bacteria responsible for periodontal infections (Kim *et al.*, 2004). In this context, systemically administered antibiotics, such as doxycycline (a semi-synthetic tetracycline), have been widely used as adjuncts to the treatment of different forms of periodontitis (Feres *et al.*, 1999; Needleman *et al.*, 2001; Ramberg *et al.*, 2001).

Doxycycline (Dox) is a broad-spectrum antibiotic with activity against a wide range of gram-positive and gram-negative microorganisms (Zhanel *et al.*, 2004). Dox shows several advantages over other tetracyclines, which include lower dosage and frequency, prolonged serum half-life, less effect of food during absorption and decreased gastrointestinal adverse-effects (Barza *et al.*, 1975).

Dox has a similar mechanism of action when compared with other tetracyclines. At clinical concentrations, Dox is bacteriostatic and inhibits the protein synthesis by binding to the 30S portion of bacterial ribosomes. This action blocks the entry of aminoacyl-transfer-RNA into the ribosome preventing the incorporation of amino acids into the peptide chains (Joshi & Miller, 1997).

In addition to its antimicrobial properties, Dox has shown the ability to inhibit a group of enzymes called matrix metalloproteinases (MMPs) which are involved in soft- and hard-tissue destruction during pathological processes such as periodontitis, osteoarthritis and other diseases (Axisa *et al.*, 2000).

However, before selecting a drug for therapeutical use, the knowledge of its pharmacokinetic (PK) and pharmacodynamic (PD) properties is very important in clinical practice. This knowledge is necessary to achieve the beneficial effects with minimal adverse effects (Freeman *et al.*, 1992; Cheymol, 2000).

The PK characteristics are useful to determine and to understand how pharmacokinetics parameters, such as absorption, distribution, metabolization, and excretion, affect the drug regimen and its clinical use. The efficacy and adverse effects are also directly PK dependent, which consequently affect the drug acceptance by patients (Patsalos, 2004).

The PD parameters include the knowledge of the spectrum of activity, potency, the minimum inhibitory concentration (MIC), and the influence of time/concentration on drug effect (Frimodt-Moller, 2002).

The integration of PK and PD indices (PK/PD) has been studied in order to optimize the effect of many antibiotics *in vivo* (Van Bambeke & Tulkens, 2001; Mouton, 2002). The most PK/PD indices used to determine efficacy of "concentration-dependent" antibiotics are the ratio between the peak of blood (or other fluids) concentration and MIC (C_{max}/MIC); and the ratio between area under concentration-time curve and MIC (AUC/MIC). In addition, the cumulative percentage of time (over a 24 h period) in which the drug concentration exceeds the MIC (T>MIC) is the most used PK/PD index for "time-dependent" antibiotics (Barger *et al.*, 2003).

Nowadays, information on PK/PD for Dox is scarce in the literature. Therefore, the aim of the present study was to investigate the PK/PD indices of Dox in plasma, GCF and saliva in patients with periodontal disease.

Material and Methods

The protocol was approved by the ethical on research committee (FOP/UNICAMP, protocol #128/2004). All subjects provided written informed consent before their participation on study.

Subject population

The study population consisted of twelve healthy volunteers (6 male and 6 female), ranging in age from 20 to 48 years (32.4 ± 8.97 years), weight from 50 to 84 kg (62.5 ± 10.88 kg) and height from 1.54 to 1.71 m (1.64 ± 0.06 m). The subjects did not present any disease except for at least two periodontal pockets ≥ 4 mm with attachment loss. The volunteers had not received neither systemic antibiotics within the last three months nor any other medication during the two weeks that preceded the study.

Antibiotic administration and clinical protocol

Single oral dose of Dox (Vibramicina® - 100 mg) was administered to the volunteers in approximately 10 h of fasting conditions.

Blood samples were collected via heparinized cannula at the following times: pre-dose, 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 hours after Dox administration. Heparinized normal saline injection solution (1 mL) was flushed after each blood sampling. Samples were collected in a centrifuge sterile tube containing 10 % EDTA solution. Non-stimulated whole saliva (± 2 mL) was collect at the same periods of time. Blood and saliva samples were centrifuged (3000 rpm x 15 min) and the supernatants were stored at -70°C until the analyses.

The gingival crevice fluid (GCF) was collected as described by Tenenbaum *et al.* (1993). Briefly, before the gingival fluid was sampled, cotton rolls were applied to prevent contamination of the sampling area with other oral fluids. The sample sites were gently dried and a standard filter paper (Periopaper, Oralflow Inc., Painview, NY, USA) were placed subgingivally for 30 seconds. The first GCF sample was discharged. Samples were obtained in duplicate at the same blood collection periods of time. All collected samples were stored in previous weighed eppendorf tubes. The volume of GCF was estimated by the difference between the weight of strips before and after fluid collection by using an analytical microbalance. The density of the GCF was considered 1.02 (Needleman *et al.*, 2001). The samples were stored at -70°C until the analyses.

Sample analyses (bioassay)

A sensitive agar diffusion method, which is frequently used to measure small volumes of antibiotics, was carried out in order to determine the concentration of Dox in plasma, GCF and saliva (Gordon *et al.*, 1981; Freeman *et al.*, 1992; Sakellari *et al.*, 2000).

A strain of *Bacillus cereus* (ATCC11778) was incubated aerobically for 24 hours at 37ºC. After this time, the broth was adjusted to an optical density of 0.15 at 550 nm (approximately 10^8 cfu/mL) and seed in 150 mm plastic Petri dishes containing tryptic soy agar (TSA).

Sterilized stainless steel cylinders (5mm-diameter) with 60 µl samples (plasma or saliva) were placed in the Petri dishes previously seeded with *B. cereus* and then incubated at 37ºC for 16 hours. A calibration curve was obtained by known amounts of Dox (0.125, 0.25, 0.5, 1.0, 2.0 and 4.0 µg/mL) on antibiotic-free plasma or saliva by using the same technique described above. This calibration curve was used to calculate the plasmatic and salivary Dox concentrations on the samples.

The concentration of Dox in the GCF was calculated according to Pascale *et al.*, (1986). Briefly, standard curves were plotted by using the inhibition area surrounding the Periopaper and known absolute amounts of antibiotics (0.5, 1, 2, 4 and 10 pg). The concentration was calculated by the proportion between absolute amount of Dox and the GCF volume on each Periopaper.

Pharmacokinetics and statistical analysis

In this study, the following Dox PK-parameters were obtained:

- C_{max}: the highest concentration observed during the 24-hour study period;
- T_{max}: the time at which C_{max} occurred;
- $T_{1/2}$: half-life elimination. The time required for the amount of a substance in a biological system to be reduced to one half of its value by biological processes.
- AUC_{0-24}: the area under the concentration-time curve from baseline to 24 h;
- AUC0-∞: the area under the concentration-time curve from baseline to 24 h plus the residual area;
- CL: clearance;
- VD: volume of distribution.

These parameters were determined by using a computer software (PK Solutions, Non-compartmental Pharmacokinetics Data Analysis Excel Template, 2001, Summit Research Services, Montrose, CO, USA).

In order to study the Dox PK/PD indices, it was considered a MIC value of 0.25 µg/mL of *P. gingivalis*, which is one of the most common pathogens for periodontal disease

(Chang *et al.*, 1994; Nichols & Rojanasomsith, 2006). The following PK/PD indices were obtained (Toutain *et al.*, 2002; Barger *et al.*, 2003):

- C_{max}/MIC: the peak level divided by the MIC;
- AUC_{0-24}/MIC: the area under the concentration-time curve over 24 h divided by the MIC;
- T>MIC: the time over a 24 h period in that the drug concentration exceeds the MIC. It is obtained by simple inspection of the simulated curve and generally expressed as a percentage of the dosage interval.

The Figure 1 represents a scheme of the mainly PK/PD indices.

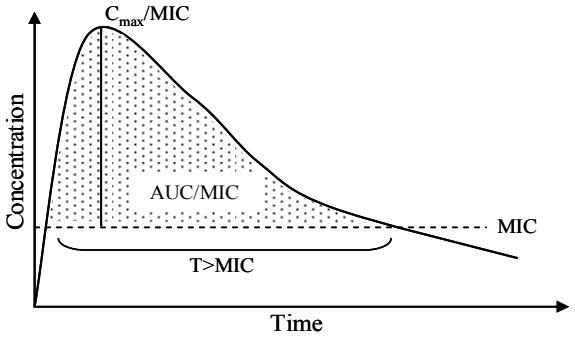

Figure 1. A representative scheme of the mainly PK/PD indices used in the therapeutics (C_{max}/MIC; AUC/MIC and T>MIC).

Results

Dox PK in plasma, GCF and saliva

The bioassay method was sensitive to quantify Dox in plasma (limit of quantification=0.125 μg/mL, R^2=0.991); GCF (limit of quantification=0.5 pg, R^2=0.986); and saliva (limit of quantification=0.125 μg/mL, R^2=0.993). Antimicrobial activity was not detected in any of the samples of baseline time (pre-dose). The mean (± SD) of plasma, GCF and saliva are shown in Table 1 and illustrated in Figure 2.

