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Gustavo Alberto Obando Pereda

"ENVOLVIMENTO DOS TLR2 E TLR4 NO RECONHECIMENTO DAS PARTÍCULAS DE TITÂNIO E ZIRCÔNIA POR MACROFAGOS MURINOS"

Dissertação apresentada ao Instituto de Biologia da Universidade Estadual de Campinas, para obtenção do Título de Doutor em Genética e Biologia Molecular, na área de Imunologia.

Orientadora: Profa. Dra. Dagmar Ruth Stach-Machado

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Dagmar Ruth Stach Machado [Orientador]
Liana Maria Cardoso Verinaud
Marcelo Marques Rocha
Sergio Braga Cruz
Jamil Awad Shibli
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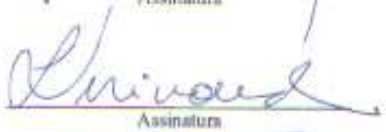
Profa.Dra. Liana Verinaud 
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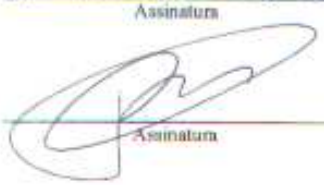
Prof. Dr. . Jamil Awad Shibli 
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TUDO PARA UMA MAIOR GLORIA DE DEUS

(Santo Ignacio de Loyola)

RESUMO

A reação biológica as particulas de desgaste é crítica para indução da perda asseptica do implante mediante a osteólise. Deste modo, foi objetivo deste trabalho avaliar o envolvimento dos receptores Toll-like receptors 2 e 4 no reconhecimento das partículas de titânio e zircônia. Mensurados em cultura de macrófagos murinos desafiados com as partículas de zircônia ou de titânio comparando a expressão de TLRs, seus adaptadores intracelulares e citocinas pró-inflamatórias. Em in vivo foram estudados a indução da osteolise utilizando o modelo de calvária e a geração da resposta inflamatoria através da indução do edema e hiperalgesia. As partículas são prontamente fagocitadas pelos macrofagos em cultura, e resultam no aumento da expressão de RNAm para TLRs 2, 3, 4 e 9, os seus adaptadores MyD88 e NF-kB e das citocinas TNF-α, IL-1β e IL-6. Contudo, o padrão de expressão de RNAm para TLRs entre as partículas é distinto, enquanto a zirconia induz um aumento significativamente na expressão de TLR2, o titânio modula a expressão signifitivamente maior de TLR3, TL4 e TLR9, respectivamente. Todavia, a expressão do RNAm para a molécula adaptadora MyD88 envolvida na sinalização intracelular de TLR é estimulada em ambas as particulas e com uma cinética de expressão semelhante. O fator de transcrição NF-kB necessário para efetuar a expressão gênica das citocinas envolvidas na resposta inflamatória apresenta uma cinética de expressão distinta entre as partículas, na zircônia a expressão é imediata e alcança o máximo da expressão após duas horas, enquanto as partículas de titânio induzem um aumento exponencial do fator de transcrição. A expressão de RNAm das citocinas inflamatórias TNF-α, IL-1β e IL-6 induzidas pelas partículas de zircônia é significativamente menor em comparação com as partículas de

titânio. Contudo a expressão proteica da citocina TNF-α é maior nas em cultura de macrofagos expostas as partículas de zircônia, enquanto as particulas de titânio induzem a expressão proteica das citocinas inflamatória IL- 6. Ambas as particulas são capazes de induzir osteólise no modelo da calvária contudo, a osteolise induzida pela zircônia assim como a perda óssea foi significativamente menor em comparação com as particulas de titânio. Assim embora ambas as particulas induzem edema e hiperalgesia nos animais de experimentação contudo as partículas de titânio uma maior sensação de hiperalgesia. Com base nos nossos resultados sugerimos que a biocompatibilidade da zircônia é maior em comparação com o titânio, e a perda asséptica é modulada pelo reconhecimento mediado pelos TLRs os quais ativam as vias de sinalização intracelular como os fatores de transcrição NF-kB levando a expressão de citocinas inflamatórias.

ABSTRACT

The biological reaction to wear debris is critical to the osteolysis underlying aseptic loosening of prosthetic implants Therefore was the aim of this study was to evaluate the involvement of Toll-like receptors 2 and 4 in the recognition of titanium and zirconia particles. Measured in cultured murine macrophages challenged with particles of zirconia or titanium by comparing the expression of TLRs, their intracellular adaptors and the proinflammatory cytokines. Particle-induced osteolysis was evaluated in mice calvaria model, whereas the inflammatory responses through induction of hind paw edema and hyperalgesia.

The particles are readily phagocytized by macrophages in culture and result in increased expression of mRNA for TLRs 2, 3, 4 and 9, its adaptors MyD88 and NF-kB, TNF-α, IL-1β and IL-6 . However, the pattern of mRNA expression TLRs is distinct for the particles, while the zirconia induces a significant increase in expression of TLR2, titanium modulates the expression significantly greater TLR3, TLR9 and TL4, respectively. However, the mRNA for the adaptor molecule MyD88 involved in intracellular signaling of TLR is stimulated in both particles and with a similar kinetic. The transcription factor NF-kB is needed to carry the gene expression of cytokines involved in the inflammatory response has a different kinetics expression between the particles, whereas zirconia induces the expression with the maximum after two hours of incubation, the titanium paricles induce an exponential increase. The mRNA expression of the inflammatory cytokines TNF-α, IL-1β and IL-6 induced by the particles of zirconia is significantly lower compared with the particles of titanium. However, the protein expression of TNF-α is greater in cultured

macrophages exposed to the particles of zirconia, while the titanium particles to induce protein expression of inflammatory cytokine IL-6. Both the particles induce osteolysis in the calvaria model however, the induced osteolysis by zirconia as well as the bone loss was significantly lower compared with titanium particles. So although both particles induce edema and hyperalgesia in animal models, titanium particles induced greater sense of hyperalgesia. Based on our results, we suggest that the biocompatibility of the zirconia is greater in comparison with the titanium and the loss is modulated by aseptic recognition mediated by TLRs which activate the intracellular signaling pathways as transcription factors NF-kB and leads to expression of inflammatory cytokines.

ABREVIATURAS

Toll-like receptors	TLRs
Nuclear factor kappa betta	NF-kB
Myeloid differentiation primary response gene (88)	MyD88
Translocation-associated membrane protein	TRAM
TIR-domain-containing adapter-inducing interferon-β	TRIF
Tumor necrosis factor alpha	TNF-α
Interleukin 1 betta	IL-1β
Interleukin 6	IL-6
Receptor activator of nuclear factor kappa-B	RANK
Receptor activator of nuclear factor kappa-B ligand	RANKL
Pathogen-associated molecular patterns	PAMPs

SUMARIO

INTRODUÇÃO

Os implantes de titânio revolucionaram a saúde oral e a saúde ortopédica, sendo que, o seu uso oral clinico bem sucedido supera os 50 anos (Palmquist et al. 2010). Esta nova técnica permite principalmente recuperar o dente ou dentes perdidos, sem a manipulação clínica de outros dentes, como a preparação dentaria para receber coroas e pónticos (prótese fixa), retém uma prótese convencional do tipo removível parcial e/ou total, além de outros benefícios como a manutenção da altura óssea, retenção de arcos faciais e ortodônticos, assim como a retenção de próteses orgânicas (Adell et al. 1981; 1986; 1988; Adell et al. 1990a; Adell et al. 1990b; Palmquist et al. 2010).

Implantes dentários estão disponíveis em diferentes materiais, sendo o mais utilizado o titânio seguido da zircônia, com diferentes tratamentos e revestimentos, diâmetros, comprimentos e plataformas. Porém o tratamento e o revestimento dado ao implante modulam muitas propriedades benéficas para a aceleração da osseointegração, cujas características são muito utilizados em termos de marketing entre empresas. Assim o clinico deve de ter em consideração as modificações na superfície do implante, no revestimento e particularidades que promovam no implante uma rápida osseointegraçao (Palmquist et al. 2010).

A inserção de um implante gera uma seqüência de eventos de cicatrização e de vascularização, conhecidas como resposta inflamatória (Thomsen et al. 1997) cuja intensidade e progressão é dependente da natureza do implante, da habilidade de reconhecimento e resposta do hospedeiro. O estabelecimento da osseointegração requer a capacidade de indução osteogênica, de modo a prover a sinalização molecular adequada que favoreça a regeneração óssea (Joos et al. 2006).

A perda do implante, classificada em perda asséptica e não asséptica (Greenfield et al. 2002), é definida pela perda ou falência da obtenção da osseointegração que resulta em dor e perda de função sendo, muitas vezes, necessária a cirurgia de revisão. Atualmente a grande maioria das perdas do implante na clinica ortopédica ocorrem devido às perdas assépticas, induzidas pela reabsorção óssea que levam a perda da fixação do implante. Este processo de osteólise é mediado por citocinas pró-inflamatórias como IL-1 α, IL-1β, IL-6 e TNF-α secretadas por células inflamatórias, como os macrófagos, no recrutamento e ativação dos precursores dos osteoclastos em resposta as partículas de desgaste (debris) produzidas pelo processo de inserção, assim como pela prótese (forças destrutivas) (Friedman et al. 1994; Greenfield et al. 2002). Estas partículas produzidas pelo desgaste são reconhecidas essencialmente pelas células do sistema imune inato e envolvem uma grande variedade de receptores celulares como os Toll-Like Receptors (TLRs) que possuem a capacidade de reconhecer padrões moleculares associados à patógenos não expressos no hospedeiro. O reconhecimento mediado pelos TLRs promove a interação destes com as moléculas adaptadoras como MyD88 (principalmente) e TIRAP, que resultam na ativação da molécula de sinalização intracelular como o fator NF-kB (fator nuclear kappa B), gerando a expressão de citocinas pró-inflamatórias que resultam na reabsorção do tecido ósseo (Xu et al. 2009), prejudicando, no caso, a osseointegração.

I. Revisão de Literatura.

Osseointegração.

A osseointegração refere-se à interfase direta entre o implante e o osso sem a interposição de tecido mole, estabelecendo uma estabilidade funcional duradoura do implante / prótese e o tecido ósseo (Branemark 1983; Puleo and Nanci 1999). O processo de osseointegração reflete um embricamento mecânico, entre as partes fixantes e inertes do implante e o osso resultando na recuperação da função do sistema substituído (Branemark 1983). O termo osseointegração descreve um estado clínico que provê uma estabilidade duradoura da prótese, contudo não é uma condição biológica para qualquer sistema de implantes (Stanford and Keller 1991). As propriedades do tecido estrutural e funcional adjacente à superfície do implante podem ser relacionadas à interação entre o material artificial e o microambiente do local onde é colocado o implante, sendo esta interação dinâmica entre os materiais artificiais e o osso inter-relacionado, afetando-se um ao outro (Joos et al. 2006). A interação osso-implante é diferente nas subáreas do preparo ósseo, desde o microambiente celular até o biofísico, como por exemplo, entre o osso cortical e medular aonde existem diferenças em relação ao travamento, sendo um travamento mais rígido no osso cortical. Assim, várias tentativas têm sido efetuadas de modo a melhorar o embricamento e estimular a formação óssea (Joos et al. 2006). Notavelmente, alterações como as características da superfície do implante e sua topografia, natureza química, (Meyer et al. 2005) e geometria do implante podem promover uma melhora na osseointegração (Meyer et al. 2003).

A taxa do sucesso clínico de um implante osseointegrado se baseia no fato da biologia óssea possuir alguns parâmetros únicos. O osso tem a habilidade de recapitular aspetos específicos do seu processo inicial de desenvolvimento e passar de um estado de regeneração a um estado de reparo "*ad integrum*" (Joos et al. 2006). A osseointegração de

um implante dentro do processo da reparação e regeneração óssea ocorre, entre a superfície do implante e o tecido que cobre o leito implantado (Joos et al. 2006). Os osteoblastos e osteócitos adjacentes ao implante produzem uma variedade de produtos morfogênicos que atuam como sinais moleculares no recrutamento e indução da proliferação e diferenciação das células esquelogênicas (Joos et al. 2006). O primeiro passo clínico da cirurgia é garantir a estabilidade primaria do implante, que consiste na fixação rígida do implante dentro do leito ósseo que impede a micromovimentação ou mesmo o deslocamento do implante (Meyer et al. 2004). Esta estabilidade primária depende das técnicas cirúrgicas, do desenho do implante e do local onde é colocado o implante (Sevimay et al. 2005), associado às propriedades bioquímicas do osso vizinho; a cortical óssea permite uma fixação mecânica do implante ao osso trabecular (Sennerby et al. 1992). O principal mecanismo que governa o processo de osseointegração nos implantes é muito similar ao que ocorre durante o reparo de uma fratura óssea envolvendo uma cascata de eventos celulares e extracelulares (Fini et al. 2004).

A resposta inicial após a inserção do implante é caracterizada pela inflamação provocada principalmente pelo trauma da cirurgia e a modificação do osso pela presença do implante. Imediatamente após o dano cirúrgico, as paredes do osso são rapidamente cobertas com sangue, assim este é o primeiro tecido em contato com a superfície do implante (Park and Davies 2000). As células inflamatórias, inicialmente os granulócitos polimorfonucleares, e em seguida os monócitos, emigram das vênulas pós-capilares e migram para o tecido adjacente que rodeia o implante. Posteriormente, as proteínas plasmáticas são absorvidas do sangue e dos fluidos teciduais, resultando na oxidação do metal utilizado no implante, evento descrito em pesquisas feitas *in vivo* e *in vitro* (Joos et

al. 2006). Muitas células inflamatórias podem ser detectadas na interfase em resposta ao estímulo da secreção de proteínas com efeito inflamatório, cicatrização óssea e reação imune produzida pela inserção do implante, podendo alterar a estrutura e as propriedades físico-químicas da superfície do implante (Thomsen et al. 1997).

Causas da não integração.

Os processos patológicos envolvidos na falha da osseointegração podem ser classificados em distúrbios de equilíbrio mecânico como sobrecarga, contaminação bacteriana (infecção) e perda asséptica**.**

- ***Equilíbrio mecânico.***

O equilíbrio mecânico é de fundamental importância durante o processo de cicatrização, e no processo de osseointegração (Albrektsson et al. 1988). Uma vez que, permite a dispersão das forças de carga da interfase osso-implante ao osso trabecular, que levam a ausência de micromovimentos. Alguns elementos são importantes para a obtenção deste equilíbrio: como a magnitude da força, o número de sobrecargas funcionais, a capacidade de reparo do osso, que é dependente da sua localização no corpo, e da área de contacto do osso com o implante (Tonetti and Schmid 1994). Se houver forças destrutivas produto de macromovimentos, ou comprometido do reparo ósseo mediado por doenças sistêmicas (diabetes entre as mais frequentes), doenças não controladas ou hábitos parafuncionais, a estabilidade mecânica ficará comprometida e haverá a perda do implante.

- ***Contaminação/Infecção bacteriana:***

Implantes dentais, em comparação com os implantes ortopédicos e dispositivos de inserção, possuem uma porção transmucosa que visa recuperar a peça dental perdida. Assim, os implantes dentários formam um complexo integrado pela intima relação entre a superfície do implante, os tecidos mucosos e o controle da placa bacteriana, requerido para a formação de um bom selado muco-implante, o qual deve ser considerado fundamental no estabelecimento de um equilíbrio entre o receptor e a placa bacteriana encontrada/presente na cavidade oral (Gristina 2004).

Assim, os implantes dentários que formam um complexo integrado pela intima relação entre a superfície do implante, requerem um bom selado entre o tecido mucoso e o implante, de modo a obter o controle efetivo da placa bacteriana, o qual é de fundamental importância para permitir o estabelecimento do equilíbrio entre o receptor e a placa bacteriana presente na cavidade oral (Gristina 2004).

A infecção/contaminação bacteriana manifesta-se numa serie de eventos inflamatórios caracterizados por dois tipos de síndromes: a lesão limitada à superfície da mucosa (mucosite periimplante), e a lesão mais grave, a periimplantite, que envolve os tecidos mucosos profundos repercutindo na porção marginal da interfase osso-implante (Tonetti and Schmid 1994).

- ***Perda asséptica:***

A perda asséptica, processo conhecido como osteólise resulta numa reabsorção extensiva de osso com a perda do implante na ausência de infecção (Harris et al. 1976). A osteólise pode ser linear: igualmente distribuída no implante; e focal: porções de osso reabsorvido em íntima relação com o implante, podendo ocorrer mesmo na ausência de

sintomas clínicos podendo o implante se manter estável (Sundfeldt et al. 2002). A teoria mais aceita que explica o processo de osteólise que resulta na perda do implante; linear, focal ou pela combinação das duas, se conhece como a teoria da partícula. As partículas produzidas pela usinagem do implante ou pela obtenção da superfície tratada na forma de plasma spray podem ser liberadas no momento da inserção do implante no leito cirúrgico, ou dispersas pelo fluido articular, por exemplo, no caso de implantes ortopédicos, ativam os macrófagos induzindo a ativação direta de osteoclastos ou mediante a ativação dos pré-osteoclastos, que iniciam a reabsorção óssea (Schmalzried et al. 1992).

Além disso, partículas de diversos biomateriais rotineiramente utilizados podem induzir uma reação biológica adversa nos tecidos periprostéticos levando a formação de granulomas produzidos pela inflamação crônica e recorrente, com a inibição da formação óssea e produção de fluidos inflamatórios que levam a perda do implante. A extensão da destruição óssea depende do número, tamanho, forma e composição das partículas. Muitas pesquisas têm sugerido que a osteólise produzida pelas partículas, pode atuar junto com outros fatores como, por exemplo, a pressão do fluido plasmático e vascular produto do edema e inflamação (Aspenberg and Van der Vis 1998). Provas histológicas em tecido periimplante mostram a formação de uma pseudomembrana contendo macrófagos e fibroblastos assim como a presença de partículas do implante com dimensões fagocitáveis (Petit et al. 2002), assim estas células parecem ser as responsáveis pelo processo inflamatório, que leva a expressão crônica uma variedade de citocinas ósseo-reabsortivas, incluindo o fator de necrose tumoral (TNF), interleucina I (IL-1), e IL-6 (Sundfeldt et al. 2006), induzidas pela fagocitose ou pelo contato direto destas partículas com superfície celular. No entanto, a natureza desta interação celular que se inicia com a fagocitose destas

partículas de desgaste e a produção posterior dos mediadores inflamatórios permanece em grande parte desconhecidas, porém os receptores Toll-like receptors (TLRs) parecem facilitar essa interação.

Uma vez fagocitadas, as partículas induzem a ativação dos macrófagos pela modulação do fator de transcrição NF-kB e a secreção de citocinas pró-inflamatorias TNF-α, IL-1β, IL-6 e IL-8. No entanto, ainda permanece sem explicação como o processo inflamatório é gerado. Embora se conheça que o TNF-α e a IL-6 sejam induzidas pela via do NF-kB, a IL-1β é produzida mediante a intervenção dos inflamassomos via caspase. A ativação inflamatória dos macrófagos leva a secreção destas citocinas induz à estimulação dos precursores de osteoclastos em osteoclastos maduros através da intervenção do RANK/RANKL que leva a exacerbação da perda óssea em tratamentos protéticos (Hallab and Jacobs 2009).

Um importante fator de transcrição de vários genes pró-inflamatórios, incluindo o TNF, é o NF-κB (fator nuclear kappa B) (Baeuerle and Baltimore 1996). Estudos prévios *in vitro* demonstraram que as partículas de titânio e polietileno são capazes de induzir/estimular a liberação/secreção/expressão de TNF-α, IL8 e IL1 em células monocíticas (Baumann et al. 2004). A importância das células da linhagem macrofágica tem sido descrito como fundamentais e intimamente associadas a indução de osteólise, uma vez que podem ser isoladas diretamente dos tecidos osteolisados; além disso, estas células multinucleadas mostram características citoquímicas e funcionais de osteoclastos (Itonaga et al. 2000). Lan *et al* demonstrou que a indução da osteoclastogênese por TNF-α ocorre como resultado da estimulação direta dos macrófagos expostos no estroma,

expressando níveis permissíveis do receptor ativador do ligante NF-κB (RANKL), cujos sinais permitem a transcrição do NF-κB (Lam et al. 2000).

As evidencias da importância do sinal de transdução do NF-κB na reabsorção óssea induzida por partículas incluem a detecção do NF-κB antes e depois da exposição das partículas do biomaterial aos macrófagos, e um decréscimo na reabsorção óssea induzida por estas partículas em camundongos knock-out para NF-κB (Schwarz et al. 2000). Clohisy et al, mostrou que a inibição do NF-κB inibe a indução de um processo inflamatório associado ás partículas de titânio e por conseqüência inibe o processo osteolítico (Clohisy et al. 2004) reafirmando a importância da molécula NF-kB no processo de reabsorção óssea.

Toll-like receptors:

A resposta imune tem sido didaticamente categorizada em resposta imune inata e adaptativa. A resposta imune adaptativa é mediada pelos linfócitos T e B, e caracterizada por sua especificidade, diversidade e pela apresentação de memória imunológica (Akira et al. 2001). A resposta imune inata, por sua vez, não possui memória imunológica, mas é capaz de efetuar o reconhecimento amplo e geral de padrões moleculares associados à patôgenos (PAMP), os quais são estruturas conservadas nos microrganismos reconhecidas por receptores de membrana conhecidas como *toll-like receptors* (TLRs) (Kaisho and Akira 2006), expressos em inúmeros tipos celulares como macrófagos e células apresentadoras de antígeno que atuam na geração da resposta imune adaptativa ou adquirida (Akira et al. 2001).

Os *toll receptors* (TRs) são proteínas transmembrana do tipo I que são evolutivamente conservadas em insetos e humanos (Gay and Gangloff 2007). O *toll* foi primeiramente identificado como uma molécula essencial no desenvolvimento embriológico da *Drosophila* mostrando subseqüentemente ser chave na imunidade antifúngica (Lemaitre et al. 1996). Famílias homólogas dos TR existentes nos mamíferos são conhecidos como TLRs (Medzhitov et al. 1997). Baseadas na similaridade nas porções citoplasmáticas (designadas como o TLR-IL-1R ou domínio TIR), os TLRs são associados ao receptor IL-1 (IL-1Rs) (Akira et al. 2001). No entanto, as porções extracelulares são diferentes: os TLRs contêm regiões de repetição ricas em leucina, onde os IL-1Rs contém três domínios parecidos a imunoglobulinas (Akira et al. 2001). Atualmente, mais de dez membros da família dos TLRs em humanos e camundongos e seus ligantes e propriedades funcionais distintas expressos diferentemente nas células imunes que parecem responder a estímulos diferentes foram descritos e caracterizados (Akira et al. 2001).

A maioria das famílias dos TLRs são expressas nas células envolvidas na primeira linha de defesa, incluindo neutrófilos, macrófagos, células dendríticas, células endoteliais da derme, e células mucosa epiteliais (Imler and Hoffmann 2001). O TLR2 e o TLR4, que são os maiores receptores para lipoproteínas e LPS, respectivamente, são também expressos nas células T e B, envolvidas na resposta imune adaptativa (Imler and Hoffmann 2001). Com exceção do TLR3 e TLR9, os demais TLRs são expressos na superfície celular. Interessantemente, a localização subcelular do TLR4 há sido mostrada ser diferente nos macrófagos e células epiteliais intestinais (Hornef et al. 2002).

A ligação via TLRs permite a ativação do NF-κB por duas diferentes vias, com a conseqüente regulação de moléculas co-estimuladoras, citocinas pró-inflamatórias e

quimiocinas. Uma via canônica resultante da ativação originada por produtos microbianos e citocinas pró-inflamatorias e uma via alternativa (não-canônica) resultante da ativação do NF-kB por citocinas da família do TNF-linfotoxina β, ligante do CD40, fator ativador de células B, e RANKL. A via canônica há sido referida em virtude da resposta imediata a sinais do TNF-α e IL-1, citocinas importantes na patogênese de doenças crônicas inflamatórias (Lawrence 2009). Outra característica deste evento permite que as células dendríticas, umas das principais células apresentadoras de antígenos que expressam TLRs, madurem e migrem para os linfonodos, aonde ocorre a ativação das células T, gerando a resposta imune adaptativa. Assim, fica claro que o NF-kB modula a resposta inflamatória a qual é mediada por vários mecanismos que afetam a magnitude e duração desta resposta.

O NF-kB possui, porém, uma importante atuação nos processos infecciosos, inflamatórios e cancerígenos no sistema esquelético, sendo sua principal função a ativação dos osteoclastos assim como a sinalização intra-celular (Karin and Greten 2005; Xu et al. 2009). Nos processos osteoliticos, onde ocorre a perda asséptica dos implantes produto do *debris* de partículas, de fato há presença de osteoclastos e por conseqüência existe evidencia da resposta do sistema imune neste processo. Uma vez que os macrófagos tenham fagocitado estas partículas, secretam citocinas, como a TNF-α, IL-1β e IL-6 capazes de estimular a mobilização, maduração e estimulação de células pré-osteoclasticas através do sinal RANKL/RANK que acentua a formação osteoclástica em doenças inflamatórias ósseas a qual pode ser amplificada pelo TNF-α quem pode incrementar a mobilização, proliferação e sobrevida dos osteoclastos amplificando o dano ósseo no local afetado (Boyce and Xing 2007), podendo levar a cronificação do processo osteolítico.

OBJETIVOS

A osteointegração de um implante requer a biocompatibilidade do material utilizado, e deve ter a capacidade osteogênica de modo, a prover a sinalização epigenética adequada que favoreça a regeneração óssea. Esta sinalização é mediada em resposta ao reparo ósseo através da secreção de citocinas e fatores de crescimento secretados pelas células inflamatórias e imunes. Os diferentes materiais rotineiramente utilizados nas confecções de implantes dentários como titânio e zircônia devem ter a capacidade de serem reconhecidos diferencialmente pelas células do sistema imune como os macrófagos. Deste modo, o objetivo deste trabalho foi :

1. Analisar a participação dos TLR 2 e 4, das moléculas adaptadoras MyD88 e transdutoras da sinalização intracelular como NF-κB no reconhecimento das partículas de zircônia e titânio.

2. Quantificar e comparar o padrão de secreção das citocinas pró-inflamatórias secretadas sob influência das partículas de zircônias e titânio.

3. Avaliar a indução da osteólise.

RESULTADOS

Os resultados obtidos nesta tese são apresentados na forma de artigo científico:

Artigo 1 : *Reaction to titanium and zirconia particles in cultured macrophages and in a model of prosthetic aseptic loosening.*

Reaction to titanium and zirconia particles in cultured macrophages and in a murine model of prosthetic aseptic loosening

Obando-Pereda, GA[1]; Bianchi, HO[1]; dos Reis, JRR[1]; Abrahão, P[1]; Fischer, L [2] & Stach-Machado, DR[1]

1. Lab of Applied Immunology - Dept of Structural and Functional Biology of Institute of Biology – IB/UNICAMP, Campinas, SP, Brasil.
2. Laboratory of Pain Physiology, Department of Physiology, Federal University of Parana, Curitiba, PR, Brazil

Corresponding author:

Dagmar Ruth Stach-Machado

Departamento Anatomia, Biologia Estrutural e Funcional

Universidade Estadual de Campinas (UNICAMP)

Caixa Postal 6109

13.083-970, Campinas, SP, Brazil

Phone: +55 19 3521 6265

Fax: +55 19 3521 6185

E-mail: dmachado@unicamp.br

Cc: obando@unicamp.br

Original Article

Abstract

The biological reaction to wear debris is critical to the osteolysis underlying aseptic loosening of joint prosthetic implants. In an attempt to reduce aseptic loosening, ceramics have been introduced as a joint surface material. However, information about the biological reaction to ceramic particles and comparison with that induced by widely used materials, such as titanium are still limited. Therefore, this study was designed to evaluate, compare and correlate the expression of TLRs, their intracellular adaptors and proinflammatory cytokines in cultured macrophages challenged with titanium or zirconia particles, as well as particles-induced osteolysis in calvaria and hyperalgesia and edema in hind paw. In cultured macrophages, particles were promptly phagocytized and increased mRNA expression for TLRs 2, 3, 4 and 9, their adaptors MyD88, TRIF and NF-κB and cytokines TNF-α, IL-1β and IL-6. In vivo experiments, particle-induced osteolysis in calvaria and hyperalgesia and edema in hind paw. There is a strong and positive correlation between TLR4, MyD88, TRIF, TNF-α, IL-1β and IL-6 expression, which were significantly higher in titanium than in zirconia challenged macrophages. These findings correlate well with the significantly higher osteolysis and hyperalgesia observed in titanium than in zirconia treated animals.

Key Words: Zirconia; Titanium; Aseptic Loosening; Toll-like Receptors, Cytokines; Osteolysis; Macrophage.

Introduction

Current estimates indicate that there are approximately 1.5 million joint replacement surgeries performed each year (Schwarz et al. 2000). Although the survival rate of these prosthesis is increasing, long-term outcomes are often limited by aseptic loosening induced by local bone resorption surrounding the implants, a process known as osteolysis (Looney et al. 2006). The key stimulus for osteolysis appears to be the phagocytosis of wear particles by macrophages and the resultant production of osteolytic inflammatory cytokines (Ingham and Fisher 2005). In fact, macrophages represent at least 70% of all cells in the interface membranes around aseptic loose periprosthetic tissues (Tamaki et al. 2009). The precise nature of cellular interaction with wear debris and the subsequent production of inflammatory mediators remains largely unknown, however, Toll-like receptors (TLRs) appear to be promising candidates to facilitate this interaction (Takagi et al. 2007; Pajarinen et al. 2009; Pajarinen et al. 2010). TLRs are transmembrane proteins of inflammatory cells, which recognize well-conserved pathogen and endogenous danger-associated molecular patterns (Kawai and Akira 2007) . After ligand binding, TLRs stimulate the recruitment of a set of intracellular adaptors, such as myeloid differentiation primary response protein 88 (MyD88) and TIR domain-containing adapter inducing interferon-ß (TRIF, also known as TICAM1). MyD88 is a universal adapter that activates inflammatory pathways; it is shared by all TLRs with the exception of TLR3. TRIF is recruited by TLR3 and TLR4, and activates an alternative pathway (MyD88-independent pathway), leading to interferon (IFN)-β production. Both pathways culminate in the activation of NF-κB, which is the master regulator of TLR induced responses. Activation of this strong proinflammatory transcription factor leads to production of several proinflammatory cytokines, including

TNF-α, IL-1β, and IL-6, responsible for periprosthetic bone resorption (Masui et al. 2005; Xu et al. 2009). Therefore, although not directly demonstrated, TLRs may mediate cytokines production associated with osteolysis.

Titanium is one of the most used joint surface materials due to its biocompatibility, lower modulus and corrosion resistance. However, these attractive properties are counterbalanced by a number of reports about aseptic prosthetic failure (Lombardi et al. 1989; Maurer et al. 2000; Harris 2001). Although studies about the biological reaction to titanium particles have been accumulated, contradictions still exist. For example, it was suggested that titanium particles stimulate TLRs expression in aseptic loose periprosthetic tissues (Tamaki et al. 2009; Lahdeoja et al. 2010), but decrease it in cultured macrophages (Takagi et al. 2007; Pajarinen et al. 2009). In an attempt to reduce aseptic prosthetic failure, ceramics have been introduced with relative successful results (Kennedy et al. 2008; Innocenti et al. 2010). Among ceramic materials, as zirconia and alumina, has made an improvement in the choice of new biomaterials for the load bearing application in dental and orthopaedic implants, because they showed mechanical resistance to high stress related to weight bearing and low debris in time (Spinelli et al. 2011). However, information about the biological reaction to zirconia particles is still limited and the response of macrophages, which are known as the major mediators of osteolysis, to ceramic particle is poorly understood. It is also not known if titanium or zirconia particles are able to initiate an immune and inflammatory response leading to pain and edema, two common features in patients with aseptic prosthetic loosening. Therefore, in this study we used cultured macrophages, largely used as an in vitro representation of the response to wear debris, to evaluate, compare and correlate the expression of TLRs, their intracellular adaptors and

proinflammatory cytokines induced by titanium and zirconia particles. Particle-induced osteolysis was evaluated in mice calvaria, a validated model of wear debris and particles-induced hind paw edema and painful response was evaluated and compared to those induced by carrageenan, a widely used inflammatory agent.

Material and Methods

Animals

Eight – ten week old male C57BL/6 mice and C57B6/ScCr10, a natural mutant for TLR4, were obtained from the Multidisciplinary Center for Biological Investigation at the University of Campinas (CEMIB- UNICAMP). All animal experimental procedures were approved by the Committee on Animal Research of the University of Campinas (protocol number 1312-1) and followed its guidelines for animal care. The animals were maintained on a temperature-controlled room (± 23ºC) and were housed in plastic cages with soft bedding on a 12:12h light cycle with food and water available *ad libitum*.

Particle preparation

Commercially pure particles of zirconia (ZrO_2) and titanium (Ti6Al4V) (Sigma – Aldrich Corp.St. Louis, MO, USA, codes 230693 and 481041) were used in this study. SEM and optical microscopic images allowed verification that 86% that titanium particles diameters (-325 mesh) were less than 5 um; same observations were seen in others studies (Pioletti et al. 1999; Kwon et al. 2000). Particles were reconstituted to a stock solution with a concentration of 1 x 10^8 particles/mL in PBS using a Neubauer chamber at 100x. The particles were initially immersed in 10 % Triton X100 on a tumbling blender overnight, then sterilized with isopropanol 100% for at least 24 h and by heat at 200ºC for more 24 hours (Xing et al. 2008; Pajarinen et al. 2009). For *in vitro* and *in vivo* experiments, particles were further diluted to cell culture medium to attain a concentration of 1 × 10^7 particles/mL.

Endotoxin test

Zirconia and titanium particles were endotoxin-free as demonstrated by the Sigma E-TOXATE assay for detection and semi-quantification of endotoxins (Sigma-Aldrich Corp. St. Louis, MO, USA). All particles used in this study contained endotoxin levels below those able to activate macrophages (< 0.001 EU/ml) (Warashina et al. 2003).

Isolation of macrophages from peritoneal exudates and cell culture

Macrophages were selective isolated from peritoneal cells following a previous described protocol: mice received an intraperitoneal injection of sodium thioglycollate 3% (1 mL), four days later, they were euthanized by CO_2 asphyxia and were submerged in 70% ethanol to clean the skin. To obtain the peritoneal cells, 5 ml of cold RPMI 1640 supplemented with 5% fetal bovine serum (FBS) and gentamicine (50μg/mL) was intraperitoneally injected. The peritoneal fluid was collected (4mL) and centrifuged for 10 min at 4^oC. Cell pellet was suspended and cell viability was > 95% as determined by Trypan blue exclusion (Wei et al. 1986).

Cells were cultured in a concentration of $1x10^7$ cells/mL in tissue culture flasks (Cellstar, Greiner bio-one, Frickenhausen, Germany) at 37ºC, 5% CO_2 with a relative humidity of 95%. After two hours, the non-adherent cells were removed by vigorous washing with warm serum free medium and adherent cells were used in experiments. Adherent cells (0,5 – 1 x 10^6 cells/mL) were verified by optical microscopy to probe the isolation of macrophages (95% macrophages per well). Macrophages were further incubated during different time periods (0.25, 2 and 24 hours), in fresh medium only (as negative control), with medium containing $1x10^7$ zirconia or titanium particles / mL or with medium containing 1 ng/mL of LPS (as positive control) . After the time periods macrophages supernatant were stocked and frozen at -80ºC until measure protein levels by ELISA and macrophages cells were performing to RNA extraction.

Oligonucleotides

Synthetic oligonucleotides were designed using the software Gene Runner (Gene Runner Version 3.05, Hastings Software Inc., Hastings, NY, USA). The mRNA sequences were obtained from the NCBI public database (http://www.ncbi.nlm.nih.gov/) (**Table 1**).