Table 1. Average concentration (± SD) in plasma, GCF and saliva following administration of a single 100 mg oral dose of Dox (12 volunteers).

Time (h)	Plasma (μg/mL)	GCF (μg/mL)	Saliva (μg/mL)
0	0.000 (± 0.00)	0.000 (± 0.00)	0.000 (± 0.00)
0.5	0.841 (± 0.57)	0.096 (± 0.11)	0.094 (± 0.08)
1	1.293 (± 0.68)	0.642 (± 0.51)	0.218 (±0.10)
2	1.772 (± 0.90)	1.337 (± 0.94)	0.352 (± 0.10)
3	1.405 (± 0.49)	1.066 (± 0.55)	0.365 (± 0.13)
4	1.140 (± 0.41)	1.001 (± 0.36)	0.272 (± 0.10)
6	1.135 (± 0.47)	1.032 (± 0.48)	0.269 (± 0.13)
8	0.968 (± 0.38)	0.972 (± 0.46)	0.239 (± 0.07)
12	0.890 (± 0.31)	0.692 (± 0.32)	0.197 (± 0.06)
24	0.520 (± 0.19)	0.467 (± 0.25)	0.132 (± 0.07)

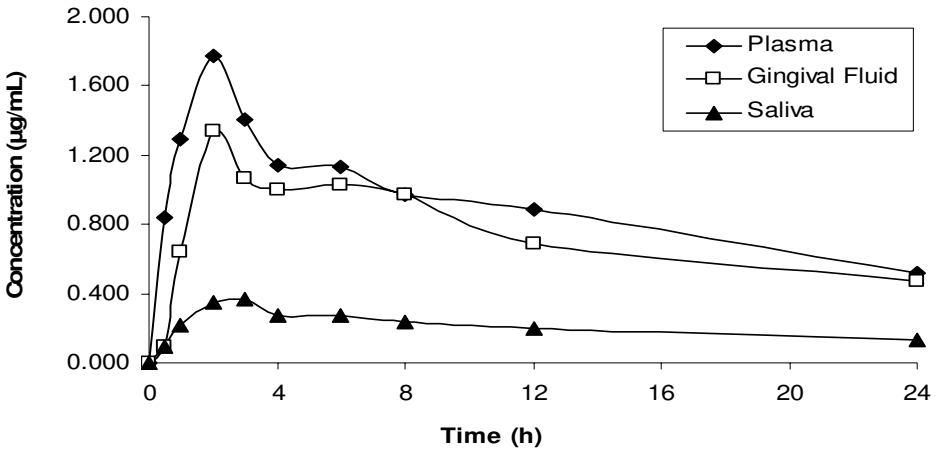

Figure 2. Concentration-time curve of Dox in plasma, GCF and saliva obtained from 12 volunteers after single oral administration (100 mg).

Dox was found 30 minutes following oral administration in all body fluid analyzed. C_{max} was achieved in plasma (1.8 μg/mL) after 2 hours, GCF (1.3 μg/mL) after 2 hours and saliva (0.4 μg/mL) after 3 h.

Dox also showed a high VD, indicating the likelihood of good penetration of biological membranes and tissue distribution. The Dox PK-parameters in the different body fluids are presented in Table 2.

Table 2. Dox PK-parameters in plasma, GCF and saliva after single 100 mg oral administration (12 volunteers).

	T_{max} (h)	C_{max} (µg/mL)	$T_{1/2}$ (h)	Cl (L/h/70kg)	Vd (L/70kg)	AUC_{0-24} (µg.h/mL)	$AUC_{0-\infty}$ (µg.h/mL)
Plasma	2.0	1.8	15.5	3.4	75.1	21.7	33.3
GCF	2.0	1.3	21.2	3.5	106.9	17.7	32.1
Saliva	3.0	0.4	20.6	12.6	375.0	5.0	8.9

PK/PD indices

Plasma showed the highest values for all PK/PD indices. C_{max}/MIC and AUC/MIC ratios were 7.2 and 86.8, respectively considering plasma; 5.2 and 70.8, respectively considering GCF; and 1.6 and 20, respectively considering saliva.

The time in that concentration of Dox in plasma, GCF and saliva exceeded the *P. gingivalis* MIC (T>MIC) were approximately 98%, 96% and 25%, respectively.

Discussion

The knowledge of the PK/PD properties is important to select the correct dose and dosage regimen. It is also important to minimize the risk to develop microbial resistance during the treatment (Bonapace, 2002; Frimodt-Moller, 2002).

Dox is readily and almost completely absorbed (95%) from the gastro-intestinal tract (Cunha, 1999). In the present study, Dox showed a relative high half-life and VD values in the three compartments studied. According to Joshi & Miller (1997), this relative high VD of Dox could be explained by its lipophilic nature allowing an excellent tissue

penetration. In addition, Dox is a well-known time-dependent antibiotic that produces moderate to prolonged post-antibiotic effect (PAE) against the majority of susceptible microorganisms (Van Bambeke & Tulkens, 2001; Craig, 2003). Therefore, the Dox time intervals could be significantly enlarged when compared to other antibiotics with little or no PAE. These data together indicate that it is possible to maintain effective antimicrobial levels during a long period and, thus, even once-daily administration could be very acceptable.

Although Dox is considered time-dependent antibiotic, it has been shown that the T>MIC parameter is not that important due to its PAE and PK properties. Thus, the goal of therapy considering Dox is to optimize the amount of drug, being the AUC/MIC index more important to determine its efficacy.

In general, the PK/PD ratios has been considered as 100 to 125 h for AUC/MIC and 8 to 12 for C_{max}/MIC considering concentration-dependent antibiotics and 60% for T>MIC considering time-dependent antibiotics (Mouton, 2002; Barger *et al.,* 2003). The present study indicated that the oral administration of 100 mg of Dox was satisfactory for T>MIC parameter in both plasma and GCF but the values of AUC/MIC and C_{max}/MIC were lower than those indicated in the literature (100 – 125 for AUC/MIC and 8 to 12 for C_{max}/MIC) for all compartment studied. Thus, in order to improve these indices, an initial dose higher than 100 mg should be used. This procedure could increase the Dox concentration in the body fluids and consequently the AUC and C_{max} values. There are very few side effects attributed to Dox and an increase on the clinical dose could be safe.

A previous study showed a higher concentration of Dox in GCF than plasma (Pascale *et al.*, 1986). However, in the present study, we found plasma levels 40% higher than GCF levels. Probably, this difference occurred because we used a single administration rather than multiple administrations. According to Golub *et al.* (1994), Dox is able to bind to the dental-root surface, favoring its slow release, while it keeps the antimicrobial activity, and therefore, it could maintain increased GCF concentrations, especially after multiple doses. However, we could not find in the present study higher levels of Dox in GCF in comparison to plasma. Probably, the methodology used to collect the GCF could be responsible for these differences.

Sakellari *et al.* (2000) observed that this high GCF levels could be related to methodology errors. Many studies used long sampling times (2 minutes or more) and, in this case, it is possible that the samples could have concentrated on the filter paper strips by evaporation. In order to minimize this effect, the GCF samples in the present study were collected in just 30 seconds. This sampling short time was shown to be adequate to collect sufficient fluid for the PK analyses by bioassay method and it minimized the evaporation since the strips were kept on sealed mini tubes.

In conclusion, the present study suggests that it could be necessary higher doses than 100 mg to improve the antimicrobial clinical outcomes considering AUC/MIC index.

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2.2 CAPÍTULO 2

Suppression of RANKL-mediated bone resorption by doxycycline

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Abstract

Inflammatory bone resorption occurs in the context of chronic infectious diseases, such as periodontal disease, septic arthritis and osteomyelitis. In such infectious bone loss lesions, receptor activator of NF-kappaB ligand (RANKL) plays a key role in the induction of osteoclast differentiation and activation. In the present study, we found that doxycycline (Dox), a semi-synthetic tetracycline antibiotic, was able to suppress RANKL-mediated bone resorption, both *in vitro* and *in vivo*. Dox inhibited RANKL-mediated osteoclast differentiation from both RAW264.7 cells and mouse bone marrow cells. Dox also inhibited matrix metalloproteinase-9 (MMP-9) activity in RANKL-stimulated RAW264.7 cells, while the total protein level of MMP-9 up-regulated by RANKL stimulation was not affected by the presence of Dox. Importantly, systemic Dox administration did not alter the viability of RAW264.7 cells as well as bone marrow cells, irrespective of RANKL stimulation, indicating that the reduction of osteoclastogenesis was not due to the loss of cell number as a result of Dox toxicity. Dox did not affect the RANKL-mediated phosphorylation of p38-, ERK- or JNK-Mitogen-Activated Protein Kinase (MAPK), key signal transduction molecules in osteoclast differentiation and activation. Focused DNA array and RT-PCR analyses revealed that Dox suppresses the mRNA expression of osteoclast differentiation/activation c-Myc promoter. Systemic Dox administration also suppressed *in vivo* induction of TRAP-positive osteoclasts, which were induced in calvaria tissues by sRANKL/LPS injection, without affecting systemic bone mineral density (BMD). The suppression function of Dox on MMP-9 enzyme activity has been previously demonstrated. More important to the present study, however, the results noted above indicated that Dox also suppresses RANKL-mediated bone resorption by interrupting the induction of osteoclast differentiation in association with the inhibition of mRNA expression for c-Myc, TRAP and cathepsin K. In conclusion, the present study indicated that the efficacy of Dox in the amelioration of inflammatory bone loss lesion seems to involve novel bio-effects of Dox which inhibit RANKL-mediated osteoclastogenesis, in addition to its previously known effects as an antibiotic and as an MMP suppressor.