Total RNA extraction, reverse transcription and real – time RT-PCR

After the incubation period of 0.25, 2 and 24 hours, macrophages were washed three times with sterile PBS following the Trizoll-reagent protocol (Invitrogen, Carlsbad, CA), the total RNA was frozen at -75°C, until further processed. After treatment with DNAse, the obtained RNAs were quantified and their integrity determined on a 1.5% agarose gel. Reverse transcription was performed using 1µg of total RNA per sample, according to the instructions of the Molone Murine Leukaemia Virus Reverse Transcriptase kit (M-MLV RT, Invitrogen, Carlsbad, CA, USA). The cDNA was quantified in an ultraviolet spectrophotometer (Biomate3, Thermo Fisher Scientific, Waltham, MA) by determining the absorbance at 260 nm, and the 260/280 nm absorbance ratio was calculated.

Real-time PCR was carried out in an ABI Prism 7000 Sequence Detection system equipped with a SYBR Green PCR Master Mix-fluorescence quantification system (Applied Biosystems, Warrington, UK). The reaction mixture contained 50 ng of cDNA, 10 pmol of forward and reverse primers and 12.5 µL of SYBR Green, adjusted with Milli-Q water to a final volume of 25 µL. The reaction cycle consisted of 40 cycles of 15 s at 95 ^{o}C for cDNA denaturing, 1 min at 60 ^{o}C for annealing and 1 min at 75 ^{o}C for elongation, followed by 10 min at 75 ^{o}C to finalize the reaction. The relative level of gene expression was calculated according to the instructions of the User's Bulletin (P/N 4303859) from Applied

Biosystems, using β-actin as reference and the cycle threshold method. Briefly, cycle threshold is the point at which the exponential increase in the signal (fluorescence) crosses a somewhat arbitrary signal level (usually 10 times higher than the background). The mean cycle threshold values of triplicate measurements were used to calculate the expression of the target gene, with normalization to an internal control (β-actin), using the delta delta Ct formula, according to the User's Bulletin (Applied Biosystems). Negative controls without RNA and without reverse transcriptase were also included. The experiments were run in triplicate and repeated three times.

ELISA

The release of IL-1β, IL-6 and TNF-α from macrophage culture medium at 24 hours was measured by the enzyme-linked immunoassay (ELISA), using commercially available kits specific for mice IL-1β, IL-6 and TNF-α (eBioscience, San Diego, CA, USA; cytokines codes: 88-7324, 88-7013, 88-7064, 88-7314, respectively). The assays were performed according to the manufacturer's instructions. The absorbance was read on Microplate Reader (Asys Expert Plus, Austria), and concentrations were calculated according to the standard curve. The lower limit for detection in each assay was 7 pg/ml for IL-1β and 3 pg/ml for IL-6 and TNF-α.

Murine Calvaria Model

We evaluated the osteolytic response induced by titanium and zirconia particles in murine calvaria, a validated model of prosthetic aseptic loosening (Warashina et al. 2003). Under general anesthesia (ketamin (70-80 mg/kg) and xylazine (5-7 mg/kg), the scalp was incised longitudinally to expose the external cranial periosteum. The periosteum was removed until coronal, sagittal and lamboid sutures of the calvaria were visible. One hundred microliters

of each particle suspended solution (1 x 10^7 particles/mL) was applied directly on the surface of the calvaria. For the control group, PBS without particles was used. The skin was then closed tightly with skin clips and sealed with Dermabond® (ETHICON, Somerville, NJ, USA) to prevent leakage of the suspended solution. At 1 week postoperatively, the animals were euthanized by CO_2 asphyxia and the calvaria were removed.

Histological analysis

The specimens were fixed in 10% phosphate-buffered formalin, decalcified in 14% EDTA for seven days, dehydrated in graded alcohols, and embedded in paraffin. Each calvarial tissue specimen was sectioned with the thickness of 7-8 μm on the sagittal plane. Three sections approximately 2–3 mm lateral to the midsagittal suture were stained with hematoxylin-eosin and the whole view of each section was scanned (x40) using a Leica microscope DM2000 coupled with Leica DFC200 digital camera. Images were analyzed using the ImageJ 1.42q free software (http://rsb.info.nih.gov/ij), the percentage of osteolytic area was indicated by the ratio of inflammatory granulation tissue in the calvaria and percentage of bone area by the ratio of osseous tissue that includes trabecular bone region (Warashina et al. 2003).

Mechanical Hyperalgesia and Edema

A common report among patients with aseptic loosening of joint prostheses is the local pain during normal daily activities (Willert et al. 2005), a condition that may be defined as hyperalgesia. Carrageenan, a mucopolysaccharide from the cell walls of the red algae, is one of the most used inflammatory agents, its administration in the hind paw induces edema and hyperalgesia mediated by cytokines production and the release of prostanoids and sympathomimetic amines (Cunha et al. 2005). In a clinical perspective, its

experimental use satisfies the criteria to simulate inflammatory pain in humans. We evaluated the ability of titanium and zirconia particles in inducing paw hyperalgesia and edema and compared to those induced by carrageenan or vehicle. Hyperalgesia and edema were evaluated two hours after treatments, when particles- induced mRNA expression in cultured macrophages was, in general, highest in C57B6 mice.

A standardized dose of carrageenan (100µg, (Cunha et al. 2005)), particles (1 x 10^7 particles/mL) or vehicle (RPMI 1640) was locally administrated in the subcutaneous dorsal tissue of the hind paw. The animals were briefly restrained and the volume of injection was 50 µl. Testing sessions were carried out during light phase (between 09:00 A.M. and 5:00 P.M.) in a quiet room at 23 °C. The Randall–Selitto nociceptive paw-withdrawal flexion reflex test (Randall and Selitto 1957) was performed using an Ugo-Basile analgesymeter (Stoelting, Chicago, IL, USA), which applies a linearly increasing mechanical force to the dorsum of the hind paw (Teixeira et al. 2010). The nociceptive threshold was defined as the force in grams at which the animal withdrew its paw. The baseline paw-withdrawal threshold was defined as the mean of three tests performed at 5-min intervals before test agents were injected. Mechanical hyperalgesia was quantified as the change in the mechanical nociceptive threshold calculated by subtracting the mean of three mechanical nociceptive threshold measurements taken 2 h after injection from the mean of the three baseline measurements (Teixeira et al. 2010).

Paw edema was assessed by measuring the paw thickness in the dorsal-plantar axis with a caliper. The paw edema index was calculated as the mean difference of paw thickness (Δ ipsi-/contralateral paw thickness) (Teixeira et al. 2010).

Statistical analysis

A two-way repeated-measures ANOVA with one between-subjects factor (i.e. treatment) and one within-subjects factor (i.e. time) was used to determine if there were significant ($p \leq 0.05$) differences among the groups in figures 1, 2, and 3. To determine if there were significant ($p \leq 0.05$) differences among treatment groups in figures 4, 5 (D and E) and 6 one-way ANOVA was performed. If there was a significant between-subjects main effect of treatment group after two or one-way ANOVA, post hoc contrasts, using the Tukey test, were performed to determine the basis of the significant difference. The relationship between the expression of *TLR4, MyD88, NF-κB, TNF-α, IL-1β* and *IL-6* within the same samples was examined using the Spearman correlation coefficient analysis. Probabilities of $p < 0.05$ were considered significant.

R**esults**

TLR mRNA expression in macrophages challenged with titanium and zirconia particles

TLR2 mRNA expression (Figure 1A): zirconia and titanium challenge significantly increased TLR2 mRNA expression at two and twenty four hours (as indicated by the symbol +). Compared with titanium, zirconia induced a significantly higher increase in TLR2 mRNA expression at two and twenty four hours (as indicated by the symbol *). There is a statistically significant interaction between material and time (p = 0,003; f = 14,509, Two Way Repeated Measure Anova). Exposition of macrophages to LPS significantly increase TLR2 mRNA expression at fifteen minutes, two and twenty four hours (7,021 $\pm$ 2,09; 7,113 $\pm$ 2,07; 19,243 $\pm$ 2,93 fold change means $\pm$ EPM for each time point respectively, data not shown).

TLR3 mRNA expression (Figure 1B): zirconia and titanium challenge significantly increased TLR3 mRNA expression at two and twenty four hours (as indicated by the symbol +). Compared with titanium, zirconia induced a TLR3 mRNA expression significantly lower at two hours and higher at twenty four hours (as indicated by the symbol *). There is a statistically significant interaction between material and time ($p < 0{,}001$; $f = 59{,}547$, Two Way Repeated Measure Anova). Exposition of macrophages to LPS significantly increase TLR3 mRNA expression at fifteen minutes, two and twenty four hours (5,469 $\pm$ 0,28; 7,773 $\pm$ 0,76; 5,419 $\pm$ 1,98, fold change means $\pm$ EPM for each time point respectively, data not shown**).**

TLR4 mRNA expression (Figure 1C): zirconia and titanium challenge significantly increased TLR4 mRNA expression at two and twenty four hours (as indicated by the symbol +). Compared with titanium, zirconia induced a significantly lower increase in TLR4 mRNA expression at two and twenty four hours (as indicated by the symbol *). There is a statistically significant interaction between material and time ($p < 0{,}001$; $f = 42{,}300$, Two Way Repeated Measure Anova). Exposition of macrophages to LPS significantly increase TLR4 mRNA expression at fifteen minutes, two and twenty four hours (160,229 $\pm$ 6,99; 1260,338 $\pm$ 3,80; 408,679 $\pm$ 3,05, fold change means $\pm$ EPM for each time point respectively, data not shown).

TLR9 mRNA expression (Figure 1D): Zirconia significantly increased TLR9 mRNA expression at fifteen minutes and two hours, whereas titanium increased it at fifteen minutes, two and twenty four the hours (as indicated by symbol +). Compared with titanium, zirconia induced a TLR9 mRNA expression significantly higher at fifteen minutes and lower at two and twenty four hours (as indicated by the symbol *). There is a

statistically significant interaction between material and time (p <0,001; f = 96,157, Two Way Repeated Measure Anova). Exposition of macrophages to LPS did not significantly increase TLR9 mRNA expression at fifteen minutes, but did at two and twenty four hours (6,607 ± 0,47; 66,943 ± 3,44; 51,349 ± 2,49, fold change means ± EPM for each time point respectively, data not shown).

Expression of mRNA for intracellular adaptors involved in TLR signaling in macrophages challenged with titanium and zirconia particles

MyD88 mRNA expression (Figure 2A): zirconia and titanium challenge significantly increased MyD88 mRNA expression at two and twenty four hours, titanium also increased it at fifteen minutes (as indicated by the symbol +). Compared with titanium, zirconia induced a MyD88 mRNA expression significantly lower at two, but higher at twenty four hours (as indicated by the symbol *). There is a statistically significant interaction between material and time (p <0,001; f = 150,234, Two Way Repeated Measure Anova). Exposition of macrophages to LPS did not significantly increase MyD88 mRNA expression at fifteen minutes, but did at two and twenty four hours (38,491 ± 2.11; 1486,714 ± 12,27; 191,335 ± 4,15, fold change means ± EPM for each time point respectively, data not shown).

NF-κB mRNA expression (Figure 2B): zirconia and titanium challenge significantly increased NF-κB mRNA expression at fifteen minutes, two and twenty four hours (as indicated by the symbol +). Compared with titanium, zirconia induced a NF-κβ mRNA expression significantly lower at fifteen minutes and twenty four hour, but higher at two hours (as indicated by the symbol *). There is a statistically significant interaction between material and time (p <0,001; f = 65,133, Two Way Repeated Measure Anova). Exposition

of macrophages to LPS significantly increase NF-κB mRNA expression at fifteen minutes, two and twenty four hours (19,706 ± 1,17; 89,068 ± 4,99; 98,004 ± 6,55, fold change means ± EPM for each time point respectively, data not shown).

Inflammatory cytokines mRNA expression in macrophages challenged with titanium and zirconia particles

TNF-α mRNA expression (Figure 3A): zirconia and titanium challenge significantly increased TNF-α mRNA expression at fifteen minutes, two and twenty four hours (as indicated by the symbol +). Compared with titanium, zirconia induced a significantly lower increase in TNF-α mRNA expression at two and twenty four hours (as indicated by the symbol *). The expression of TNF-α mRNA at fifteen minutes after particle exposure, is compatible with the well known role of this cytokine, reported to be an early expressing molecule secreted in the early stage of the osteolytic process. There is a statistically significant interaction between material and time ($p<0,001$; $f = 66,444$, Two Way Repeated Measure Anova). Exposition of macrophages to LPS did not significantly increase TNF-α mRNA expression at fifteen minutes, but did at two and twenty four hours (24,183 ± 0,88; 873,164 ± 38,63; 148,88 ± 15,01, fold change means ± EPM for each time point respectively, data not shown).

IL-1β mRNA expression (Figure 3B): zirconia and titanium challenge significantly increased IL-1β mRNA expression at two and twenty four hours (as indicated by the symbol +). Compared with titanium, zirconia induced a significantly lower increase in IL-1β mRNA expression at two and twenty four hours (as indicated by the symbol *). There is a statistically significant interaction between material and time ($p<0,001$; $f = 113,516$, Two

Way Repeated Measure Anova). Exposition of macrophages to LPS significantly increase IL-1β mRNA expression at fifteen minutes, two and twenty four hours (10,318 ± 2,31; 50,618 ± 1,41; 26,712 ± 0,48, fold change means ± EPM for each time point respectively, data not shown).

IL-6 mRNA expression (Figure 3C): zirconia and titanium challenge significantly increased IL-6 mRNA expression at two and twenty four hours (as indicated by the symbol +). Compared with titanium, zirconia induced a significantly lower increase in IL-6 mRNA expression at two and twenty four hours (as indicated by the symbol *). There is a statistically significant interaction between material and time ($p < 0,001$; $f = 52,141$, Two Way Repeated Measure Anova). Exposition of macrophages to LPS significantly increase IL-6 mRNA expression at fifteen minutes, two and twenty four hours (822,602 ± 35,65; 2859,936 ± 136,71; 1267,784 ± 168,32, fold change means ± EPM for each time point respectively, data not shown).

Inflammatory cytokines expression in macrophages challenged with titanium and zirconia particles

TNF-α protein expression (Figure 4A): Zirconia, but not titanium particle challenge significantly increased TNF-α expression (as indicated by the symbol +). However, the increase in TNF-α expression induced by zirconia was not significantly higher than that induced by titanium. Exposition of macrophages to LPS significantly increases TNF-α expression (1839,967 ± 29,267, data not shown).

IL-1β protein expression (Figure 4B): Titanium, but not zirconia particle challenge significantly increased IL-1β expression, which was significantly lower in macrophages

challenged with zirconia than in those with titanium particles (as indicated by the symbol *). Exposition of macrophages to LPS significantly increases IL-1β expression (158,200 ± 12,500, data not shown).

IL-6 protein expression (Figure 4C): Titanium, but not zirconia particle challenge significantly increased IL-6 expression, which was significantly lower in macrophages challenged with zirconia than in those with titanium particles (as indicated by the symbol *). Exposition of macrophages to LPS significantly increase IL-6 expression (2693,217 ± 147,767, data not shown).

Data correlation

Table 2 shown that there are strong and positive correlations between TLR4, NF-kB, IL-1β and IL-6 mRNA expression (as indicated by the symbol *). Protein levels of IL-1β and IL-6 also correlates well with TLR4, NF-kB, IL-1β and IL-6 mRNA expression (as indicated by the symbol *). Table 3 shown that there a positive correlation between TLR2, TLR3 and TLR9 with IL-6 mRNA expression (as indicated by the symbol *). TNF-α protein level correlates well with TLR2 mRNA expression (as indicated by the symbol *).

Histological analysis

A coronal section of mice calvaria showed a little inflammatory reaction and a well conserved trabecular bone in animals that received PBS (Figure 5 A, sham group). In contrast, in those that received zirconia particles inflammation with small osteolysis is visible (Figure 5 B), large inflammatory reaction and osteolysis is visible titanium treated group (Figure 5 C).Tissue reactions were quantitatively evaluated and express as percentage of osteolytic and bone area.

Osteolysis (Figure 5D): Both particles induced significantly osteolysis (as indicated by the symbol +); however that induced by zirconia was significantly lower than that induced by titanium (as indicated by the symbol *).

Bone area (Figure 5 E): Both particles significantly reduced bone area (as indicated by the symbol +); however it was significantly less reduced in animals exposed to zirconia than in those exposed to titanium particles (as indicated by the symbol *).

Particle-induced hyperalgesia and edema

Hyperalgesia (Figure 6A): Carrageenan and both particles induced hyperalgesia (as indicated by the symbol *). Titanium particles induced a hyperalgesic response similar to that of carrageenan and significantly higher than that induced by zirconia particles (as indicated by the symbol +).

Edema (Figure 6B): Carrageenan and both particles induced edema (as indicated by the symbol *) and it was similar in titanium and zirconia treated groups.

Discussion

This study demonstrated that cultured macrophages challenged with zirconia or titanium particles phagocyte them and express increased mRNA levels for TLRs, their intracellular adaptors and inflammatory cytokines. In vivo, zirconia and titanium particles induced significant osteolysis in calvaria and hyperalgesia and edema in hind paw. These data show that the simple exposition to zirconia, as well as to titanium particles, in the absence of any other inflammatory or stimulatory factor, initiates an immune response mediated by cytokines production and associated with osteolysis and inflammatory hyperalgesia and

edema. However, important quantitative differences are evident, since, in general, zirconia-induced cytokines expression, osteolysis and hyperalgesia were lower than those induced by titanium.

Toll- like receptors and their adaptors

Macrophages challenged with titanium or zirconia particles presented an increase in TLR 2, 3, 4 and 9 mRNA expression. However, in general lines, this increased TLRs mRNA expression may not be directly associated with increased cytokines production and osteolysis, since titanium-induced a consistent higher cytokines expression and osteolysis than zirconia particles, while induced a lower mRNA expression for TLR 2, 3 and 9 in at least one time point analyzed. In accordance with our data, a recent clinical study have found increased TLR 2, 4 and 9 mRNA expression in aseptic loose periprosthetic tissues (Tamaki et al. 2009). However, the same authors have previously found decreased TLRs mRNA expression in cultured macrophages challenged with titanium (Takagi et al. 2007; Pajarinen et al. 2009). The reasons for the discrepancies in literature remain to be elucidated. However, it is important to note that in addition to an upregulation in mRNA expression for TLRs, we have also demonstrated an upregulation for their intracellular adaptors, MyD88, TRIF and NF-Kβ.

Interestingly, mRNA expression for TLR4 and it adaptor MyD88 was consistently higher in titanium than in zirconia challenged macrophages. Although this increased expression does not allow to conclude that TLR4 signaling mediate cytokines production associated to aseptic loosening, we can suggest it based on some facts. First, the higher mRNA expression for TLR4 and it adaptor MyD88 correlates well with the higher osteolytic

cytokines expression, osteolysis and hyperalgesia induced by titanium than by zirconia particles. Second, a recent study suggested an essential role of TLR4 in aseptic loosening of joint prosthetic implants. It was shown that after particle exposure, macrophages presented increased TLR4 mRNA expression, associated with increased production of osteolytic cytokines, which was consistently blocked by the blockade of TLR4 expression by antisense oligonucleotide (Hao et al. 2010). These findings support our suggestion that TLR4 mediates cytokines production associated to aseptic loosening. Finally, it is known that the transcription of the genes encoding proteins involved in an active signaling pathway is upregulated. In accordance with this idea, we have shown that the mRNA most increased by LPS (used as positive control) was that of TLR4, which is essential to LPS recognition (Poltorak et al. 1998; Hoshino et al. 1999). Therefore, the upregulation of mRNA for TLR4 and its adaptors is compatible with their involvement in cytokines production and osteolysis. At this point, it is important to consider that the particles used in this study was processed to remove any contaminant and then tested negative for LPS. Therefore, in the absence of molecular patters that could be recognized by TLRs in the particles, it is reasonable to suggest that these receptors are activated in response to cellular mechanisms initiated by the phagocytic process. In fact, it was suggested that the production of osteolytic cytokines mediated by TLR4 is dependent of an endogenous heat shock protein generated in response to particles phagocytosis (Hao et al. 2010).

Cytokines

Titanium and zirconia particles significantly increased the expression of TNF-α, IL-1β and IL-6 in cultured macrophages. At the mRNA level, the expression of the three cytokines were significantly higher in titanium than in zirconia challenged macrophages, while at the

protein level, only IL-1β and IL-6 were significantly higher in titanium than in zirconia challenged macrophages. Compared with not challenged macrophages, only zirconia increased TNF-α expression, while only titanium increased IL-1β and IL-6 expression. These findings indicate that the osteolytic pathway initiated by each particle may be distinct, since TNF-α is known to acts directly on the osteoclast precursors, while IL-1β and IL-6 both primarily stimulate bone resorption indirectly by increasing RANKL (receptor activator of NF-κB ligand) production (Merkel et al. 1999; Kobayashi et al. 2000; Ragab et al. 2002). Although further studies are needed to clarify this issue, previous studies suggested that the biological reaction induced by titanium is mainly mediated by IL-1β and IL-6 (Warashina et al. 2003), while that induced by zirconia is mainly mediated by TNF-α (Hatton et al. 2003). The reasons for the discrepancies between the expression of cytokines at the mRNA and protein level are unknown, however it may be due to post-transcriptional processing, since it is a well known mechanism to prevent excessive and harmful inflammatory responses. In accordance with our data, it was shown that the expression of TNF-α, IL-1β and IL-6 is increased at the interface membrane surrounding loosened prostheses (Jiranek et al. 1993; Kim et al. 1994; Goodman et al. 1998) and in particle-challenged macrophages (Catelas et al. 1999; Merkel et al. 1999; Azuma et al. 2000).

Osteolysis

The osteolytic lesions induced by titanium in mice calvaria were significantly greater than that induced by zirconia particles, suggesting that the biological reaction and bone deterioration induced by ceramics are less extensive than those induced by titanium. Although clinical reports have shown osteolysis around ceramic joint prosthesis (Mahoney and Dimon 1990; Wirganowicz and Thomas 1997), experimental studies comparing the

effects of ceramic and metal debris have suggest a better biocompatibility of ceramics as joint surface materials (Catelas et al. 1999; Warashina et al. 2003) . In addition, the wear rate of ceramic prosthesis is described as being much lower than that of metal on polyethylene (Dorlot et al. 1989). These observations and the current results suggest that ceramics is a promising joint surface material.

Hyperalgesia and edema

A common report among patients with aseptic loosening of joint prostheses is the local pain, which initially presents during normal daily activities and then becomes continuous and severe (Willert et al. 2005). A painful sensation elicited by a low-intensity stimulus may be defined as hyperalgesia and is mediated by sensitization of primary nociceptors by inflammatory mediators delivered during inflammatory response. In this study, we demonstrated for the first time, to our knowledge, that titanium and zirconia particles directly injected in hind paw induced hyperalgesia and edema. This finding suggests that particles per se are able to initiate a strong inflammatory response that leads to nociceptor sensitization and increased vascular permeability. The previous findings from this study support the idea that particles are phagocytozed by macrophages and induce cellular responses that culminate with osteolytic cytokine production. Although our data does not permit exclude other mechanisms, this same pathway may be involved in particle-induced hyperalgesia and edema, since it is known that TNF-α, IL-1β and IL-6 induce hyperalgesia (Cunha et al. 2005) and edema (Cerami 1992). In this sense, it would be expected that particle-induced cytokine expression would lead to some degree of hyperalgesia and edema. However, the intensity of particle-induced hyperalgesia and edema was surprisingly observed. Titanium-induced hyperalgesia and titanium and zirconia-induced edema were

similar to those induced by a high dose of carrageenan (100µg), which is one of the most widely used models in animal inflammation research. Titanium induced greater hyperalgesia and a non-significantly greater edema than zirconia particles. These findings correlate well with the higher cytokines expression as well as with the higher osteolysis induced by titanium than by zirconia particles. Further studies are necessary to evaluate the long term effects of particles on hyperalgesia and edema.

Correlations

There are strong and positive correlations between the expression of TLR4, it intracellular adaptor NF-kB and osteolytic cytokines. These findings from cultured macrophages correlate well with those of osteolysis and hyperalgesia from in vivo experiments. Compared to titanium, zirconia induced significantly lower expression of TLR4, it adaptor NF-kB and osteolytic cytokines, as well as significantly lower osteolysis and hyperalgesia. Based on these findings and in their important correlations, we suggest that the biocompatibility of zirconia is greater than that of titanium and that aseptic loosening of prosthetic implants is at least partially mediated by TLR4, which activate NF-kB pathway to induce osteolytic cytokines production.

Conclusion

In summary, this study demonstrated that cultured macrophages challenged with zirconia or titanium particles, express higher levels of TLRs, their intracellular adaptors and inflammatory cytokines. In vivo, particles induced osteolysis in calvaria and hyperalgesia and edema in hind paw. Compared with titanium, zirconia induced lower expression of TLR4, it intracellular adaptor NF-kB and osteolytic cytokines in cultured macrophages and

induced lower osteolysis in calvaria and hyperalgesia in hind paw. These findings suggest that the biocompatibility of zirconia is greater than that of titanium. Although further studies are needed to understand the mechanisms underlying aseptic loosening of prosthetic implants, the correlations among our data suggest that TLR4 mediates, at least partially, aseptic loosening by activating NF-kB pathway to induce osteolytic cytokines production.

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Figure legends

Figure 01 Expression of TLRs mRNA in macrophages wild type (red) and mutant for TLR4 (black) challenged with titanium (▼) and with zirconia (○) particles. Real time RT PCR, with normalization to β-actin using the ΔCt method. **(A)** TLR2; (**B**) TLR3; (**C**) TLR4; (**D**) TLR9. The symbol + indicates a statistically significant increase in TLRs mRNA expression in comparison to not challenged macrophages. The symbol * indicates a statistically significant difference in comparison to macrophages challenged with titanium. There is a statistically significant interaction between time and material in all cases (Two Way Repeated Measure Anova).

Figure 02 Expression of mRNA for intracellular adaptors molecules involved in TLR signaling in macrophages wild type (red) and mutant for TLR4 (black) challenged with titanium (▼) and zirconia (○) particles. Real time RT-PCR, with normalization to β-actin using the Ct method. **(A)** MyD88; (**B**) NF-KB. The symbol + indicates a statistically significant increase in expression of mRNA for intracellular adaptors in comparison to not challenged macrophages. The symbol * indicates a statistically significant difference in comparison to macrophages challenged with titanium. There is a statistically significant interaction between time and material in all cases (Two Way Repeated Measure Anova).

Figure 03 Expression of mRNA for inflammatory cytokines in macrophages wild type (red) and mutant for TLR4 (black) challenged with titanium (▼) and zirconia (○) particles. Real time RT-PCR, with normalization to β-actin using the Ct method. **(A)** TNF-α; (**B**) IL-1β; (**C**) IL-6. The symbol + indicates a statistically significant increase in cytokines mRNA expression in comparison to not challenged

macrophages. The symbol * indicates a statistically significant difference in comparison to macrophages challenged with titanium. There is a statistically significant interaction between time and material in all cases (Two Way Repeated Measure Anova).

Figure 04 TNF-α, IL-1β and IL-6 protein expression in macrophages wild type (red) and mutant for TLR4 (black) challenged with titanium (▼) and zirconia (○) particles, determined by ELISA. **(A)** TNF-α; (**B**) IL-1β; (**C**) IL-6. The symbol + indicates a TNF-α expression significantly higher than that of not challenged macrophages. The symbol * indicates an IL-1β and IL-6 expression significantly higher than of the other groups. One way Anova and Tukey test, $p<0.05$.

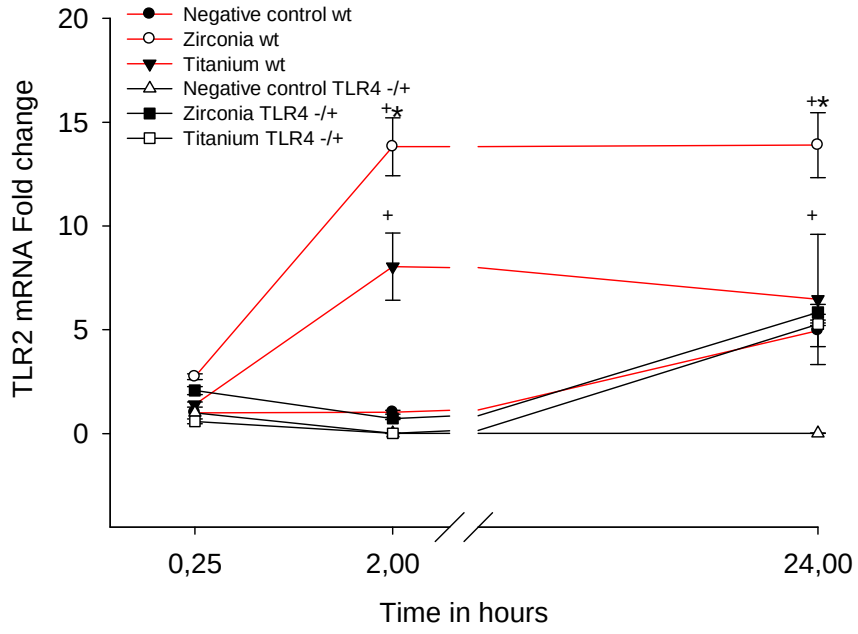
Figure 05 Histological appearance of murine calvaria (stain: hematoxylin and eosin; magnification: ×200). (**A**) In sections obtained from PBS treated animals (sham group) no inflammatory and osteolytic changes are visible. (**B**) In sections obtained from zirconia treated animals inflammation with small osteolysis is visible. (**C**) In sections obtained from titanium treated animals a marked inflammatory reaction and large osteolysis is visible. The quantitative evaluation of tissue reactions was expressed as percentage of osteolytic (**D**) and bone area (**E**). The symbol + indicates a tissue reaction significantly different to that of sham group and the symbol * indicates a tissue reaction significantly different to that of titanium treated group. One way Anova and Tukey test, $p<0.05$.

Figure 06 Particles-induced hind paw hyperalgesia and edema. (**A**) hyperalgesia and (**B**) edema. The symbol + indicates a response significantly greater than that induced by vehicle and the symbol * indicates a response significantly lower than that of titanium and carrageenan treated group. One way Anova and Tukey test, $p<0.05$.

Figures

Figure 1

A



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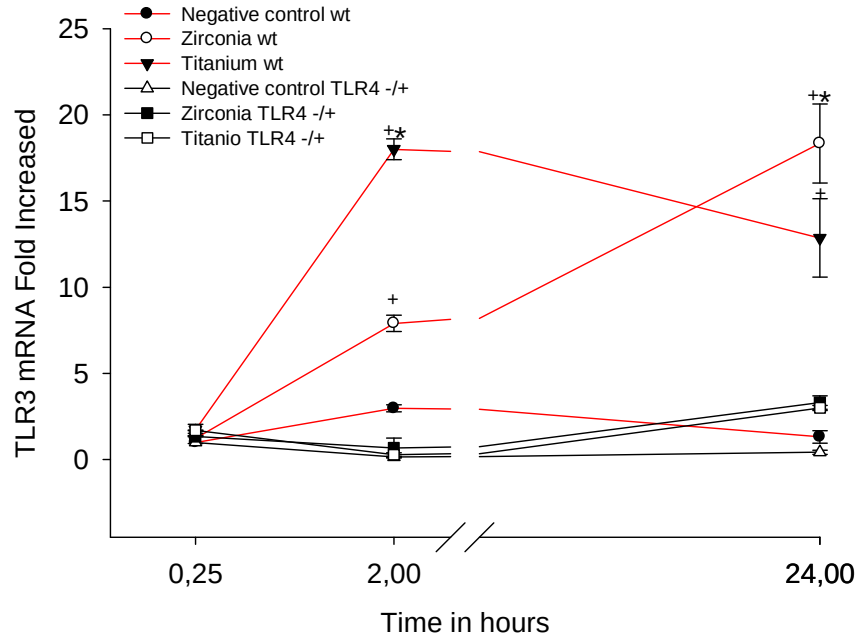

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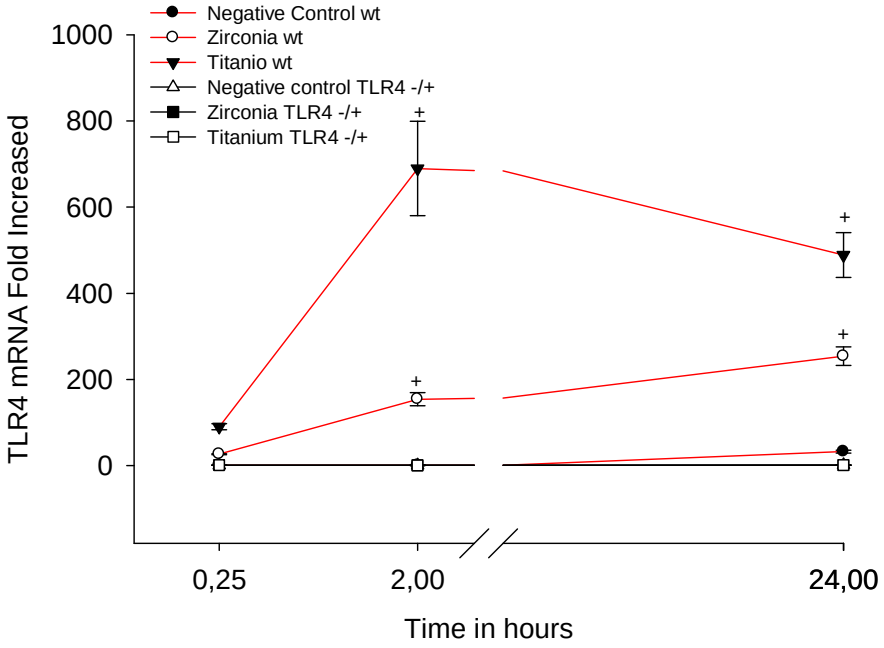
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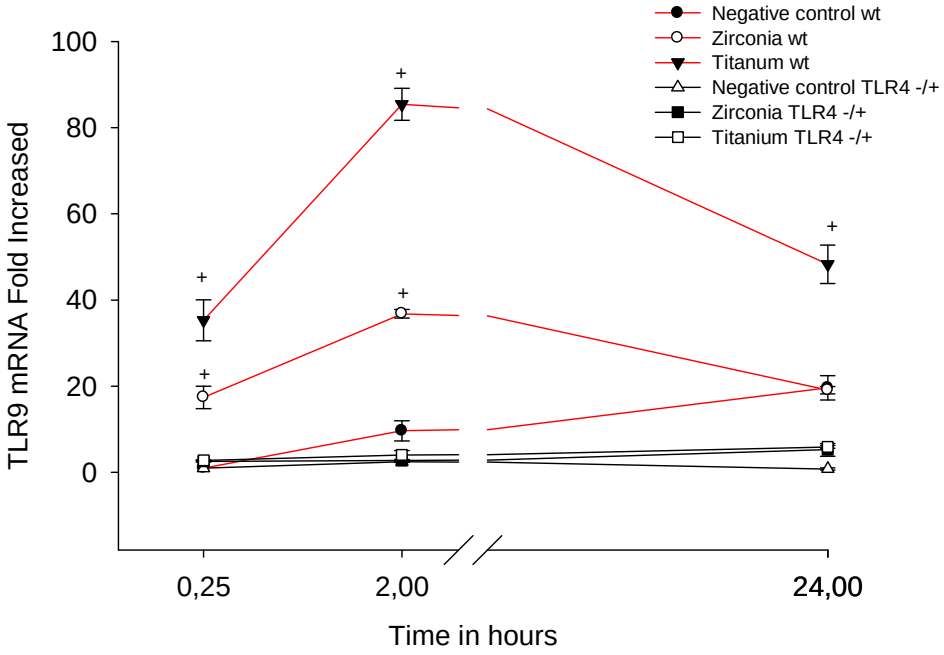
Zirconia wt
Titanio wt
Negative control TLR4 -/+
Zirconia TLR4 -/+
Titanium TLR4 -/+
TLR4 mRNA Fold Increased
1000
800
600
400
200
0
0,25
2,00
24,00
Time in hours

D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Zirconia wt
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Zirconia TLR4 -/+
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80
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40
20
0
0,25
2,00
24,00
Time in hours