Keywords: Doxycycline therapeutics, RANKL, Osteoclasts, MMP-9, c-Myc.

Introduction

Bone resorption is facilitated by osteoclasts, multinucleated giant cells, which are derived from mononuclear precursor cells present in bone marrow. These cells not only play an important role in the maintenance of homeostatic bone remodeling, but they also cause pathological bone loss in several diseases, including rheumatoid arthritis, osteomyelitis, periodontal disease and postmenopausal osteoporosis (9, 39).

Differentiation and activation of osteoclast cells depend on the stimulation of osteoclast precursor cells with receptor activator of nuclear factor ligand (RANKL) and its permissive cytokine M-CSF (2). RANKL is a member of the tumor necrosis factor family and is expressed by osteoblast/stromal cells, as well as by activated lymphocytes (2, 14, 32, 37, 38), under regulation of a complex system of co-stimulatory signals generated by other cell surface receptors (16). In addition to RANKL, tumor necrosis factor alpha (TNF-α) was also found to stimulate osteoclastogenesis in an RANKL-dependent or -independent manner (15, 18).

Host-derived enzymes regulate the remodeling of extracellular matrix proteins which are important components in both soft tissue and bone. A group of enzymes, called matrix metalloproteinases (MMPs), is intimately involved in the degradation of extracellular matrixes, and 26 enzymes are currently known to belong to the MMP family (22). The importance of MMPs, especially MMP-9 (gelatinase B), derives from their involvement in the physiologic and pathologic decomposition of hard tissue, particularly bone, which is composed of both extracellular matrix proteins and calcified mineral compounds. Originally, MMP-9 was found to be secreted by macrophages and neutrophils, as these cells migrate towards the inflamed tissues by a process of degrading the matrix components in the basal membrane (6). Later, MMP-9 was demonstrated to be involved in the invasive activity of osteoclasts by causing the degradation of extracellular matrix collagens (12, 29, 43). It is true that MMP-9 (gelatinase B) is recognized as the most abundant gelatinolytic MMP in mature osteoclasts (25, 44). However, in spite of previously accumulated knowledge, the specific mechanism underlying matrix metalloproteinase inhibitors (MMPIs)-mediated inhibition of bone resorption is yet to be elucidated,

specifically whether MMPIs inhibit bone loss solely by suppressing the MMP activities without affecting the differentiation or activation of osteoclasts.

MMPIs can inhibit alveolar bone loss induced by local LPS injection in a rat model of experimental periodontal disease (24). Among clinically applied MMPIs, the use of doxycycline (Dox), a semi-synthetic tetracycline antibiotic, has been growing in clinical practice for a range of diseases (28). This essentially results from its robust inhibitory effects on MMPs, especially MMP-9 (26, 30). Based on the clinical efficacy of Dox, as demonstrated in its effects on rheumatoid arthritis and periodontal disease, which both involve inflammatory bone loss, the non-antimicrobial effects of Dox and its derivatives are therefore indicated as having anti-inflammatory action that involves inhibition of MMPs (8, 28). The inhibitory effect of Dox on bone loss has been attributed to its ability to inhibit MMP-9 activity and to possibly suppress osteoclast differentiation (8, 27). Still, the precise mechanism underlying Dox-mediated bone loss inhibition is unclear. Most importantly, although it has become apparent that RANKL plays a key role in the induction of osteoclast differentiation and activation of mature osteoclasts, the effects of Dox on the RANKL-dependent osteoclast differentiation mechanism are totally unknown. To address these questions, the present study investigated the influences of Dox on RANKL-mediated osteoclastogenesis, both *in vitro* and *in vivo*, that result in bone resorption.

Materials and Methods

Antibiotics

Doxycycline (Dox), Amoxicillin (Amo), Vancomycin (Van), Bacitracin (Bac), Spectinomycin (Spc) and Gentamicin (Gen) used in this study were purchased from Sigma-Aldrich Corp., St. Louis, MO, USA.

In vitro osteoclast differentiation and cellular viability assays

A murine macrophage cell line (RAW264.7) ($5x10^3$ cells/well) and BALB/c mouse bone marrow cells ($2x10^5$ cells/well) were stimulated with 50 ng/ml of soluble RANKL (sRANKL, mouse recombinant, PeproTech Inc., Rocky Hill, NJ, USA) in α-MEM

supplemented with 15% Fetal bovine serum (FBS) and containing 100 units/ml penicillin and 10 μg/ml streptomycin (α-MEM complete medium). For the bone marrow culture, M-CSF (10 ng/ml, PeproTech) was supplemented with sRANKL. The following antibiotics were additionally applied to this α-MEM complete medium: Dox (0.2 and 2 μg/mL); Amo (0.5 and 5 μg/mL); Van (2 and 20 μg/mL); Bac (0.2 and 2 μg/mL); Spc (10 and 100 μg/mL) and Gen (0.5 and 5 μg/mL). The cells were incubated for 6 days at 37°C in 5% CO_2 atmosphere. The half volume of culture supernatant was replaced with fresh medium containing sRANKL (and M-CSF for bone marrow), in the presence or absence of antibiotics, every three days. After the appropriate incubation time (RAW264.7 cells - 6 days; bone marrow cells - 8 days), the medium and nonadherent cells were removed by rinsing with phosphate buffered saline solution (PBS). The remaining cells in each well were fixed with formaldehyde saline and subsequently stained by tartrate-resistant acid phosphatase (TRAP-staining assay) (13, 14, 40). Osteoclast-like cells with three or more nuclei were identified as TRAP-positive ($TRAP^+$) cells.

In addition, the effect of antibiotics on the viability of RAW264.7 cells and bone marrow cells was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl-tetrazolium bromide (MTT) assay. After incubation of cultured cells in 96-well culture plates with MTT for 4 hours, the blue formazan that developed in the viable cells was dissolved by isopropanol, and the intensity of dissolved blue formazan was measured using (OD at 575 nm). The production of formazan from the treated and control (non-treated) cells was then compared.

Pit formation assay

In order to determine the bone resorption activity by mature osteoclasts, a pit formation assay was carried out using dentin discs (diameter = 5 mm; ALPCO Diagnostics, Windham, NH, USA) (16). The RAW264.7 cells ($5x10^3$ cell/well) were cultured on dentin discs in 96-well culture plates for 6 days with sRANKL (50 ng/mL), with or without Dox (2 μg/mL). The half portion of culture medium was replaced with fresh medium containing respective additive every three days. After incubation, the cells were removed by washing

with 10% sodium hypochlorite, and the dentin discs were then stained by a 0.5% Toluidine Blue solution (14).

Gelatin Zymogram

For the measurement of gelatinase activity, a gelatin zymography was utilized. The amount of total protein in each sample was measured using Quant-iT™ protein assay kit (Invitrogen, Carlsbad, CA, USA). Based on the results of Quant-iT™ protein measurement, an equal amount of sample protein was separated by electrophoresis in 10% Tris-Glycine zymogram gelatin gel (Invitrogen) under non-reducing conditions. After renaturing the proteins separated in the gel, the zymogram gel was developed in Developing Buffer supplied by the manufacturer (Invitrogen). Subsequently, the developed gelatin gel was stained with colloidal blue stain (Invitrogen), allowing the proteases to be visualized as clear bands on the gelatin gel.

Western blot analysis

Total proteins were extracted from the cultured cells with lysis buffer-A that contains 25 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-100, 0.1% SDS, 1% NP-40, 1 mM PMSF, protease inhibitor cocktail (Sigma) and phosphatase inhibitor I and II (Sigma) (10^6 cells/100 ul of lysis buffer). Insoluble cellular components were removed by centrifugation at 10,000 × g at 4°C for 20 min. The cell lysates were further dissolved in SDS-PAGE loading buffer (Invitrogen, Carlsbad, CA, USA) containing 2-mercaptoethanol. After boiling for 10 min, the cell lysates were subjected to SDS-PAGE, and the proteins separated in the gel were blotted onto a nitrocellulose membrane. After blocking with 5% skim milk, the membrane was incubated with specific primary antibodies to MMP-9 (goat polyclonal, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and to α-Tubulin (mouse monoclonal, clone B-7, Santa Cruz), followed by incubation with peroxidase-conjugated rabbit anti-goat IgG and peroxidase-conjugated goat polyclonal anti-mouse IgG (Sigma), respectively. The bands recognized by the specific antibody were visualized using the chemiluminecsence-based ECL™ system (Amersham Biosciences, Piscataway, NJ) and exposed to an X-ray film (Eastman Kodak, Rochester, NY). In order to

investigate the effect of Dox on mitogen-activated protein kinase (MAPK) signaling pathways, phosphorylations of p38, ERK and JNK induced in RAW264.7 cells upon stimulation with sRANKL were examined using Western blot.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cultured cells by using RNA-Bee (TEL-TEST, Inc., Friendswood, TX). Complementary DNA (cDNA) was synthesized from the freshly isolated total RNA (100-500 ng) using SuperScript™-II transcriptase (Invitrogen) following the manufacturer's instructions. The resulting cDNA was reacted and amplified with specific PCR primers in the presence of Taq polymerase (Hot Start Taq, Qiagen, Valencia, CA). The primer sequences are as follows: TRAP, 5'-ACACAGTGATGCTGTGTGGCAACTC and 3'-CCAGAGGCTTCCACATATATGATGG (26); cathepsin K, 5'-CTGAAGATGCTTTCCCATATGTGGG and 3'- GCAGGCGTTGTTCTTATTCCGAGC (26); c-Myc, 5'-ACGTCTCCACTCACCAGCACAACT and 3'-GCTCCTCCTCGAGTTAGGTCAGTT; β-actin, 5'-GCTTCCTGGGCATGGAATCCTG and 3'-GGAGGAGCAACAATCTTGATCTTC. The amplified PCR products were separated by electrophoresis in 1.5% agarose gels. The PCR products in the gels were visualized by SYBR safe™ (Invitrogen) and scanned using AlphaImager® (San Leandro, CA).