Figure 2

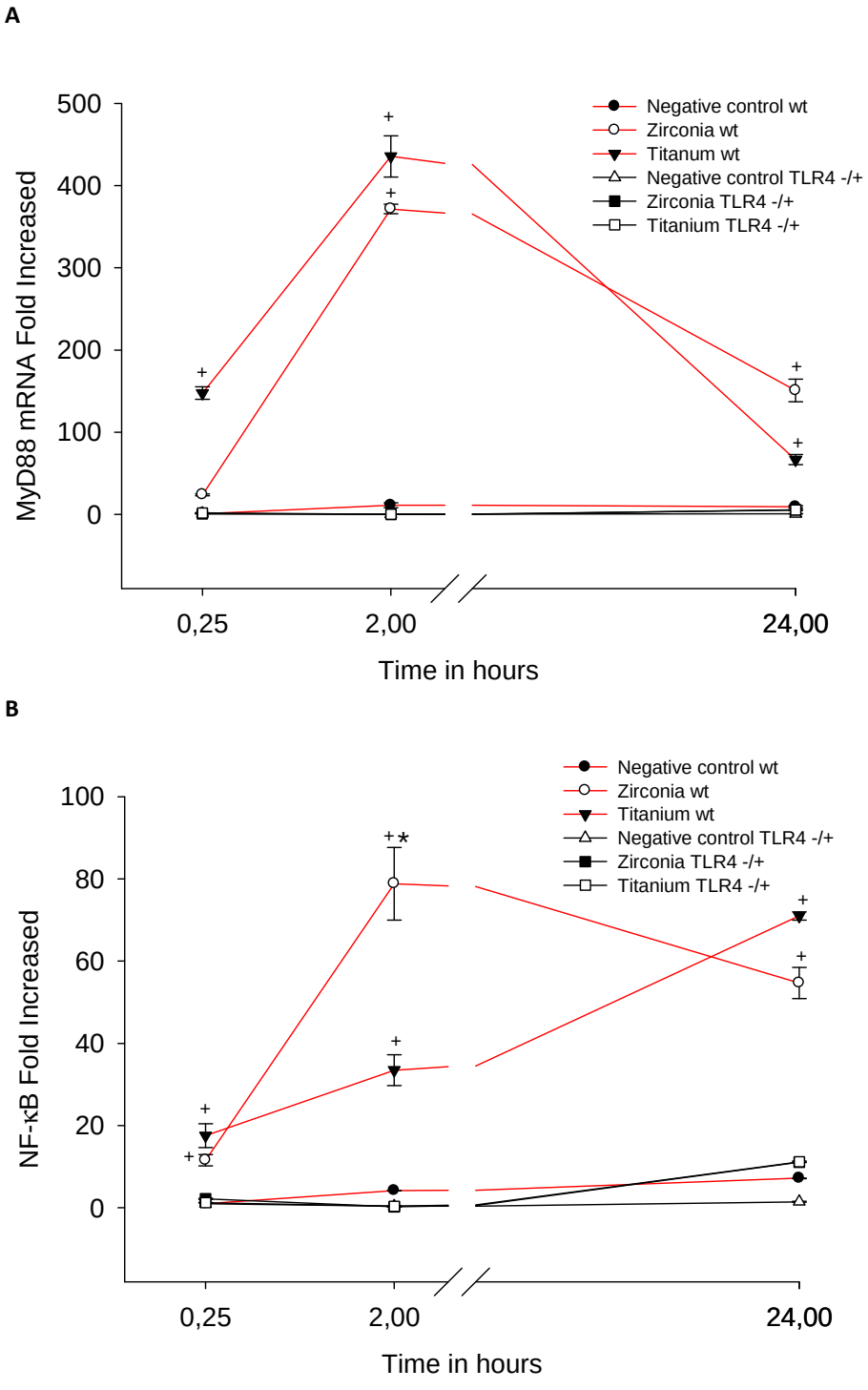
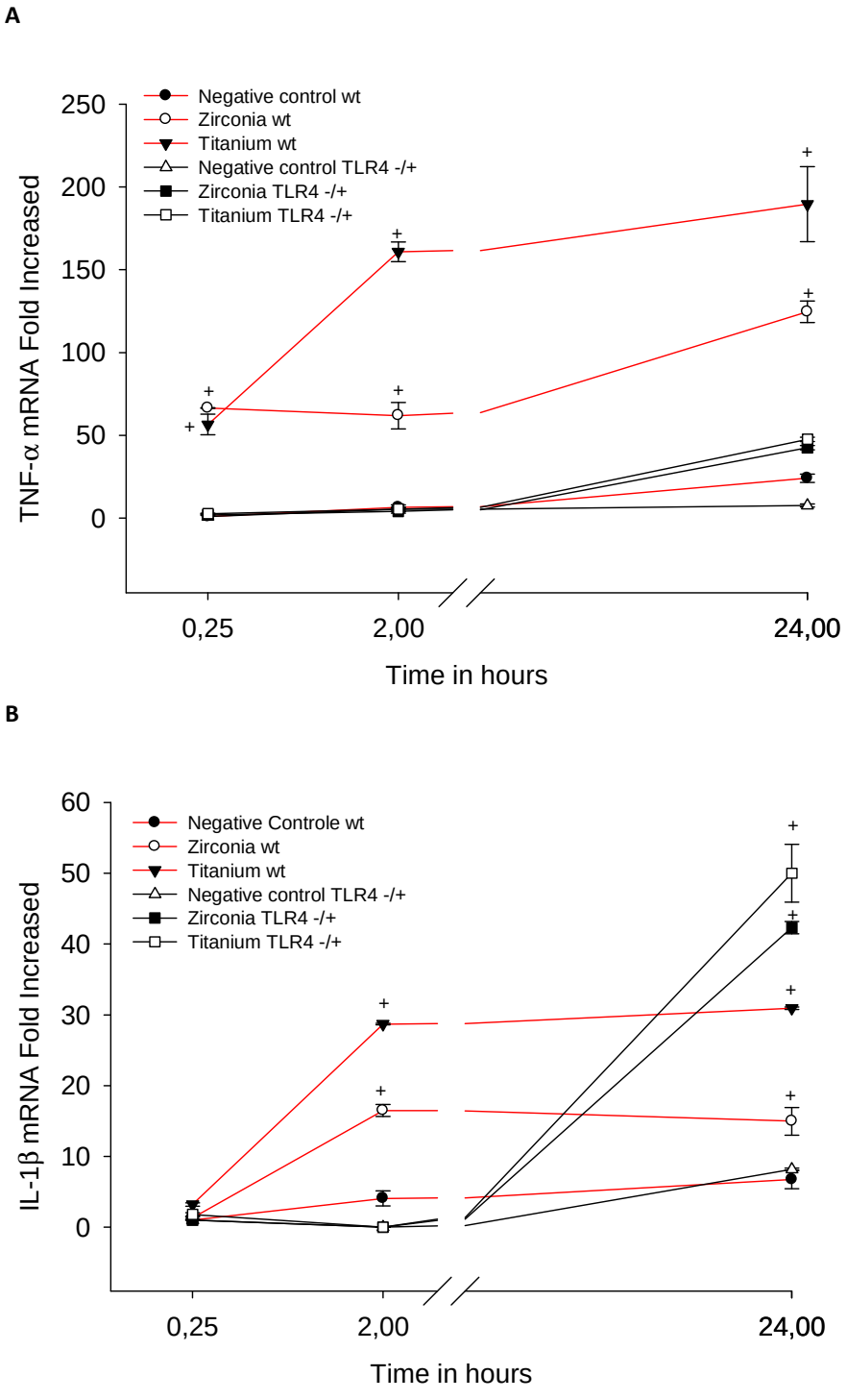
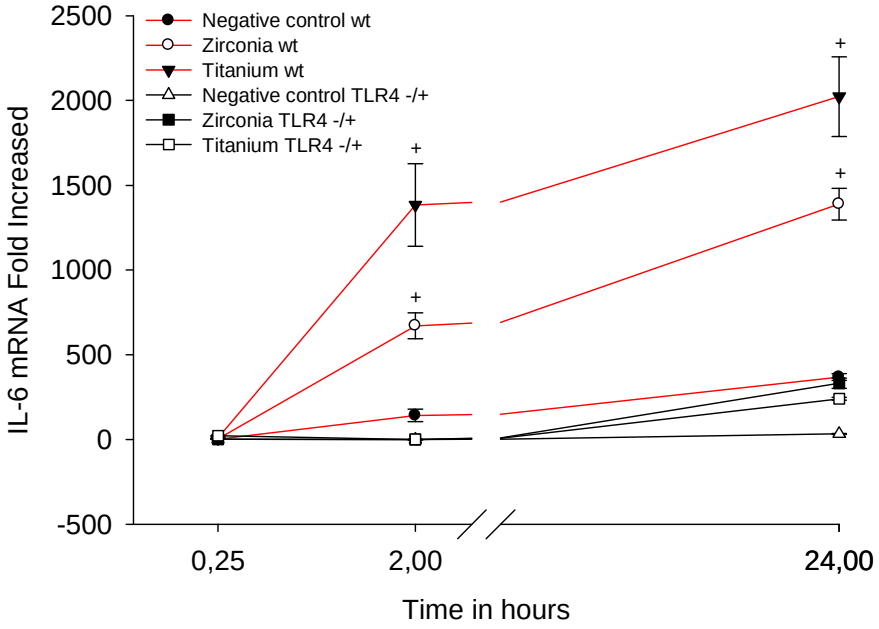

A
MyD88 mRNA Fold Increased
500
400
300
200
100
0
Negative control wt
Zirconia wt
Titanum wt
Negative control TLR4 -/+
Zirconia TLR4 -/+
Titanium TLR4 -/+
0,25
2,00
24,00
Time in hours
B
NF-κB Fold Increased
100
80
60
40
20
0
Negative control wt
Zirconia wt
Titanium wt
Negative control TLR4 -/+
Zirconia TLR4 -/+
Titanium TLR4 -/+
0,25
2,00
24,00
Time in hours

Figure 3.


A
Negative control wt
Zirconia wt
Titanium wt
Negative control TLR4 -/+
Zirconia TLR4 -/+
Titanium TLR4 -/+
TNF-α mRNA Fold Increased
250
200
150
100
50
0
0,25
2,00
24,00
Time in hours
B
Negative Controle wt
Zirconia wt
Titanium wt
Negative control TLR4 -/+
Zirconia TLR4 -/+
Titanium TLR4 -/+
IL-1β mRNA Fold Increased
60
50
40
30
20
10
0
0,25
2,00
24,00
Time in hours

c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Zirconia wt
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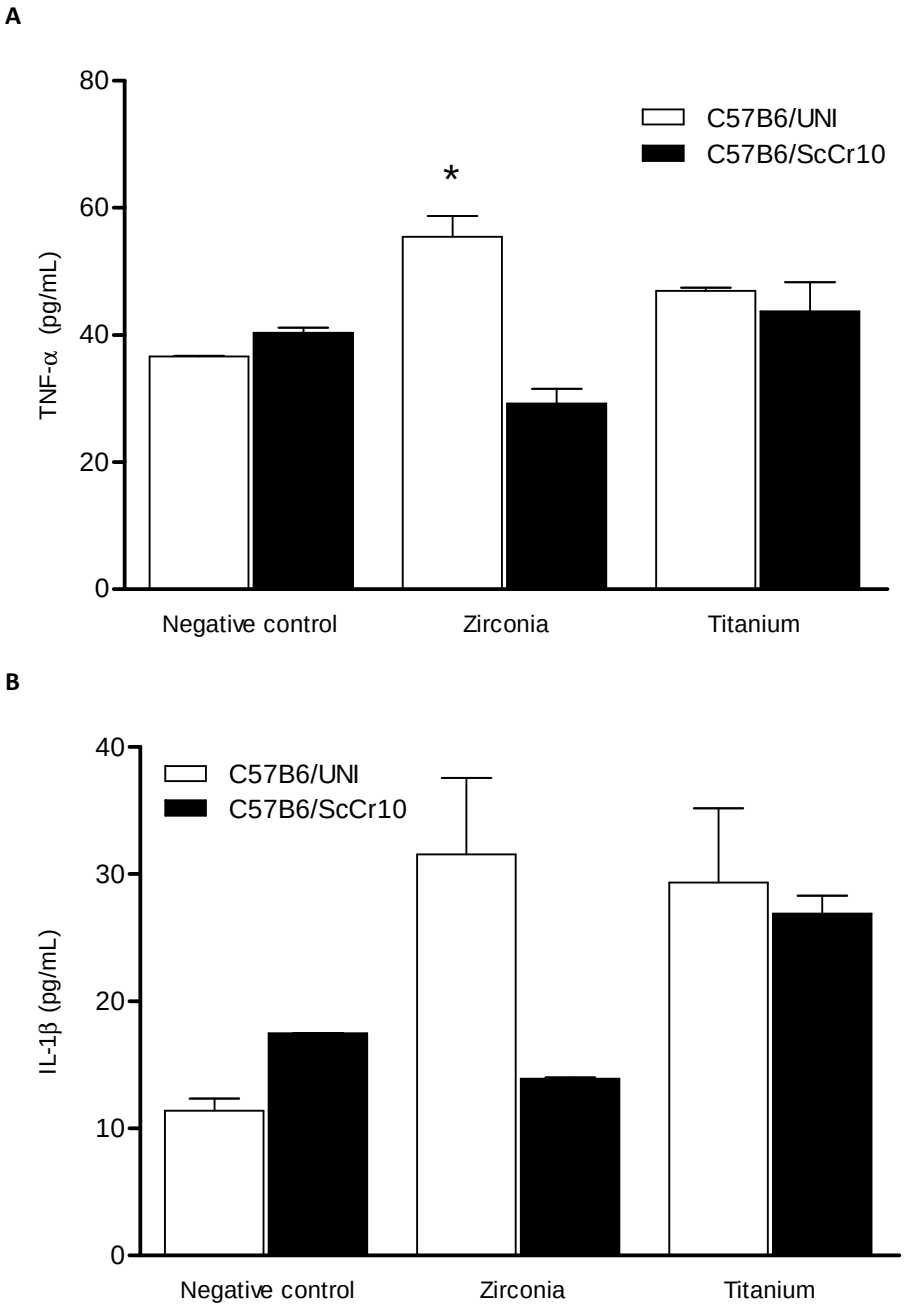
IL-6 mRNA Fold Increased
2500
2000
1500
1000
500
0
-500
0,25
2,00
24,00
Time in hours
+
+
+
+

Figure 4.


A
80
60
40
20
0
TNF-α (pg/mL)
C57B6/UNI
C57B6/ScCr10
*
Negative control
Zirconia
Titanium
B
40
30
20
10
0
IL-1β (pg/mL)
C57B6/UNI
C57B6/ScCr10
Negative control
Zirconia
Titanium

C

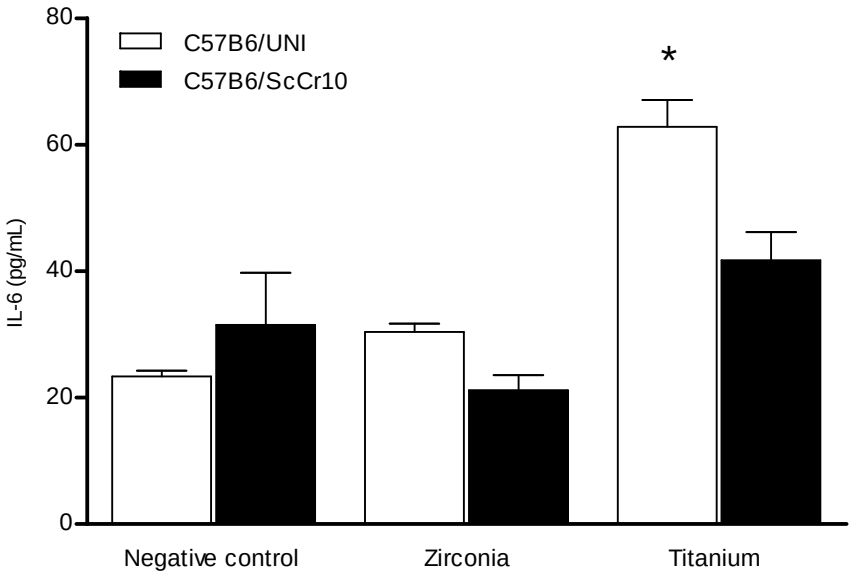
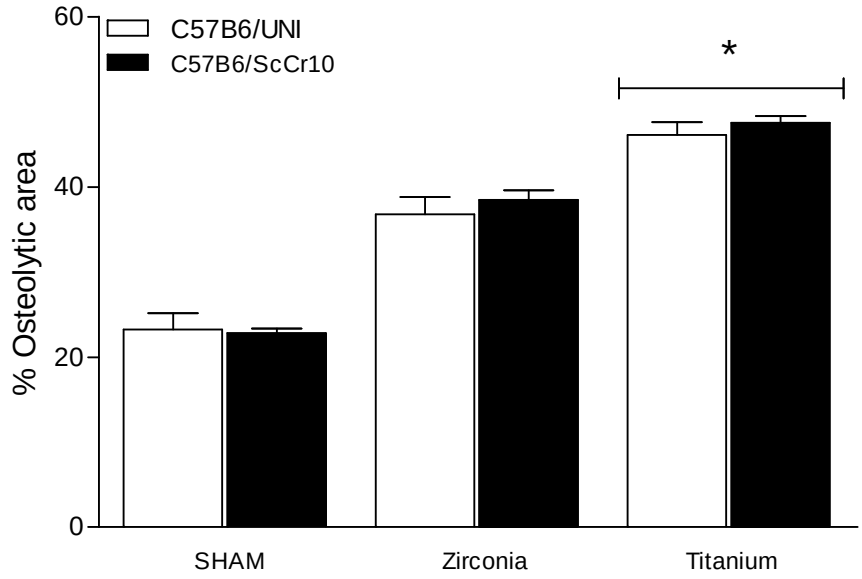

80
60
40
20
0
IL-6 (pg/mL)
C57B6/UNI
C57B6/ScCr10
*
Negative control
Zirconia
Titanium

Figure 5.


A- SHAM
B- Zirconium
C- Titanium

D


60
40
20
0
% Osteolytic area
C57B6/UNI
C57B6/ScCr10
*
SHAM
Zirconia
Titanium

E

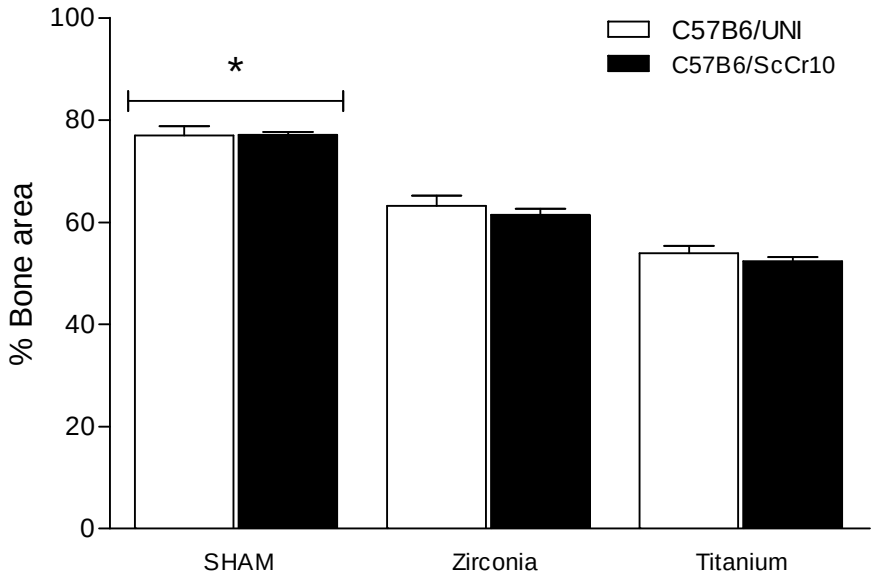

100
80
60
40
20
0
% Bone area
*
C57B6/UNl
C57B6/ScCr10
SHAM
Zirconia
Titanium

Figure 6.

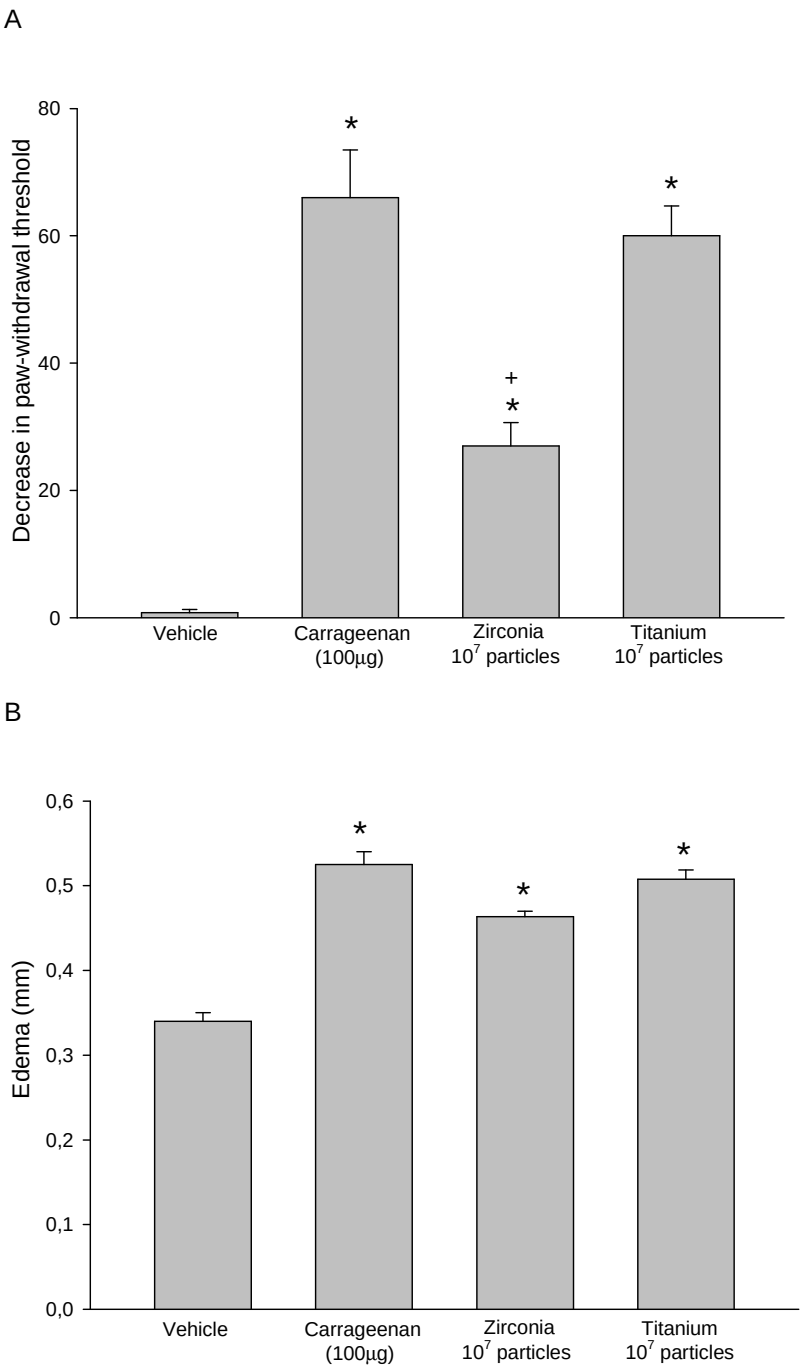

A
Decrease in paw-withdrawal threshold
80
60
40
20
0
*
*
+
*
Vehicle
Carrageenan (100µg)
Zirconia 10^7 particles
Titanium 10^7 particles
B
Edema (mm)
0,6
0,5
0,4
0,3
0,2
0,1
0,0
*
*
*
Vehicle
Carrageenan (100µg)
Zirconia 10^7 particles
Titanium 10^7 particles

Table 1 - Oligonucleotides design

Gene	Primer forward (5'-3')	Primer reverse (3'-5')	Número de acesso (NCBI)
TLR2	***CGCCCTTTAAGCTGTGTCTC***	***TCCAGTTCGTACTTGCACCA***	***NM_011905.2***
TLR3	***ATATGCGCTTCAATCCGTTC***	***TCAGGGGGAAGCCATAATAA***	***NM_126166.4***
TLR4	***GGTTGAGAAGTCCCTGCTGA***	***ATTCGAGGCTTTTCCAT***	***NM_021297.2***
TLR9	***ACAGCCTGCGCGTTCTCTTCAT***	***GTTGGCGGGGCACCTTTGTGAG***	***AF348140***
MyD88	*CGCGCATCGAGGAGGACTG*	*GGGGTCATCAAGGGTGGTGA*	***NM_010851.2***
NF-κB	***GAAGAGGAAGAAAATGGCGGAGTT***	***TCCTCCGAAGCTGAACAAACA***	***AY521463***
TNF-α	***TCGTAGCAAACCACCAAGTG***	***CCTTGAAGAGAACCTGGGAGT***	***NM_013693***
IL1-β	***AGCCCATCCTCTGTGACTCAT***	***CATTGAGGTGGAGAGCTTTC***	***NM_008361***
IL-6	***CTTGGGACTGATGCTGGTG***	***TTCCACGATTTCCCAGAGA***	***NM_031168***
β-actina	***TCCTGTGGCATCCATGAAACTA***	***CCAGGGCAGTAATCTCCTTCTG***	***NM_031144.2***

All oligonucleotides were design to 150 pairs bases using Primer3 and were tested for self complementary and primer dimers using GeneRunner free software. Temperature gradient was performed to evaluate the best annealing temperature for PCR reactions. The best temperature for all primers was at 60ºC.

Table 2- Spearman correlation coefficient

	MyD88 mRNA		NF-kB mRNA		TNF- α mRNA		IL-1β mRNA		IL-6 mRNA		TNF- α protein		IL-1β protein		IL-6 protein	
	rho	p value	*rho*	p value	*rho*	p value	*rho*	p value	*rho*	p value	*rho*	p value	*rho*	p value	*rho*	p value
TLR4	0.486	0.356	0.886*	0.033	0.886*	0.033	0.886*	0.033	0.829*	0.058	-0.085	0.919	0.886*	0.033	0.829	0.058
MyD88			0.486	0.356	0.486	0.356	0.486	0.356	0.429	0.419	0.657	0.175	0.486	0.356	0.429	0.419
NF-κB					1.000*	0.002	1.000*	0.002	0.943*	0.016	-0.085	0.919	0.886*	0.033	0.943*	0.016
TNF- α							1.000*	0.002	0.943	0.016	-0.085	0.919	0.886*	0.033	0.943*	0.016
IL-1β									0.943	0.0167	-0.085	0.919	0.886*	0.033	0.943*	0.016
IL-6											0.0857	0.919	0.943*	0.016	1.000*	0.002

Spearman's correlation coefficients (*rho*) and the corresponding *P* values, the symbol * indicates significant correlation. TLR4, Toll-like receptor 4; MyD88, myeloid differentiation factor 88; NF-kB, Nuclear factor kappa B; TNF- α, Tumor necrosis factor alpha; IL-1 β, Interleukin 1 betta; IL-6, interleukin 6.

Tabla 3- Coeficiente de correlação de Spearman

	MyD88 mRNA		NF-kB mRNA		TNF- α mRNA		IL-1β mRNA		IL-6 mRNA		TNF- α protein		IL-1β protein		IL-6 protein	
	rho	p value	*rho*	p value	*rho*	p value	*rho*	p value	*rho*	p value	*rho*	p value	*rho*	p value	*rho*	p value
TLR2	0,943*	0,01	0,429	0,419	0,543	0,297	0,429	0,419	0,886*	0,033	1,00*	0,002	-0,429	0,419	-0,143	0,803
TLR3	0,771	0,103	0,600	0,242	0,429	0,419	0,600	0,242	0,829*	0,058	0,657	0,175	-0,314	0,564	-0,028	1,000
TLR9	0,771	0,103	0,600	0,242	0,600	0,242	0,600	0,242	0,714*	0,136	0,600	0,242	-0,143	0,803	-0,085	0,919
MyD88			0,371	0,497	0,486	0,356	0,371	0,497	0,943*	0,016	0,943*	0,016	-0,486	0,356	-0,200	0,714
NF-κB					0,886*	0,033	1,000*	0,002	0,429	0,419	0,429	0,419	0,486	0,356	0,543	0,297
TNF- α							0,886*	0,033	0,543	0,297	0,543	0,297	0,486	0,356	0,429	0,41
IL-1β									0,429	0,419	0,429	0,419	0,486	0,356	0,543	0,297
IL-6											0,886*	0,033	-0,429	0,419	0,028	1,00

Coeficiente de correlação de Spearman (*rho*) e os correspondentes valores P, o símbolo * indica correlação significante. TLR2, Toll-like receptor 2; TLR3, Toll-like receptor 3; TLR9, Toll-like receptor 9; MyD88, myeloid differentiation factor 88; NF-kB, Nuclear factor kappa B; TNF- α, Tumor necrosis factor alpha; IL-1 β, Interleukin 1 betta; IL-6, interleukin 6.

DISCUSSÃO

Implantes dentários são amplamente utilizados como substitutos de dentes na terapia dentária para restaurar ou mesmo substituir a função em indivíduos parcial ou totalmente desdentados. No implante dentário a biomecânica, bioquímica, as exigências funcionais e estéticas do material são de fundamental importância para garantir o sucesso clinico (Adell et al. 1981; 1986; 1988; Adell et al. 1990a; Adell et al. 1990b; Palmquist et al. 2010). E este estado clínico funcional pressupõem a osseointegração, que consiste da interação e contato direto do implante com o tecido ósseo. Inúmeros tipos celulares, citocinas e fatores de crescimento estão envolvidos de forma coordenada nas diferentes fases do remodelamento e cicatrização óssea (Palmquist et al. 2010; Telleman et al. 2011; Tomisa et al. 2011).

No implante dentário e ortopédico o titânio é amplamente utilizado, pois é um material altamente biocompátivel, com boa resistência a corrosão e não apresenta toxicidade para macrófagos e fibroblastos e não induz no tecido do peri-implante uma resposta inflamatória.

Vários dados na pratica dental reportam que o sucesso clínico dos implantes dentais de titânio superam o 90%, sendo a taxa de sucesso de 98% em implantes colocados na mandíbula e 94% em implantes colocados na maxila. E assim, dependendo da técnica cirúrgica, dos hábitos de higiene e de próteses bem adaptadas estes implantes não apresentarão falhas na sua osseointegração (Branemark et al. 1977; Adell et al. 1981; Adell et al. 1990a; Tonetti and Schmid 1994; Gapski et al. 2003; Telleman et al. 2011). No entanto, esta pequena porcentagem de fracassos pode ser resultante principalmente dos hábitos de higiene inadequados, próteses mal adaptadas (Tonetti and Schmid 1994) e por infecções provocadas por tratamentos dentários nos dentes vizinhos (Laird et al. 2008), ou ainda a reações alérgicas ao titânio (Siddiqi et al. 2011). No entanto, na clinica dental o

titânio apresenta algumas desvantagens, sendo a principal de caráter estético devido a cor cinza escura do implante que transparenta a mucosa peri-implante (Ozkurt and Kazazoglu 2011). Outras desvantagens descritas são a imunomodulaçao não especifica, reações autoimunidade (Stejskal and Stejskal 1999), reações de hipersensibilidade (Siddiqi et al. 2011), correntes galvânicas (Tschernitschek et al. 2005), e formação de placa bacteriana (Burgers et al. 2010).

Já na clinica ortopédica, as falhas na osseointegraçao dos implantes são mais frequentes e variam numa porcentagem entre 65 a 84% (Kaczmarek et al. 2010) (van der Weegen et al. 2011), sendo a causa mais comum deste fracasso a perda asséptica (van der Weegen et al. 2011). Por esta razao, inúmeras alterações e tratamentos na superfície dos implantes tem sido testados de modo a aumentar a biocompatibilidade das próteses ortopédicas. Dentre os inúmeros materiais testados, a zircônia tem sido considerada a mais promissória (Hernigou et al. 2009). Este material cerâmico por sua natureza é mais resistente à abrasão, produzindo deste modo menos *debris* ou partículas de desgaste e tende a se osseointegrar mais rapidamente que o titânio (Kraay et al. 2006; Spinelli et al. 2011). A zirconia, já é amplamente utilizada tanto em implantes ortopédicos quanto em implantes dentários, pois possui uma boa emergência marginal que contribuem com a estética (Ozkurt and Kazazoglu 2011). (Lee et al. 2010). Não entanto, a zircônia apresenta menor resistencia fisica e sob estresses mecânicos pode sofrer fraturas. Esta desvantagem faz com que os implantes dentários sejam produzidos num diâmetro único (4,3mm) encontrada em todas as marcas disponíveis (Himmlova et al. 2004). Outra desvantagem destes implantes está relacionada com a necessidade da inserção cirúrgica ser efetuada numa boa posição anatômica, pois não permite correções de mal posicionamento da prótese (Andreiotelli and Kohal 2009).

A causa mais freqüente da perda de um implante ortopédico se deve a perda asséptica, a qual tem sido atribuída à inflamação local crônica gerada em resposta ao reconhecimento dos *wear debris* ou partículas de desgaste produzidos na interface do implante quando ocorre sua inserção no tecido ósseo principalmente quando se produz a abrasão do implante por função (Drees et al. 2007; Hallab and Jacobs 2009). A resposta imune a estas partículas de desgaste é mediada pela ativação dos macrófagos iniciada pela fagocitose (Maloney et al. 1996; Catelas et al. 1998; Rader et al. 1999; Warashina et al. 2003; Grandjean-Laquerriere et al. 2007; Taki et al. 2007; Xing et al. 2008; Pajarinen et al. 2009; Greenfield et al. 2010; Hirayama et al. 2011), em cirurgias de revisão aproximadamente 60 a 80 % das populações celulares encontradas nas pseudomembranas são macrófagos (Hallab and Jacobs 2009).

Como mostram os nossos resultados, às partículas de titânio e zircônia são fagocitadas pelos macrófagos do lavado peritoneal, contudo apresentam uma cinética fagocitária é distinta. As partículas de zircônia são fagocitadas mais rapidamente e eficientemente alcançando o platô de 70 % de fagocitose, após 2 horas de incubação, enquanto as partículas de titânio são fagocitadas mais lentamente alcançando o platô de 60% de fagocitose, somente após 24 horas de incubação (Anexo 1). Interessantemente, a cinética de fagocitose das partículas de titânio é idêntica entre as células do lavado peritoneal da linhagem C57B6/ ScCr10, mutantes para o receptor TLR4, a linhagem selvagem (wt), contudo a cinética de fagocitose das partículas de partículas de zircônia é totalmente diferente em comparação com a linhagem wt. A fagocitose ocorre mais lentamente alcançando o platô de 50 % após 24 horas e que é muito menor quando comparada com a linhagem wt sugerindo um provável envolvimento deste receptor para mediar a fagocitose das partículas de zircônia.

Os *Toll-like receptors* (TLRs) efetuam o reconhecimento de padrões moleculares associados à patogenos e sinais de perigo, mediando a ativação da imunidade inata e a modulação da resposta adaptativa. Estes receptores têm sido associados na modulação da resposta inflamatória quando células imunes são tratadas com partículas de titânio (Grandjean-Laquerriere et al. 2007; Takagi et al. 2007; Pajarinen et al. 2009; Hirayama et al. 2011), liberando conseqüentemente citocinas pró-inflamatorias capazes de em modelos animais o processo osteolítico (Warashina et al. 2003).

Sabemos que qualquer tipo de implante gera produtos de degradação como partículas DE DESEGASTE ou *wear debris* com tamanhos e formas variadas, e a resposta a estas partículas é essencialmente dependente dos macrófagos que possuem a capacidade de efetuar a ingestão de partículas menores que 150 nm mediante endocitose ou pinocitose, enquanto partículas maiores até 10 µm são fagocitadas. A fagocitose pode ser subdividida em etapas distintas, mas requer como etapa inicial o reconhecimento via receptor celular. Os macrófagos expressam inúmeros receptores celulares entre eles os TLRs, uma família de receptores evolutivamente conservada capaz de reconhecer padrões moleculares associados aos patogenos (PAMPs) expressos em microrganismos. A especificidade de reconhecimento dos TLRs se deve a sua base estrutural extracelular de múltiplos módulos de resíduos de leucina que se ligam diretamente aos PAMPs ou as moléculas acessórias/adaptadoras se ligam aos PAMPs.