In vivo bone resorption assay - Induction of osteoclasts in mouse calvaria tissue

In vivo, the effect of Dox on MMP activity and on osteoclastogenesis was evaluated using mouse calvaria (21). BALB/c mice (6-week-old males – 5 animals/group) received a supra-calvaria injection (50 μl/site/day) of a combination of sRANKL (5 μg/ml) and LPS (500 μg/ml), or control PBS, for three consecutive days. The animals were treated with daily administration of Dox (30 mg/kg), or PBS by gavage, for 10 days. Zymography and TRAP staining were carried out on the calvaria tissues removed from the killed animals on Day 10. All experimental procedures using mice were approved by the Institutional Animal Care and Use Committee (IACUC) at The Forsyth Institute.

A half portion of calvaria was homogenized in lysis buffer-B (0.1% SDS, 1% NP-40, 1% Triton X-100, 25 mM Tris and 150 mM NaCl) without protease inhibitors. After adjusting the concentration of protein using the Quant-iT™ protein assay kit, calvaria tissue homogenates were subjected to gelatin gel zymography and Western blot analyses for MMP-9 and α-tubulin, as previously described.

The calvariae were fixed and decalcified in 10% EDTA solution for 21 days. These calvariae were embedded in Tissue-Tek (Sakura, Torrance, CA), and coronal sections (8 μm in thickness) were obtained using a cryostat. The sections were stained for TRAP, and the nuclei were counter-stained with methyl green.

Measurement of TRAP activity in the calvaria homogenates

The calvaria tissue was homogenized in the lysis buffer-B. The samples and standard recombinant TRAP were reacted with p-nitro-phenol-phosphate in 150 mM tartrate buffer (pH 5.5) supplemented with 1 mM $MgCl_2$. After 2 h of incubation at 37°C, the reaction was stopped by the addition of 2N NaOH. The absorbance at 405 nm was measured by a spectrophotometer. This assay system only showed specificity to TRAP, but did not show reactivity to alkaline phosphatase (ALP). Consequently, ALP activity was determined by the parallel assay using ALP (Sigma) as a control group, which did not show color development under the conditions described above.

Effect of Dox on bone mineral density (BMD) – in vivo assay

BALB/c mice (8-week-old males, 5 mice/group) were treated with either Dox (30mg/kg/day in water solution) or with control water for 30 days (p.o. every single day). Afterwards, the animals were killed, and the measurement of BMD (g/cm^2) of whole body and femur was performed using PIXImus™ bone mineral densitometer (Lunar Corp., Madison, WI).

Statistical analysis

The results were expressed as mean ± standard deviations (SD). Statistical differences between the mean values of control and experimental groups were analyzed by using ANOVA or Student's *t* test at a significance level of 5%.

Results

Dox suppressed RANKL-mediated osteoclast differentiation

To examine the effects of Dox on RANKL-mediated osteoclastogenesis, RAW264.7 cells were stimulated with sRANKL in the presence or absence of different antibiotics (Figure 1 A). Compared to the number of $TRAP^+$ multinuclear cells induced in the positive control of RAW264.7 cells, which were stimulated with sRANKL alone, Dox concentration at both 0.2 and 2 μg/ml significantly reduced the number of $TRAP^+$ multinuclear cells. The other antibiotics, including Amo, Van, Bac, Spc and Gen, did not show any suppressive effect on the RANKL-mediated osteoclastogenesis induced in RAW264.7 cells.

Dox treatment also inhibited the induction of $TRAP^+$ multinuclear cells in bone marrow cells upon sRANKL stimulation in the presence of M-CSF. Since Dox also inhibited the induction of $TRAP^+$ multinuclear cells from bone marrow cells stimulated with sRANKL alone, Dox appeared to inhibit the RANKL-mediated differentiation of both osteoclast precursor cells, as well as intermediately differentiated pre-osteoclasts (Figure 1B). The images of TRAP staining of both RAW264.7 cells and bone marrow cells stimulated with sRANKL in the presence or absence of Dox are shown in Figure 2 (A-C, RAW264.7 cells; and D-F, bone marrow cells). Importantly, the increased number of $TRAP^+$ mononuclear cells was observed in both RAW264.7 cells, as well as bone marrow cells, which were stimulated with sRANKL in the presence of Dox (Fig. 2 C and F), suggesting that Dox allowed osteoclast precursor cells to differentiate to $TRAP^+$ pre-osteoclast cells.

The bone resorption activity of mature osteoclasts induced in RANKL-stimulated RAW264.7 cells was analyzed on dentin slice surfaces (Fig. 2, G-H).

Stimulation of RAW264.7 cells with sRANKL remarkably increased decalcified areas on the dentin discs (H) compared to the control non-stimulated RAW264.7 cells (G), which showed few or no resorption pits. The addition of Dox to the culture of sRANKL-stimulated RAW264.7 cells inhibited the formation of resorption pits on dentin discs (I). These results indicated that Dox not only suppresses RANKL-mediated osteoclast differentiation, but also inhibits bone resorption activity.

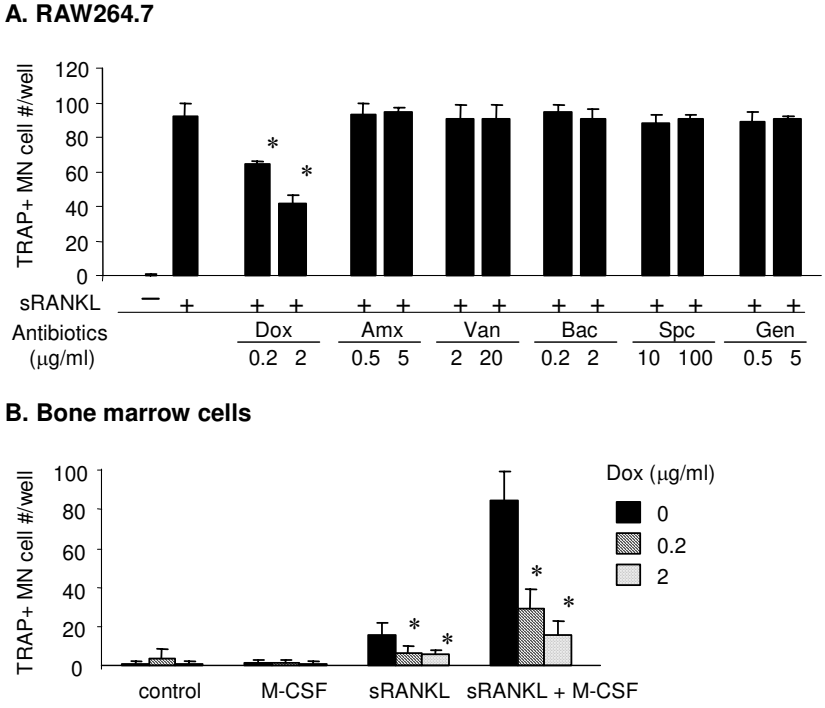

Fig. 1. Effects of antibiotics on the RANKL-dependent induction of TRAP$^+$ multinuclear cells. A) RAW264.7 cells were stimulated with sRANKL (50 ng/ml) for 6 days in the presence or absence of antibiotics shown in the figure. B) BALB/c mouse bone marrow cells (6-week-old) were cultured with sRANKL and/or M-CSF in the presence or absence of two different doses of Dox (0.2 and 2 μg/ml) for 8 days. A half portion of culture medium was replaced every three days. After respective periods of incubation, the cells were fixed with formalin and subjected to TRAP staining. TRAP$^+$ cells with nuclei equal to, or exceeding, three were counted. Data represent mean ± SD of triplicate cultures. *Significantly lower than sRANKL or sRANKL+M-CSF stimulation by Student's *t* test (p<0.05).

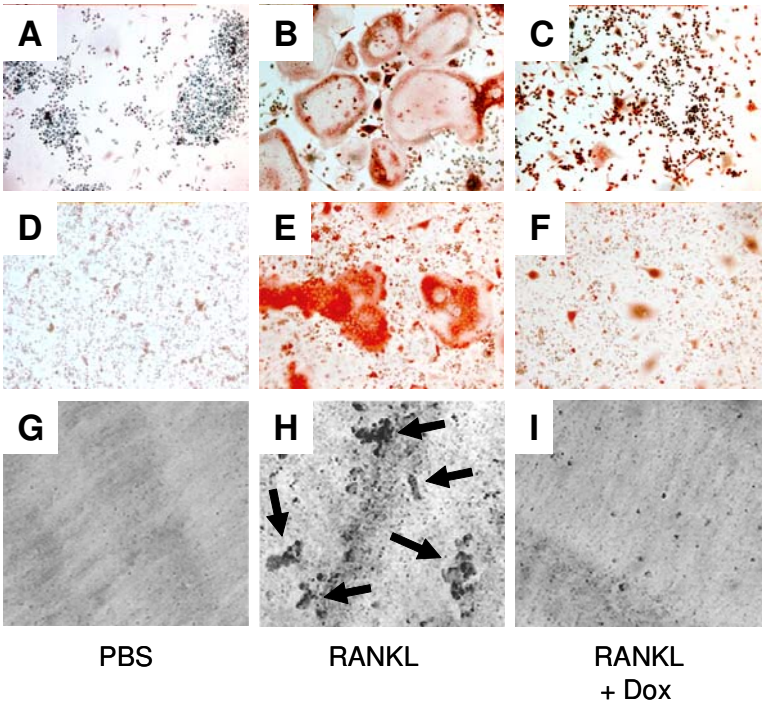

Fig. 2. Microscopic analyses of the effects of Dox on the appearance of osteoclasts induced by sRANKL and pit formation assay to determine the effect of Dox on the osteoclasts' bone resorption function (Magnification, 20x). RAW264.7 cells (A-C) and bone marrow cells (D-F) were stimulated with or without sRANKL in the presence or absence of Dox (2 μg/ml), following the same incubation protocol described in Fig. 1. After respective periods of incubation (RAW264.7 cells - 6 days; bone marrow cells - 8 days), the cells were fixed and stained for TRAP, followed by nuclear staining with methyl green. Pit formation assay (G-H) was carried out on the dentin slices using RAW264.7 cells. After incubation for 6 days, the cells were removed by washing with 10% sodium hypochlorite, and the dentin discs were stained by a 0.5% Toluidine Blue solution. The arrows (H) indicate the decalcified pits formed by activated mature osteoclasts.

In order to evaluate whether the suppression effects of Dox on RANKL-mediated osteoclastogenesis is due to its toxicity, MTT assay was carried out on the RAW264.7 cells and bone marrow cells stimulated with sRANKL in the presence or absence of Dox (Fig. 3). Dox did not decrease the viability of either RAW264.7 cells or

bone marrow cell cultures, irrespective of the presence of sRANKL. These results indicated that the suppression of RANKL-mediated osteoclast differentiation/activation by Dox was not due to the presence of any Dox toxicity.

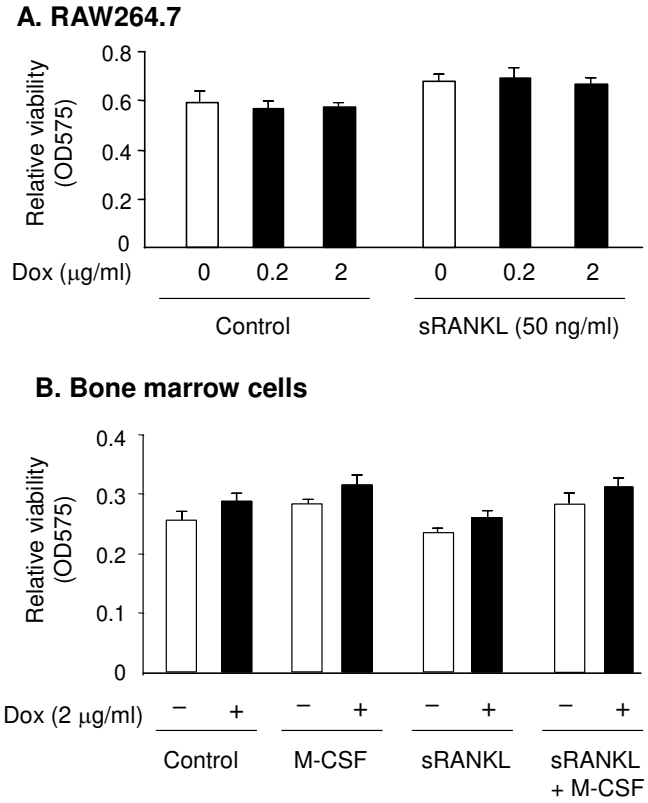

Fig. 3. MTT assay for toxic influence of Dox on sRANKL-stimulated RAW264.7 cells and bone marrow cells. MTT testing was carried out to evaluate the effect of Dox and sRANKL on cell viability. A) RAW264.7 cells were cultured with or without sRANKL (50 ng/ml) in the presence or absence of Dox for 24 hours. B) Bone marrow cells were cultured with or without sRANKL (50 ng/ml) and/or M-CSF (10 ng/ml). All groups of cells were subjected to MTT assay, and relative viability of cells in each well was expressed by OD575 of dissolved formazan. The Dox treatment groups did not show any statistically significant difference compared to the control non-treatment groups. Solid or open columns indicate “with” or “without Dox treatment”, respectively.

Dox suppressed MMP-9 gelatinase activity in the RANKL-stimulated RAW264.7 cells

The non-antibiotic property of Dox is implicated in the inhibition of MMP activities, especially MMP-9 (4). Therefore, the influence of Dox on the MMP-9 activity of RAW264.7 cells stimulated by sRANKL was analyzed in gelatin gel zymography (Fig. 4 A). Stimulation of RAW264.7 cells with recombinant sRANKL induced an up-regulation of MMP-9 activity, whereas Dox nearly abolished this up-regulation (Fig. 4 A).

The influence of Dox on MMP-9 protein expression in RAW264.7 cells was examined by Western blot (Fig. 4 B). This analysis demonstrated that sRANKL stimulation up-regulated MMP-9 protein expression. More importantly, the addition of Dox to the RAW264.7 cell culture did not affect sRANKL-induced up-regulation of MMP-9 protein expression in the RAW264.7 cells. These results indicated that Dox suppressed MMP-9 activity, but not MMP-9 protein expression (Fig. 4). In summary, stimulation of RAW264.7 cells with sRANKL up-regulated the amount of MMP-9 protein, irrespective of the presence of Dox, whereas MMP-9 activity was blocked by Dox.

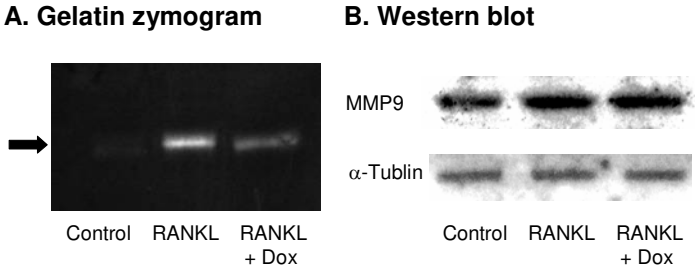

Fig. 4. Gelatin gel zymography and Western blot analyses for the MMP-9 expressed in RAW264.7 cells. RAW264.7 cells were incubated with sRANKL in the presence or absence of Dox for 24 hours. The amount of protein isolated from harvested cells was adjusted and subjected to gelatin gel zymography (A) and Western blot for MMP-9 and α-Tubulin (B). Mature MMP-9 (MW 86 kD) is indicated by arrow shown in the zymogram gel.

Dox administration "in vivo" suppressed osteoclast differentiation and MMP-9 activity

In order to examine the effects of Dox on in vivo osteoclast differentiation, osteoclastogenesis was induced by supra-calvaria injections with a mixture of sRANKL and LPS. Since bacterial inflammatory components, such as LPS, are present in the context of chronic infectious diseases with bone resorption, LPS was applied together with sRANKL. Ten days after sRANKL/LPS injection, animals were sacrificed, and decalcified calvaria tissues were analyzed by histochemistry using TRAP staining. The sRANKL/LPS injection induced a remarkably increase in the number of $TRAP^+$ osteoclast-like cells in the calvaria bone lacunae compared to the control mice, which received placebo (PBS) injection (Fig. 5 A, control PBS; B, sRANKL+LPS injection), whereas systemic administration of Dox reduced the induction of $TRAP^+$ osteoclast-like cells in the calvaria tissues receiving sRANKL/LPS (Fig. 5 C). The injection of sRANKL alone also induced relatively fewer $TRAP^+$ osteoclast-like cells in calvaria than did the sRANKL/LPS injection (not shown), indicating that inflammatory stimulation caused by LPS augments RANKL-mediated osteoclastogenesis in calvaria, but that, significantly, Dox can inhibit the RANKL-mediated osteoclastogenesis in the context of inflammation.