Os nossos resultados mostram que o co-cultivo ou exposição das células aderentes do lavado peritoneal dos animais *wt* às partículas de titânio e zircônia induz a expressão dos TLR 2, 3, 4 e 9, contudo, a expressão não é idêntica entre as partículas. As partículas de titânio induzem um aumento substancial na expressão gênica dos TLR 4, 3 e 9 enquanto as

partículas de zircônia estimulam uma maior expressão de TLR2. Embora haja modulação na expressão gênica destes receptores nos animais mutantes para TLR4, a linhagem C57B6/ScCr10, ela é muito menor quando comparada com os animais *wt.* Este dado sugere que a ativação do TLR4 inicia a resposta imune contra estas partículas mais eficientemente que o TLR2 e inclusive pode até amplificar esta resposta.

Um aumento na expressão destes receptores, também foi encontrado na interface de implantes (Takagi et al. 2007), contudo, a sua participação exata no reconhecimento das partículas ainda não está totalmente elucidado (Grandjean-Laquerriere et al. 2007; Pajarinen et al. 2009; Hirayama et al. 2011). Algumas pesquisas sugerem que, a ativação na verdade é mediada pela contaminação das partículas com LPS e até o momento esta possibilidade não pode ser totalmente excluída. Endotoxinas podem aderir às partículas e variando de acordo com a sua composição química (titânio ou zircônia) afetar diferentemente a sua atividade biológica (Greenfield et al. 2005; Greenfield et al. 2010; Hirayama et al. 2011). Contudo trabalho recentes de Cui e colaboradores mostram de forma inequívoca, que a exposição às nanopartículas de titânio causam danos oxidativos e disfunção hepática *in vivo* com aumento da expressão gênica de TLR2 e 4 e outros genes relacionados a resposta inflamatória (Cui et al. 2011).

O reconhecimento mediado pelos TLRs está ligado a uma cascata de eventos que promovem à resposta inflamatória, ativação da resposta imune inata e a indução da resposta imune adaptativa. A interação com o TLR induz a ativação das vias de transdução de sinal intracelular que são iniciadas pelo recrutamento de uma ou mais moléculas ou melhor proteínas adaptadoras que culminam com a resposta transcricional dos genes alvo. As moléculas adaptadoras entre elas o MyD88 *(myeloid differentiation primary response*

proteína 88), TIRAP (*TIR domain containing adaptor protein*), TRIF (*TIR domain-containing adaptor protein inducing IFNβ*) e TRAM (TRIF – *related adaptor molecule*) se associam com o domínio citoplasmático do TLR. Com exceção do TLR3, todos os outros TLRs utilizam como molécula adaptadora o MyD88, e somente TLR4 é o único receptor que ativa duas vias de transdução de sinalização: a MyD88 e o TRIF (Akira 2006).

Interessantemente a expressão do mRNA para o adaptador MyD88 foi consistentemente mais elevada para as particulas de titânio comparadas as particulas zircônia. Este dado pode sugerir que a sinalização proveniente do TLR4 modula a resposta inflamatória associada à perda asséptica, primeiro, pela alta expressão nos níveis de TLR4 e sua molécula adaptadora MyD88, e segundo, pela relação atribuída ao TLR4 no processo osteolítico já que se tem observado que o bloqueio do TLR4 por técnicas *anti-sense* o processo osteolítico fica diminuído (Hao et al. 2010). Por conseqüência a regulação do TLR4 e suas moléculas adaptadoras são compatíveis com a expressão e liberação de citocinas pro-inflamatórias e osteólise. É importante lembrar que as partículas foram tratadas para excluir níveis de LPS capazes de ativar os macrófagos, assim, na ausência deste PAMP que pode ser reconhecido pelo TLR4, se sugere que este receptor seja ativado devido a processos celulares relacionados à fagocitose destas partículas.

Todo processo inflamatório possui evidencia de citocinas inflamatórias. A tríade mais conhecida no processo osteolítico são as citocinas TNF-α, IL-1β e IL-6, responsáveis de manter o processo inflamatório e a reabsorção óssea (Chiba et al. 1994; Grandjean-Laquerriere et al. 2005; Taki et al. 2007). No nosso estudo mostra que estas citocinas são expressas (tanto geneticamente como em proteína) pelos macrofagos co-cultivados com as partículas, embora o titânio induza uma expressão maior destas citocinas.

A indução da perda óssea foi avaliada utilizando o modelo da calvária e nossos resultados confirmam os resultados descritos por Warashina et al (2003), evidenciando uma maior perdida óssea e formação de tecido de granulação pelo titânio em comparação com a zircônia. Estes resultados estão de acordo com inúmeras pesquisas que sugerem que o titânio induz maior perda perda óssea que implantes feitos ou tratados com superfície cerâmica (Rader et al. 1999; Stea et al. 2000; Bi et al. 2001; Warashina et al. 2003; Sundfeldt et al. 2006; Pajarinen et al. 2009).

A dor é uma característica do processo osteolítico provocado pela perda asséptica (Willert et al. 2005). Diversas pesquisas têm mostrado que clinicamente os pacientes sentem dor local que vai aumentando com o decorrer do processo de osteolise (Schulte et al. 1993; Berry et al. 1995; Willert et al. 2005). No nosso trabalho confirmou este dado experimentalmente, *in vivo*, pela primeira vez sugerindo que as partículas são capazes de iniciar uma resposta inflamatória forte que permite a sensibilização dos nociceptores com aumento da permeabilidade vascular avaliada através do teste de edema de pata. Este processo é mediado pelas citocinas inflamatórias (TNF-α, IL-1β and IL-6) que induzem hiperalgesia e edema. as particulas de titanio induzem maior hiperalgesia e edema o qual pode ser explicado pela indução e secreção maior das citocinas inflamatórias como IL-1β and IL-6, contudo a zircônia também induz ambos contudo estimula maior produção das citocinas tnf-α e il-1β, demonstrando que a presença desta partículas de desgaste são capazes de gerar dor e edema in vivo confirmando os dados clínicos descritos.

Todos estes dados permitem sugerir que o processo osteolítico produzido por partículas de titânio e zircônia é originado por seu reconhecimento pelos TLRs. Devido a uma alta modulação do TLR4 nos experimentos sugerimos que este TLR está relacionado com o

reconhecimento de estas partículas, e pode ainda ter cooperação com outros TLRs externos ou internos; e uma vez que este TLR4 tenha sido ativado, envia-se uma sinal intracelular utilizando a via dependente de MyD88 para o NF-kB a qual inicia a transcrição de citocinas inflamatórias (TNF-α, IL-1β e IL-6) as quais darão inicio ao processo inflamatório destrutivo, a hiperalgesia e edema, sendo mais evidenciado no titânio que na zircônia. Estes resultados apoiam, outras pesquisas, a utilização de materiais cerâmicos na confecção de implantes para a diminuição dos processos osteolíticos (Warashina et al. 2003; Kraay et al. 2006; Sundfeldt et al. 2006; Hernigou et al. 2009; Spinelli et al. 2011).

CONCLUSAO GERAL

Segundo os dados obtidos podemos concluir que:

1. As partículas de titânio e zircônia são reconhecidas e fagocitadas pelos macrófagos do lavado peritoneal;
2. Modulam a expressão dos receptores TLR2, 3 ,4 e 9;
3. O TLR4 modula uma resposta imune quando macrófagos peritoneais são cultivados com partículas de zircônia e titânio, respectivamente, produzindo a secreção de citocinas pró-inflamatórias, principalmente a secreção de IL-6 no titânio, quem apresenta maior reabsorção óssea, em comparação com a zircônia, quando é colocada na calvária murina.
4. Outro receptores da família dos TLRs (TLR2, 3 e 9) em cooperação com o TLR4 participam na resposta imune.

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ANEXO

In vitro expression of inflammatory mediators and bone regulators in murine macrophages under exposure of commercial and experimental MTA

Autores: Obando-Pereda, GA [A]; Cornejo-Fudinaga, AC [B] ; Salas-Beltrán HE [B]; Peroni LA[A] & Stach-Machado, DR[A].

A. Department of Anatomy, Cell Biology and Physiology and Biophysics, Institute of Biology, University of Campinas, Campinas, SP, Brazil.
B. Department of Cariology and Endodontology, School of Dentistry, Saint Mary Catholic University, Arequipa, Perú.

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BACKGROUND:

MTA has used in a variety of surgical and nonsurgical endodontic applications. The aim of this study is to evaluate the gene expression and protein production of TNF-α, IL-1β, IL-6as well as the gene expression of RANKL and OPG using both a commercial and an experimental mineral trioxide aggregates in macrophage cell cultures.

METHODS:

Peritoneal macrophage cell culture was performed. Viability, gene expression of cytokines, RANKL, and OPG, and protein levels in experimental- and commercial-grey mineral trioxide aggregates co-cultured with peritoneal macrophages was determinate by tryptan blue, Real time-PCR and ELISA.

RESULTS:

The expression of TNF-α for both commercial and an experimental MTA were higher while, the expression of IL-1β and IL-6 were similar when compared to the negative control. At protein expression level, no differences were observed between negative control and cements. RANKL did not show a significant improvement in gene expression when compared with negative control, but OPG expression in cement samples was higher when compared to negative control.

CONCLUSIONS:

This study suggests that commercial and experimental MTA promotes anti-inflammatory processes, as well bone healing capacity.

Introduction:

Mineral trioxide aggregate (MTA) is an optimal material used mainly for regeneration purposes in endodontic procedures such as endodontic apical surgery, perforation repair, and apexification treatment[1]. These substances can be compared to the materials used in the construction industry for building foundations such as cement and mortar[1]. It is use was pioneered by Dr. Torabinajad of the University of Loma Linda in California, USA, who has discovered it to have exceptional biologically compatible properties[2-3]. Since the material is very alkalinic (pH 12), it is expected to irritate body tissues[4-5].However, nature can be very surprising, leading, in this case, that the hard tissues of the body welcome this material[6] alone or mixed with other dental [7-9] or natural [10] materials.

Periradicular lesions are characterized by the inflammation of the connective tissue which is accompanied by bone destruction around the infected root-end of the teeth [11-12]. Many different types of cells have been detected in these lesions. Some of the most important, however, are macrophages and T and B lymphocytes[13-14]. These cells play a central role in the pathogenesis of inflammation and, among other things, generate cytokine production [15].

Macrophages are among the first cells to come in contact with foreign bodies, and play the main role in the pathogenesis of the inflammatory process [15]. They produce several cytokines which promote the initiation, perpetuation, and directing of the immunological response. However, at the same time, they also cause the inhibition of the immunological response process [16].

The inflammation of endodontic lesions is characterized by the presence of certain cytokines such as IL-1, IL-6, tumor necrosis factor (TNF), and other inflammatory mediators [17-18]. The cytokines IL-1β and TNF-α are responsible for the majority of the bone-reabsorbing activity in chronic periapical lesions in humans [19].Likewise, IL-1β is known to account for the majority of the bone reabsorbing activity within infection induced periapical lesions in lab rats [20]. Similarly, IL-6 accounts for periradicular inflammation and bone reabsorption in response to antigens and cytokines like TNF-α and IL-1β [21].

Investigations in the field of molecular biology have led to an increased understanding of the mechanisms and proteins involved in bone reabsorption. This process is controlled by a system comprised of three key proteins, receptor-activator of nuclear factor kappa beta (RANK), its receptor-activator of nuclear factor kappa beta ligand (RANKL), and a decoy receptor osteoprotegerin (OPG). This system is regulated by many osteotropic hormones and cytokines (mainly TNF-α, IL-1β) which reduce the OPG/RANKL ratio[22]. RANKL/RANK signaling regulates osteoclast formation, activation, and survival in normal bone modeling and remodeling, as well as in a variety of pathological conditions characterized by increased bone turnover. OPG protects bone from excessive reabsorption by binding to RANKL and preventing it from binding to RANK. Thus, the relative concentration of RANKL and OPG in bone material is a major determinant of bone mass and strength [23].

Because MTA has only recently been used as an endodontic material, there are few studies which evaluate its effects on cytokines, RANKL, and OPG mRNA expression. The goal of this study was to evaluate the mRNA expression of RANKL and OPG and the expression and releasing of cytokines using both a commercial MTA and an experimental MTA in macrophage cell cultures.

Materials and Methods

All experiments were conducted in accordance with the ethical guidelines established by the Colégio Brasileiro de Experimentação Animal (COBEA) and were approved by the Ethics Committee on Animal Experimentation (CEEA) of the Institute of Biology, UNICAMP (protocol number 1312-1).

Preparation of MTA and eMTA solutions

Both grey MTA-Ângelus (Odontológika, Londrina, PR, Brazil) and an experimental MTA (eMTA)[24]were used. The cements were prepared with a mixture of MTA powder and distilled water in according to the MTA-Ângelus manufacturer specifications. Their handling was carried out in a laminar flow hood under aseptic conditions, and packed onto the bottom of 24-well dishes (Nunclon, Nunc) to create a uniform surface area [25]. The freshly prepared samples were allowed to set for 24 hours in a cell incubator at 37℃ and 100% humidity. After 24 hours, 1 ml of RPMI supplemented with 10% fetal bovine serum and gentamicine (50µg/mL) was added to each well. The materials were incubated with the culture medium at 37℃ and 100% humidity. The m aterial solutions were collected after 24 hours and passed through a sterile 0.2 µm Millipore™ filter, aliquoted into 1.5-ml Eppendorf tubes, and stored at -20℃ until use [26].

Mice Peritoneal Isolation and cell culture

Peritoneal murine macrophages were collected from 7 week old male C57BL/6 WT mice[27]. The mice were injected with 1 mL solution of sodium thioglycollate 3%. After 4 days mice were asphyxiated using CO_2gas and were submerged in 70% ethanol solution to clean the skin. Following this, the peritonium was exposed. To obtain the peritoneal cells the mice were injected with a 5 mL solution comprised of coldstandard cell culture medium (RPMI),

5% fetal bovine serum (FBS), and gentamicine (50µg/mL).After a gentle massaging of the peritonium, 4 mL of peritoneal content was extracted and collected in a sterile 15 mL culture tube. The cells were then counted using a Neubauer chamber and diluted to final concentration of 1×10^6 cells/mL.

1 mL of the aforementioned cells was cultivated in 12of the 24 wells of a Nunclon™ well-dish having a concentration of $1x10^6$ cells/mL in an environment with 5% CO2 and a relative humidity of 95% for two hours in order to allow the macrophages to adhere to the well. After that, the cells in each well were washed separately three times with sterile PBS in order to free the non-adhered cells. The seeded macrophages were treated with cements solutions (1:10 dilution)[26] and controls at 37ºC with 5% CO2 and a relative humidity of 95% for 24 hours.The 12 wells were separated, as mentioned before, into 4 groups: MTA, eMTA, the negative control which was the cell culture medium, andthe positive control which was a solution of the cell culture medium with lipopolysaccharide (which elicits strong immune responses by activating macrophages) at a concentration of 1 ng/mL.

Viability

To evaluate the cellular viability, 1×10^6 macrophages/mL were seeded in 24-well plates (Nunclon) together with circular slides for microscopy (Glasscyto, HDA Instruments, China) and treated with cement solutions or control solution and incubated for 24 hours. The viability analysis was performed with an inverted microscope, following the addition of 100 µL of 0.5% tryptan blue solution (Sigma Chemical Co.) in PBS[28]. The counting were performed at 40x and 100 cells per lecture was observed. Dead cells (blue stained) were tabulated using free software Image J (http://rsbweb.nih.gov/ij/). The lectures were done in triplicate.

Total RNA isolation and reverse transcription

After 24 hours of stimulation with cements solutions and controls, the macrophages were washed three times with sterile PBS and treated with Trizoll-reagent (Invitrogen, Carlsbad, CA). Extracted total RNA was then frozen at -75℃ until further use. Nucleic acid yields were measured using spectrophotometry at 260/280 nm (Biomate3, Thermo Fisher Scientific, Waltham, MA). Before the reverse transcription, all of the RNA samples taken of the macrophage cells were treated with DNAse. After initial denaturation at 65℃ for 10 min, cDNA was produced by reverse-transcription using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

Real Time RT - PCR

All of the primers were designed with a freeware program called Primer3 to be used withβ-actin, RANKL, OPG, and proinflammatory cytokines (Table nº1). The length of each primer was 150 base pairs. These primers were then revised with GeneRunner, a free program, to detect such problems as self complementary and primer dimmers. Because β-actin worked to 100% satisfaction as a housekeeping gene, all results were standardized to it. For each of the 4 cDNA samples, 25 µL of RT-PCR mix was made using 3 µL of the respective cDNA, 0.5 µLof each primer, 12.5 µLof syber green master mix (qPCR – Sybr Green Rox Plus, LGC Biotecnologia, SP, Brazil), and 8.5 µL of ultrapure water. Real time PCR was carried out in an ABI Prism 7000 Sequence Detection system (Applied Biosystems, Warrington, UK), and the program used was 1 - 2 minute cycle at 50ºC for the initial denaturation, 1 - 10 minute cycle at 95ºC, 45 - 15 second cycles at 95ºC, and 1 - 1 minute cycle at 60ºC. For melt curve the program used was 15 sec at 95ºC, 30 sec at 95ºC, and 10 sec at 95ºC. Each of these tests was performed 3 times.

Enzyme-Linked Immunosorbent Assays (ELISAs)

Measurements of TNF-α, IL-1β, and IL-6 cytokines in co-culture supernatant were determined by ELISA using commercially available kits (eBioscience, San Diego, CA, USA; cytokines codes: 88-7324, 88-7013, 88-7064, 88-7314) according to the manufacturer's instructions. The sensibilities of ELISAs were TNF-α: > 8 pg/ml, IL-1β: > 8 pg/ml, and IL-6: > 4 pg/ml. The results are expressed as histograms of cytokine (–SD) per microliter of supernatant, from duplicate measurements.

Data analysis and Statistical analysis

For the mRNA expression, data was calculated by the cycle threshold (Ct) method according to the instructions in the User's Bulletin (P/N 4303859) from Applied Biosystems. The mean Ct values of the triplicate measurements for each cDNA were used to calculate the expression of the target gene by normalizing to an internal control (β-actin) using the ΔCt formula according to the User's Bulletin. Results are presented as the arithmetic mean and standard deviation (SD). The normal distribution of the data was tested using Shapiro-Wilk test, and an analysis of variance (ANOVA) was performed using Tukey test to evaluate the level of significance of differences between the experimental groups and Kruskal-Wallis for viability test. Probabilities of $p< 0.05$ were considered significant.