The TRAP enzyme activities measured in the homogenates of calvaria tissues also demonstrated a similar trend in the histochemical evaluation (Fig. 5 D). In this analysis, the sRANKL/LPS injection significantly up-regulated the TRAP activities in the calvaria homogenates compared to the control PBS-injected calvaria tissues, while Dox administration abrogated such TRAP activities induced by sRANKL/LPS injection (Fig. 5 D).

The gelatinase activity of MMP-9 in the calvaria tissue homogenates was also examined using Zymography (Fig. 5 E). Dox treatment abrogated the gelatinase activities of MMP-9 induced in the calvaria tissues receiving sRANKL/LPS injection. Total MMP-9 protein was similarly increased in the calvaria tissues, irrespective of Dox administration. These data indicated that systemic Dox administration can inhibit the RANKL-mediated *in vivo* osteoclastogenesis accompanied by reduced MMP-9 activities. These results, in turn, supported the *in vitro* findings that Dox inhibits sRANKL-mediated osteoclastogenesis, as

well as MMP-9 activities, while allowing the expression of MMP-9 protein expression in $TRAP^+$ mononuclear cells (Fig. 5 F).

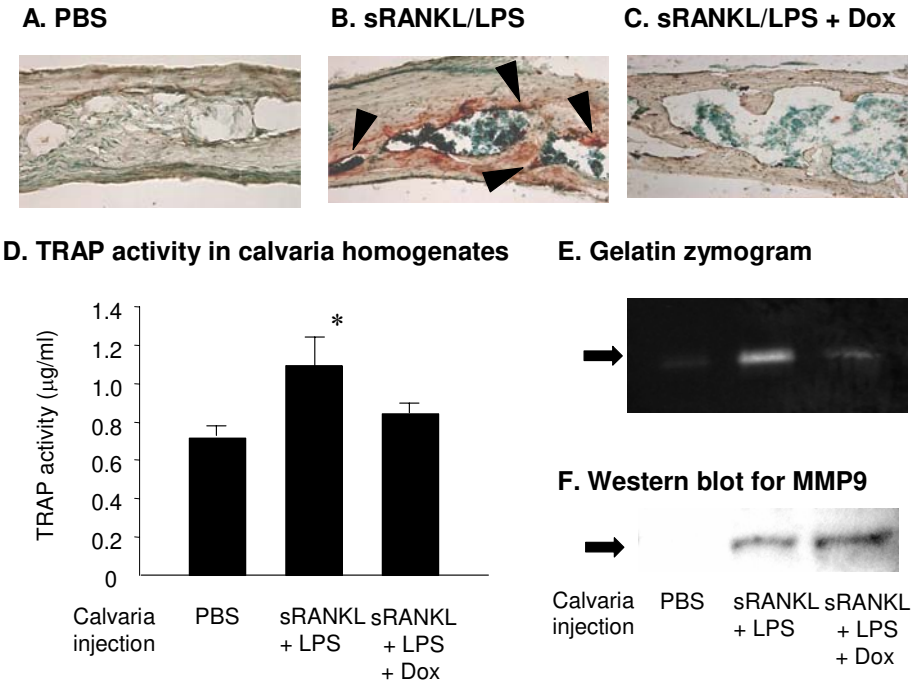

Fig. 5. Influence of Dox on the *in vivo* osteoclastogenesis induced in mouse calvaria tissues. After ten days of supra-calvaria injections with a combination of sRANKL (5 μg/ml) and LPS (500 μg/ml), or control PBS for three consecutive days, calvaria tissues were decalcified and stained for TRAP. sRANKL/LPS-injected groups received systemic Dox administration (30mg/kg/day) or placebo PBS for 10 consecutive days. Histochemical results of calvaria tissues stained for TRAP are shown: A) control PBS injection, B) sRANKL/LPS injection and C) sRANKL/LPS injection and systemic administration with Dox (20x magnification; arrows indicate $TRAP^+$ cells). TRAP enzyme activities in the calvaria tissue homogenates from 10-day-old samples were determined (D). *Significantly higher than control PBS placebo injection by Student's *t* test ($p<0.05$). Using identical samples of calvaria tissue homogenates, gelatin gel Zymography (E) and Western blot for MMP-9 (F) were carried out, respectively. Mature MMP-9 (MW 86 kD) is indicated by arrow in the zymogram gel.

Systemic administration of Dox shows no effects on systemic bone mineral density

Since Dox could inhibit *in vivo* RANKL-mediated osteoclastogenesis, the influence of Dox on homeostatic bone remodeling was examined (Fig. 6). Normal male BALB/c mice were treated with Dox or control water for 30 days. Bone mineral density (BMD) of whole body or femur measured on Day 30 did not show any significant differences between Dox-treated mice and control mice, indicating that Dox does not influence systemic physiologic bone metabolism during a time period of less than 30 days.

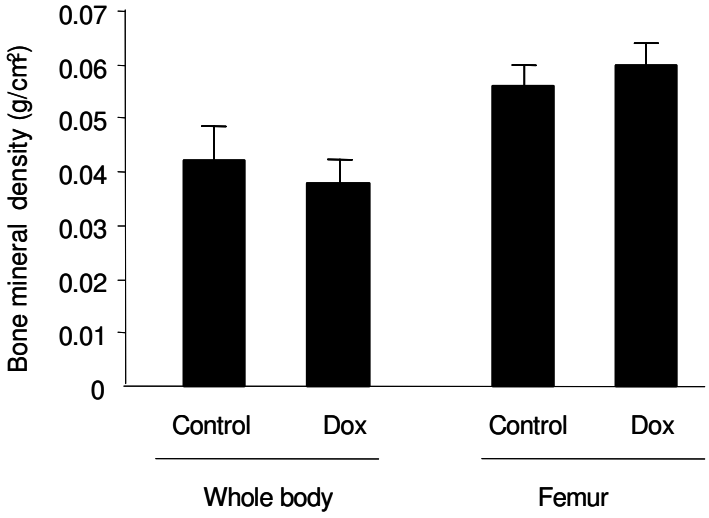

Fig. 6. Influence of Dox on systemic bone mineral density of mice. BALB/c mice (8-week-old males, n = 5 mice/group) were treated with either Dox (30mg/kg/day in water solution) or with control water for 30 days (p.o. every single day). Afterwards, the animals were sacrificed, and the measurement of BMD (g/cm^2) of whole body and femur was performed using PIXImus™ bone mineral densitometer (Lunar Corp., Madison, WI). The group treated with Dox did not show any statistically significant difference compared to the control placebo group receiving PBS.

Modulation of c-Myc expression by Dox.

RANKL ligation to its receptor (RANK) elicits the ubiquitination of the signaling adaptor molecule, TNF receptor-associated factor 6 (TRAF6), which transduces the activation signals through NF-kB and MAPK pathways. Such activation signals induce

the expression of transcription factors required for osteoclast differentiation, including c-Fos, c-Myc and nuclear factor of activated T-cells c1 (NFATc1) (1, 23, 31, 33, 35). In order to examine the effects of Dox on the signaling pathways during RANKL-mediated osteoclastogenesis, the phosphorylation of MAPK pathway molecules, including p38, ERK and JNK, were examined by Western blot analysis (Fig.7 A). However, phosphorylation of these three molecules in RAW264.7 cells upon activation by RANKL was not affected by the presence of Dox, indicating that Dox affects either the downstream signaling of these three key molecules or other signaling pathways that are cooperative to MAPKs in RANKL-mediated osteoclastogenesis, such as Ca^{2+}/Calcineurin or NF-kB pathways.

RT-PCR analysis, as shown in Fig. 7B, demonstrates that Dox down-regulated c-Myc and mRNA expressions, as well as cathepsin K and TRAP. These translated proteins play key functional roles in the mature osteoclasts. Importantly, c-Myc is required in the up-regulation of mRNA expression of cathepsin K and TRAP during osteoclast differentiation (1). Therefore, the Dox-mediated, down-regulated mRNA expression of cathepsin K and TRAP appeared to be associated with the diminished expression of c-Myc in RANKL-stimulated RAW264.7 cells. These data indicate that the inhibitory effects of Dox on RANKL-mediated osteoclastogenesis are, at the very least, attributed to its suppression of c-Myc gene expression. To confirm if Dox suppresses c-Myc during RANKL-mediated osteoclastogenesis, focused gene expression arrays were employed. The results revealed that Dox did suppress the gene expression of c-Myc in RAW264.7 cells upon stimulation with sRANKL, whereas Dox did not suppress gene expression of c-Fos, which was also increased by RANKL stimulation in RAW264.7 cells (data not shown).