Results

Cellular viability to MTA solutions

The mean cellular viability was higher to 97% in all macrophage cultures. There was no difference in the percentages of live cells between the MTA-Ângelus, eMTA and negative control (Fig. 1). However, the percentage of live macrophage cells exposed for 24h to LPS was statistically minor than the others groups ($P > 0.05$).

Cytokines mRNA expression and protein production in macrophages cell culture

In order to evaluate the effects of MTA and eMTAon the expression of pro-inflammatory cytokines, peritoneal macrophages were cultivated in different groups: a positive control, a negative control, and 2 different types of cements. The expression of mRNA for TNF-α of MTA and eMTA (Figure 2a)did not show statistical differences (19.44 ± 0.48 and 13.52 ± 0.24 respectively) but they expression were increased in comparison of negative control (p 0.05).The positive control showed high mRNA expression levels (152.8 ± 3.98) when compared with the cements and the negative control (p 0.001).The IL-1β mRNA expression observed in MTA did not show a statistical difference with eMTA (1.337 ± 0.20 and 1.104 ± .024, respectively) and the negative control (Figure 2b). The positive control, showed the highest levels (352.1 ± 1.38) when compared with the cements and the negative control (p 0.001). The mRNA expression of pleiotropic IL-6 did not show statistical differences between the cements (2.307 ± 0.273, 1.347 ± 0.225) and the negative control (Figure 2c). On the other hand, however, the positive control showed the highest levels (302.9 ± 36.67) when compared with the cements and the negative control (p 0.001).

Release of proinflammatory cytokines from macrophages occurred at 24 hours following commercial and experimental MTA

exposure. TNF-α did not show statistical differences between negative control, MTA and eMTA (166.00 ± 1.414; 164.667 ± 8.327; 172.00 ± 12.767 respectively), however, positive control showed great values of protein production (2766.667 ± 249,154,p 0.001) in compare with negative control, MTA and eMTA (figure 3a). IL-1β production for MTA, eMTA and negative control (figure 3b) was not statistically different (155.00 ± 35.35; 223.33 ± 15.275; 250.00 ± 45.826 respectively). The positive control showed great values of IL-1b production (716.667 ± 25.166, p 0.001). For IL-6 (figure 3c), the positive control

showed the most great values (4800.00 ± 700.00, p □ 0.001) in compare to negative control, MTA and eMTA (145.00 ± 7.07; 403.33 ± 49.329; 250.00 ± 79.373, respectively).

The Expression of RANKL and OPG mRNA in macrophages cell culture

The mRNA expression of RANKL for MTA and eMTA (Figure 4a) did not show statistical differences between them and the negative control (1.153 ± 0.02, 0.873 ± 0.02 and 1 respectively). But, in comparison, the positive control showed a greater mRNA expression(6.337 ± 0.15, p □ 0.0001).

The mRNA values for OPG in MTA and eMTA (Figure 4b) did not show statistical differences (5.001 ± 0.0001 and 5.438 ± 0.05 respectively). The positive control, on the other hand, showed elevated mRNA values (10.16 ± 0.51) when compared with the cements and the negative control (p □ 0.0001).

Discussion

Since its introduction as a commercial endodontic cement[29], MTA has been studied *in vivo*[30-32]and in vitro [33-35]with many interesting results. However, in the dental industry, there exist other types of MTA made by different commercial groups which are currently the under research[5, 36]. It has been reported from all of these studies that these cements have excellent biocompatibility, excellent bone and periodontal regeneration, and great deposition of dentinal and cemental tissues[31, 36].

During this study, an experimental MTA made with Peruvian Portland cement was used. This material has been the subject of many odontological theses whose results have been promising. In this study, neither the Commercial MTA nor the experimental MTA produced elevated expressions of cytokines or RANKL when compared with the positive control. However, these cements did produce a significant expression of OPG when compared

with RANKL which indicates that these materials possess good bone regenerative properties.

Macrophages viability was observed at 24 hours when cells were exposed to cements solutions and controls. In these conditions, viability was higher than 97% for all treatments in accordance with previous studies performed with macrophages[28], fibroblasts[32, 37-38] and osteoblasts[38-39].Viability were decreased in LPS group, however the LPS concentration applied in all experiment did not produce a high decrement of viability but produce a strong immune responses by activating macrophages.

In this study macrophages were exposed to MTA and an experimental MTA solutions *in vitro* for 24 hours in order to observe the mRNA expression of the cytokines TNF-α, IL-1β and IL-6, which are responsible for promoting inflammation and bone reabsorption. TNF-α plays an important role in the response to tissue damage and infection by promoting inflammation and recruiting lymphocytes and monocytes to the infected areas. They also stimulate endothelial cells to express adhesion molecules and secrete chemokines[40], as well as inducing pulp inflammation and bone resorption in inflamed periapical tissues [41].

Our data showed that levels of TNF-α for both cements were elevatedwhen compared with the negative control, however for protein production, values of TNF-α showed same levels for both cements and negative control. According to Silva et al (2008), the levels of TNF-α were also elevated in later stages during an inflammatory response explain the great values of mRNA of TNF-α for MTA and eMTA. Similar kinetics were observed by Rezende et al (2005) who denoted large quantities of TNF-α after incubation with MTA for 24 hours. The results presented herein are in agreement with this suggestion. Rezende et al (2005) also observed the same TNF-α kinetic of these quantities in groups without MTA, which suggests that this response may be due to the peritoneal macrophage extraction

procedure. Our data showed that macrophages, without any contact with MTA, did not produce relative expressions of this cytokine.

IL-1β is a cytokine that mediates the bone reabsorption[42]. It is synthesized by various cells and macrophages close to the bone reabsorption and the osteoclasts[43]. Our data showed that the expression levels of IL-1β were similar to the negative control for both cements, which indicates that this cytokine did not reach the threshold necessary to observe a biological function[44].For the IL-1β production, both cements and negative control were similar in compare with positive control. Some studies [30, 45] observed that MTA produced an increment of IL-1β levels when compared to others endodontic materials in fibroblasts cell cultures. This conduct may be due to the differences in the epigenetic background of macrophages and fibroblasts. However the levels showed for both cements can be suggest the repair function of IL-1β inducted macrophages to clean the injured site according with Ferreira et al (2009).

IL-6 is a pleitropic proinflammatory cytokine secreted by: T cells, monocytes, fibroblasts, epithelial cells, and macrophages, in response to antigens and other cytokines such as IL-1 and TNF-α[46].It has also been implicated in bone reabsorption by osteoclast activation[47]. Our data showed that the expression and protein production of IL-6 for both cements was similar to the negative control, however, the IL-6 from MTA reached the threshold necessary to perceive biological functions[44]. According to data from Gomez-Filho et al (2009) and Silva et al (2008), we agree with the suggestion that the amount of IL-6 was not statistically different from the control group, which shows that they can play an important role in controlling the inflammation and promoting the healing process.

The RANK/RANKL/OPG pathway is critically involved in the maturation and activation of osteoclasts during inflammation and other pathological processes [48-49]. Previous studies have found that the regulation of RANKL and OPG expression by LPS appears to be complex and biphasic. In laboratory experiments, it has been seen that osteoblast and bone marrow cells treated with LPS showed an increased RANKL and suppressed OPG [50]. On the other hand, mice injected with LPS had decreased serum RANKL levels and increased OPG levels [51]. However, in periodontal ligament fibroblasts, LPS led to elevated expressions in both factors [52].

Our data showed that the expression of RANKL for both cements was similar to the negative control, however, the OPG expression showed a significant expression when compared with the negative control. Data from Coon et al (2007) showed that expression of RANKL are diminished suggesting that MTA did not stimulate any significant osteoclast formation. Therefore, we agree with this information. However, Renzede et al (2008) showed that the expression of RANKL was elevated which demonstrated that the MTA can influence the RANKL induced osteoclastogenesis which were produced by the T-Cells in the periradicular lesion. According to Coon, the OPG expression showed levels similar to RANKL, which suggests a delayed healing in periradicular lesions [26]. Nevertheless, our data suggests that both cements present a potential capacity for bone healing.

Further in vivo studies should be performed to determinate the bone healing capacities of MTA and eMTA.

Conclusion

Data presented here showed that both MTA-Ângelus and experimental MTA did not promoting the expression and secretion of proinflammatory cytokines suggesting a great anti-inflammatory capacity; in the other hands both cements did not promoting the

expression of RANKL but promoting the expression of OPG suggesting a positive regulation for bone healing.

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Figure 1

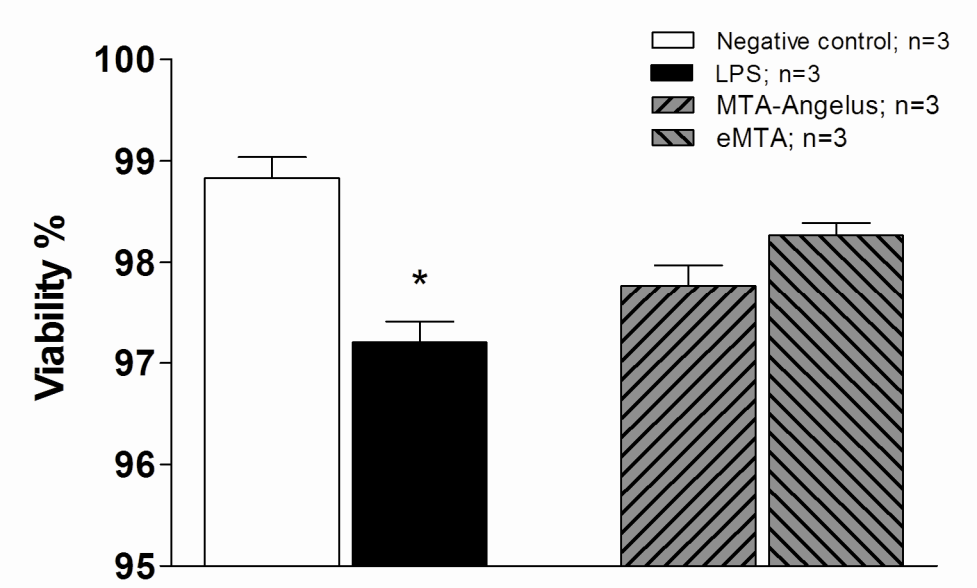

Figure 1. Percentage of live macrophages 24 hours after incubation in the presence of MTA, eMTA solutions and controls. Bars represent the mean of experiment performed in triplicate. * represent statistical difference $P < 0.05$

Figure 2

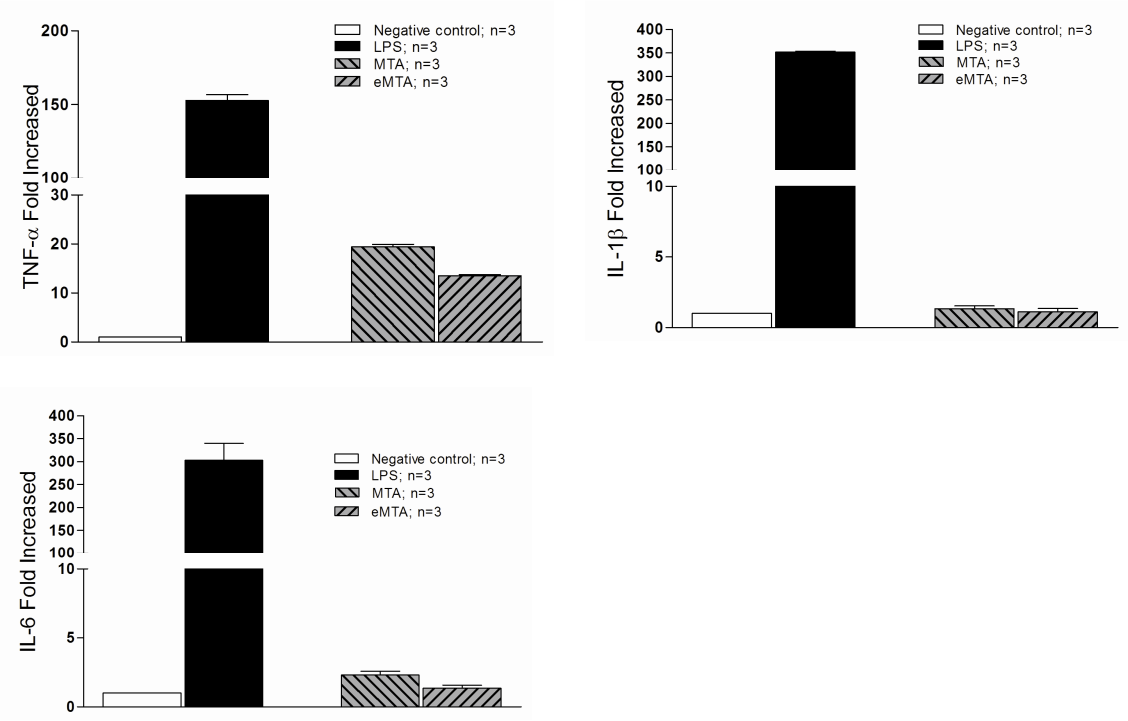

Figure 2. mRNA expression of cytokines: levels of TNF-α (a), IL-1β (b) and IL-6 (c) for MTA and eMTA were measured with real time-PCR. β-actin were used like a endogen control. *Significant difference from control values with $P < 0.0001$

Figure 3

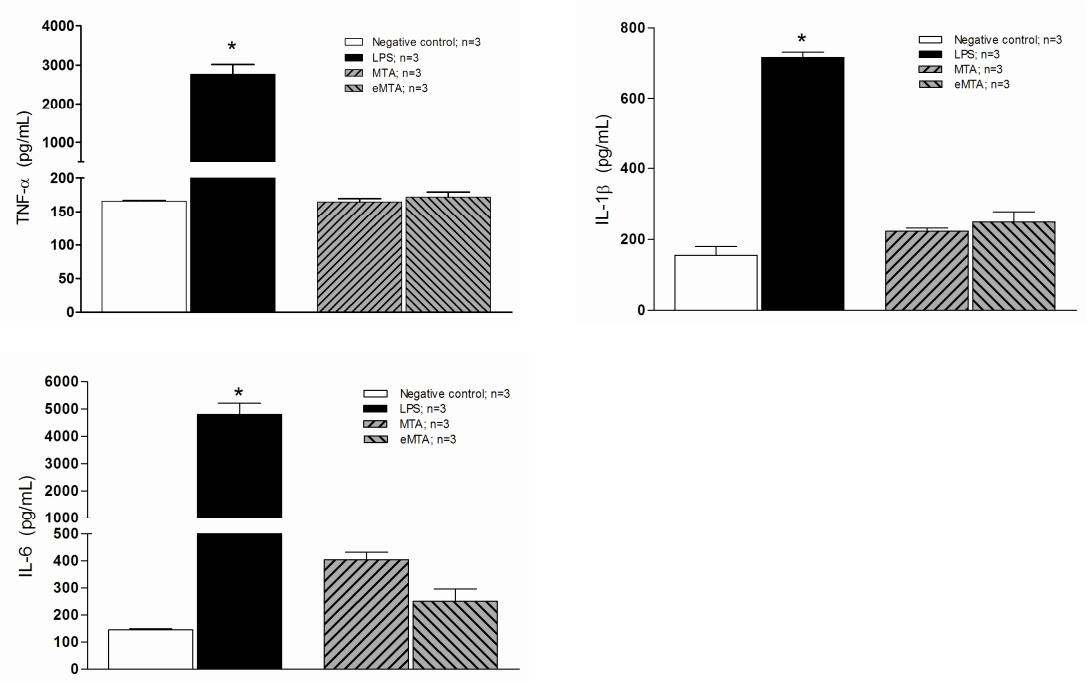

Figure 3. Release of cytokines: levels of TNF-α (a), IL-1β (b) and IL-6 (c) for MTA and eMTA were measured with ELISA. * Significant difference from control values with $P < 0.0001$

Figure 4

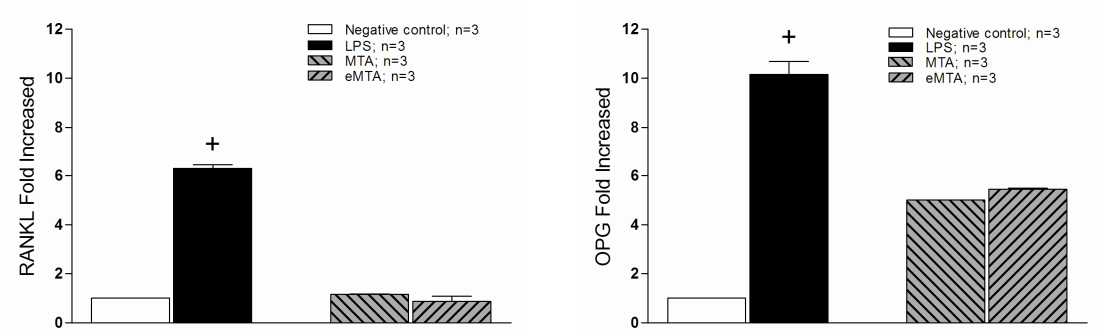

Figure 4. mRNA expression of RANKL/OPG: levels of RANKL (a) and OPG (b) for MTA and eMTA were measured with real time-PCR. β-actin were used like a endogen control. *Significant difference from control values with $P < 0.0001$

TABLE Nº1 - Oligonucleotides design

Gene	Primer foward (5'-3')	Primer reverse (5'-3')	
TNF-α	TCGTAGCAAACCACCAAGTG	CCTTGAAGAGAACCTGGGAGT	NM_013693
IL-1β	AGCCCATCCTCTGTGACTCAT	CATTGAGGTGGAGAGCTTTC	NM_008361
IL-6	CTTGGGACTGATGCTGGTG	TTCCACGATTTCCCAGAGA	NM_031168
RANKL	CTGGAAGTGGGGGAGAACGACA	TCAGGGGACACGGGCATAGAGT	NM_009399.3
OPG	TCCCTTGCCCTGACCACTCTTA	AACGCCCTTCCTCACACTCACA	NM_008764.3
β-actin	TCCTGTGGCATCCATGAAACTA	CCAGGGCAGTAATCTCCTTCTG	NM_031144.2

All oligonucleotides were design to 150 pairs bases using Primer3 and were tested for self complementary and primer dimers using GeneRunner free software. Temperature gradient was performed to evaluate the best annealing temperature for PCR reactions. The best temperature for all primers was at 60ºC.