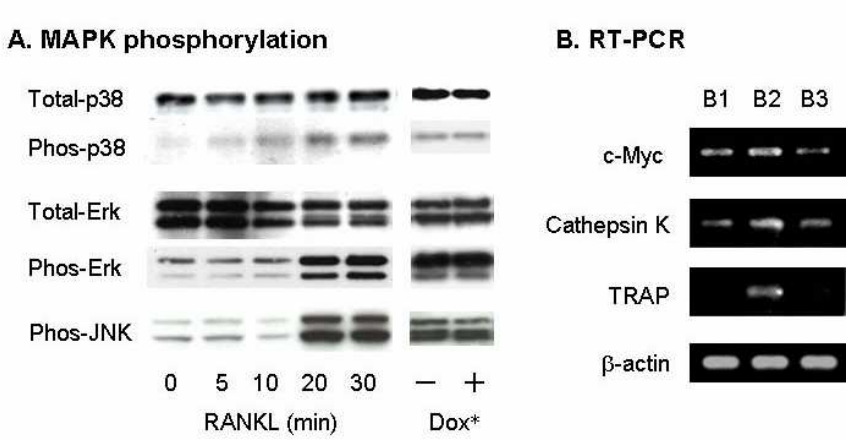

Fig 7. Effects of Dox on the phosphorylation of p38, ERK and JNK-MAPK and mRNA expression of c-Myc by RANKL-stimulated RAW264.7 cells. (A) Western blot for MAPK was carried out for p38-, ERK-, JNK-MAPK. The left hand panel shows kinetic experiments performed from 5-30 min after stimulation with sRANKL (50 ng/ml). Results of RAW264.7 cells stimulated with sRANKL (50 ng/ml) in the presence or absence of Dox (2 μg/ml) for 30 min are shown in right hand panel. (B) RT-PCR for mRNA of c-Myc; cathepsin K, TRAP and β-actin of RAW264.7 cells stimulated with sRANKL for 48 h. (B1) negative control; (B2) sRANKL (50 ng/ml); (B3) sRANKL with Dox (2 μg/ml).

Discussion

The growing use of Dox in clinical practice is due to its inhibitory activity against MMPs in addition to its antibiotic activity. Although Dox is thought to inhibit bone resorption primarily through its ability to inhibit MMPs (8, 28), its direct influence on sRANKL-mediated osteoclastogenesis has been unclear. In the present study, however, our results demonstrate that Dox can inhibit both *in vitro* and *in vivo* RANKL-mediated osteoclastogenesis in association with its suppression of c-Myc gene expression. Moreover, the present study showed that the inhibitory effects of Dox in RANKL-mediated osteoclastogenesis, while unrelated to the presence of any Dox toxicity, do, in fact, result from both the blocking of MMP-9 enzyme activity and suppression of osteoclast differentiation, especially the multinucleation process from $TRAP^+$ pre-osteoclast cells.

Our results further demonstrated that Dox down-regulated the expression of c-Myc mRNA along with the diminished mRNA expression for TRAP and cathepsin K (Fig. 7 B), as well as TRAP activity (Fig. 4 A), while Dox lacks such inhibitory effects on MMP-9 protein expression (Fig. 4 B). These results were supported by the study of Battaglino et al. (1) who found that expression of c-Myc, a transcription factor, is up-regulated in response to sRANKL and is required for osteoclast differentiation. They demonstrated this by the introduction of dominant negative c-Myc expression in RAW264.7 cells which show remarkably reduced RNA expressions for TRAP and cathepsin K upon stimulation with RANKL (1). Therefore, it is plausible that the Dox-mediated suppression of TRAP and cathepsin K mRNA expression found in the present study may be due to the c-Myc down-regulation induced by Dox.

Three enzymes, including TRAP, cathepsin K and MMP-9, are all considered functional phenotypic markers for mature osteoclasts. As such, the expression of all three molecules plays important, but different, roles in osteoclast bone resorption activity (17, 44). TRAP is engaged in bone destruction by dephosphorylation of noncollagenous matrix proteins in the bone. Cathepsin K functions in solubilization of hydroxyapatite crystals in the bone that results in exposure of the collagenous bone matrix, creating resorption lacunae after collagen degradation. MMP-9 degrades collagen types I, III, IV, and V, as well as gelatins. Therefore, MMP-9 produced by osteoclasts plays a pivotal role in digesting collagen types I, III and IV, which are present in the bone. Importantly, then, the inhibitory function of Dox on mRNA expression for cathepsin K and TRAP, accompanied by suppression of MMP-9 enzyme activity strongly appears to be responsible for the diminished pit formation activity by RANKL-stimulated RAW264.7 cells (Fig. 2 I).

A number of molecular mechanisms underlying the differentiation, function, and survival of osteoclasts have been elucidated in the last few years (16, 34, 36, 41). Holmes et al. (11) showed that Dox can reduce osteoclast differentiation induced from human peripheral blood mononuclear cells (PBMC) upon stimulation with sRANKL and M-CSF. They also demonstrated that Dox at lower concentrations (ranging between 0.25 to 2.5 μg/ml), which are similar to the ranges of Dox used in the present study, reduces osteoclast formation without induction of apoptosis in PBMC stimulated with sRANKL

and M-CSF, while higher concentrations of Dox, those of more than 5 μg/ml, induce apoptosis in maturely differentiated osteoclast cells (11). This supports the present study in which we hypothesize that Dox inhibits osteoclastogenesis independent of its toxic effects inducing cell death. We demonstrated that Dox was not toxic to RAW264.7 cells or bone marrow cells, irrespective of RANKL stimulation. Furthermore, the results of DNA array analysis for RAW264.7 cells showed that Dox suppressed one of the apoptosis induction genes, Bax, induced by RANKL stimulation (data not shown). Additionally, the present study appeared to show that the inhibitory effects of Dox on sRANKL-mediated osteoclastogenesis are partly attributable to the inhibition of c-Myc gene expression (Fig. 7) and not to the induction of cell death (Fig.3). It is, however, still to be elucidated which molecule is the direct target of Dox that results in the inhibition of c-Myc gene expression, as well as cathepsin K and TRAP mRNA, in the RANKL-mediated osteoclastogenesis.

Excessive MMP activity, with particular reference to collagenase and/or gelatinase, has been noted in many disease processes, including rheumatoid and osteoarthritis, metastatic cancer, aneurysm, adult periodontitis, osteoporosis, corneal abrasion, and others (20). Specifically, with respect to periodontitis, it has been reported that MMPI can inhibit alveolar bone loss induced by local LPS injection in a rat model of experimental periodontal disease (24). Interestingly, although Dox did not affect the expression of MMP-9 protein in RAW264.7 cells and calvaria tissue upon stimulation with RANKL, gelatinase activity of MMP-9 in RAW264.7 cells and calvaria tissues was, in fact, remarkably decreased by Dox. The inhibition of this activity is most likely due to the pharmacological action of Dox, which binds to the primary and secondary zinc and calcium ions within the catalytic domain of the MMP (7). Therefore, a decreased activity of MMP-9 would lead to a reduction in both connective tissue degradation and bone resorption, which are key events involved in the pathogenesis of osteoarthritis, periodontitis, and other diseases (5, 19, 24). It is true that MMP-9 is produced not only by osteoclast cells, but also by fibroblasts (10) that comprise the major cellular populations in connective tissues. Therefore, inhibition of MMP-9 by Dox most likely protects both hard and soft tissues from destruction.

Finally, Dox administered orally (30mg/kg/day for 30 days) did not physiologically interfere with BMD during the entire 30 days of systemic administration, while the same dose of Dox did inhibit the osteoclastogenesis induced within 10 days after RANKL injection of calvaria tissues. Brandt et al. studied the effects of Dox on 431 women with unilateral osteoarthritis of the knee (3). They reported that, after 30 months of treatment following a regimen of doxycycline, patients experienced reduced progression of minimum joint space width (JSW) in the index knee compared to the placebo group, whereas JSW of the contralateral knee was not affected by Dox treatment (3). They concluded that pathogenic mechanisms in the contralateral knee joint were different from those in the index knee. Since, even in the same knee lesion, the mechanism of bone destruction differs, it is conceivable that the mechanism underlying local inflammatory bone resorption would be different from that of systemic bone resorption in homeostatic bone remodeling. Although Dox treatment in the present study did not affect systemic BMD within 30 days, it could, however, affect BMD in long-term administration. This hypothesis is supported by a study which shows that an 8-week administration of minocycline (a Dox family agent) prevents the decrease of BMD in ovariectomized rats (42). To summarize, treating infectious inflammatory bone loss diseases with Dox may be additionally advantageous in that only bone resorption at the site of the inflamed lesion is affected, while systemic BMD remains unaffected by Dox.

In conclusion, the present study indicated that the efficacy of Dox in the amelioration of infectious inflammatory bone loss lesion seems to involve novel bio-effects of Dox which inhibit RANKL-mediated osteoclastogenesis, in addition to its previously known effects as an antibiotic and as an MMP suppressor.

Acknowledgments

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3. CONCLUSÃO

1- Os resultados da análise dos índices PK/PD sugerem que uma dose inicial maior do que 100 mg poderia resultar em melhores resultados clínicos.

2- Os resultados referentes ao efeito da Dox sobre a MMP demonstraram que embora a Dox não interfira na produção desta enzima, a atividade da MMP foi significantemente diminuída na presença deste fármaco;

3- A eficácia clínica observada da Dox, quando de seu emprego como modulador da atividade do hospedeiro, parece não se limitar à inativação da MMP, mas estar relacionada também, a propriedades adicionais deste fármaco sobre os processos de osteoclastogênese e ativação osteoclástica mediada por RANKL, incluindo a modulação da expressão de c-Myc, TRAP e Catepsina K.

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* De acordo com a norma da FOP/UNICAMP, baseadas na norma do International Committee of Medical Journals Editors – Grupo de Vancouver. Abreviaturas dos periódicos em conformidade com o Medline.

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Anexo 1 - Informação CCPG/002/06

INFORMAÇÃO CCPG/OO2/06[6]

Tendo em vista a necessidade de revisão da regulamentação das normas sobre o formato e a impressão das dissertações de mestrado e teses de doutorado e com base no entendimento exarado no Parecer PG n° 1985/96, que trata da possibilidade do formato alternativo ao já estabelecido, a CCPG resolve:

Artigo 1º - O formato padrão das dissertações e teses de mestrado e doutorado da UNICAMP deverão obrigatoriamente conter:

I. Capa com formato único ou em formato alternativo que deverá conter informações relativas ao nível (mestrado ou doutorado) e à Unidade de defesa, fazendo referência à Universidade Estadual de Campinas, *sendo o projeto gráfico das capas definido pela PRPG.*
II. Primeira folha interna dando visibilidade à Universidade, a Unidade de defesa, ao nome do autor, ao título do trabalho, ao número de volumes (quando houver mais de um), ao nível (mestrado ou doutorado), a área de concentração, ao nome do orientador e co-orientador, ao local (cidade) e ao ano de depósito. No seu verso deve constar a ficha catalográfica.
III. Folha de aprovação, dando visibilidade à Comissão Julgadora com as respectivas assinaturas.
IV. Resumo em português e em inglês (ambos com no máximo 500 palavras).
V. Sumário.
VI. Corpo da dissertação ou tese dividido em tópicos estruturados de modo característico à área de conhecimento.
VII. Referências, formatadas segundo normas de referenciamento definidas pela CPG da Unidade ou por critério do orientador.
VIII. Todas as páginas deverão, obrigatoriamente, ser numeradas, inclusive páginas iniciais, divisões de capítulos, encartes, anexos, etc... As páginas iniciais poderão ser numeradas utilizando-se algarismos romanos em sua forma minúscula.
IX. Todas as páginas com numeração "impar" serão impressas como "frente" e todas as páginas com numeração "par" serão impressas como "verso".

§ 1º - A critério do autor e do orientador poderão ser incluídos: dedicatória; agradecimento; epígrafe; lista de: ilustrações, tabelas, abreviaturas e siglas, símbolos; glossário; apêndice; anexos.

§ 2º - A dissertação ou tese deverá ser apresentada na língua portuguesa, com exceção da possibilidade permitida no artigo 2° desta Informação.

§ 3º - As dissertações e teses cujo conteúdo versar sobre pesquisa envolvendo seres humanos, animais ou biossegurança, deverão apresentar anexos os respectivos documentos de aprovação.

Artigo 2º - A critério do orientador e com aprovação da CPG da Unidade, os capítulos e os apêndices poderão conter cópias de artigos de autoria ou de co-autoria do candidato, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, escritos no idioma exigido pelo veículo de divulgação.

§ único - O orientador e o candidato deverão verificar junto às editoras a possibilidade de inclusão dos artigos na dissertação ou tese, em atendimento à legislação que rege o direito autoral, obtendo, se necessária, a competente autorização, deverão assinar declaração de que não estão infringindo o direito autoral transferido à editora.

Artigo 3º - Dependendo da área do conhecimento, a critério do orientador e com aprovação da CPG da Unidade, a dissertação ou tese poderá ser apresentada em formato alternativo, desde que observados os incisos I, II, III IV, V e VII do artigo 1º.

Artigo 4º - Para impressão, na gráfica da Unicamp, dos exemplares definitivos de dissertações e teses defendidas, deverão ser adotados os seguintes procedimentos:

§ 1º - A solicitação para impressão dos exemplares de dissertações e teses poderá ser encaminhada à gráfica da Unicamp pelas Unidades, que se responsabilizarão pelo pagamento correspondente.

§ 2º - Um original da dissertação ou tese, em versão definitiva, impresso em folha tamanho carta, em uma só face, deve ser encaminhado à gráfica da Unicamp acompanhado do formulário "Requisição de Serviços Gráficos", onde conste o número de exemplares solicitados.

§ 3º - A gráfica da Unicamp imprimirá os exemplares solicitados com capa padrão. Os exemplares solicitados serão encaminhados à Unidade em, no máximo, cinco dias úteis.

§ 4º - No formulário "Requisição de Serviços Gráficos" deverão estar indicadas as páginas cuja reprodução deva ser feita no padrão "cores" ou "foto", ficando entendido que as demais páginas devam ser reproduzidas no padrão preto/branco comum.

§ 5º - As dissertações e teses serão reproduzidas no padrão frente e verso, exceção feita às páginas iniciais e divisões de capítulos; dissertações e teses com até 100 páginas serão reproduzidas no padrão apenas frente, exceção feita à página que contém a ficha catalográfica.

§ 6º - As páginas fornecidas para inserção deverão ser impressas em sua forma definitiva, ou seja, apenas frente ou frente/verso.

§ 7º - O custo, em reais, de cada exemplar produzido pela gráfica será definido pela Administração Superior da Universidade.

Artigo 5º - É obrigatória a entrega de dois exemplares para homologação.

Artigo 6º - Esta Informação entrará em vigor na data de sua publicação, ficando revogadas as disposições em contrário, principalmente as Informações CCPG 001 e 002/98 e CCPG/001/00.

Campinas, 13 de setembro de 2006

Profa. Dra. Teresa Dib Zambon Atvars
Presidente
Comissão Central de Pós-Graduação

Anexo 2- Certificado do Comitê de Ética em Pesquisa (Capítulo 1)

UNICAMP

COMITÊ DE ÉTICA EM PESQUISA
UNIVERSIDADE ESTADUAL DE CAMPINAS
FACULDADE DE ODONTOLOGIA DE PIRACICABA

CERTIFICADO

Certificamos que o Projeto de pesquisa "Estudo de biodisponibilidade relativa entre a vibramicina® (drágeas de doxiciclina 100 mg, pfizer) e clordox® (drágeas de doxiciclina 100 mg, teuto brasileiro)", protocolo CEP nº ***128/2004***, dos Pesquisadores ***Gilson Cesar Nobre Franco*** e ***Pedro Luiz Rosalen***, está de acordo com a Resolução 196/96 do Conselho Nacional de Saúde - MS e foi aprovado pelo Comitê de Ética em Pesquisa da Faculdade de Odontologia - UNICAMP.

We certify that the research project "Relative biodisponibility study of vibramicina® (tablets of doxicycline 100 mg, pfizer) and clordox® (tablets of doxicycline 100 mg, teuto-brasileiro)", register number ***128/2004*** of ***Gilson Cesar Nobre Franco*** and ***Pedro Luiz Rosalen***, is in agreement with the recommendations of 196/96 Resolution of the National Health Committee - Brazilian Health Department and was approved by the Research Ethics Committee of the School of Dentistry of Piracicaba - State University of Campinas - UNICAMP.

Piracicaba - SP, Brazil, July 02 2004

Profa. Dra. Cinthia Pereira Machado Tabchoury
Coordenadora
CEP/FOP/UNICAMP

Prof. Dr. Jacks Jorge Júnior
Coordenador
CEP/FOP/UNICAMP

Anexo 3 - Certificado do Comitê de Ética em Animais (Capítulo 2)

THE FORSYTH INSTITUTE

Institutional Animal Care & Use Committee

June 2, 2005

To: Dr. Toshihisa Kawai

From: Richard L. Pharo, Interim Chairman

Re: Animal Protocol Amendment Application # 04-006

The amendment application for use of vertebrate animals in research:

Submitted: 5/22/05

Entitled: Role of oral-bacterial tolerance in periodontal inflammation

has been reviewed and given FULL APPROVAL by the Executive Committee of the Forsyth Institute's Animal Care and Use Committee on June 1, 2005

Protocol Approval Date: 6/2/04

Amendment Approval Date: 6/1/05

Animal Assurance Number: A3051-01

The protocol approval date and animal assurance number must appear on your grant application.

Enclosed is a copy of the signed amendment for your records.

RLP:om
cc Animal Quarters

ANEXO 4 – Comprovante de submissão à publicação do Capítulo 2

BONE-S-06-00847[1].pdf>>

From: BONE (ELS)
Sent: Tuesday, November 21, 2006 1:14 PM
To: Kawai, Toshi
Subject: Submission Confirmation

Dear Toshi,

Your submission entitled "**Suppression of RANKL-mediated bone resorption by doxycycline.**" has been received by Bone

You may check on the progress of your paper by logging on to the Elsevier Editorial System as an author. The URL is http://ees.elsevier.com/bone/.

Your manuscript will be given a reference number once an Editor has been assigned.

Thank you for submitting your work to this journal.

Kind regards,
Elsevier Editorial System
Bone

ANEXO 5 – Direitos autorais

Fls. Nº 37
Proc. Nº 01D-8807-04

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FACULDADE DE ODONTOLOGIA DE PIRACICABA

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DECLARAÇÃO

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Piracicaba, 21 de Dezembro de 2006.

GILSON CESAR NOBRE FRANCO
RG: 27780345-7
Autor(a)

PEDRO LUIZ ROSALEN
RG: 11.185.975
Orientador(a)