MARIANA DA SILVA QUINTEIRO

EVALUATION OF PERIPHERAL EFFECT OF 15d-PGJ_2 ON INFLAMMATORY PROCESS INDUCED BY RHEUMATOID ARTHRITIS INTO RATS' TEMPOROMANDIBULAR JOINT

AVALIAÇÃO DO EFEITO PERIFÉRICO DA 15d-PGJ_2 NO PROCESSO INFLAMATÓRIO INDUZIDO PELA ARTRITE REUMATOIDE NA ARTICULAÇÃO TEMPOROMANDIBULAR DE RATOS

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Faculdade de Odontologia de Piracicaba

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Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do título de Doutora em Odontologia, na área de Fisiologia Oral.

Orientadora: Profa. Dra. Juliana Trindade Clemente Napimoga

Este exemplar corresponde à versão final da tese defendida pela aluna, e orientada pela Profa. Dra. Juliana Trindade Clemente Napimoga.

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ABSTRACT

Inflammation of the temporomandibular joint (TMJ) induced by Rheumatoid Arthritis (RA) have often resulted in persistent pain and caused distress to many patients. The 15-desoxi-$^{\Delta 12,14}$-prostaglandin J_2 (15d-PGJ_2) has demonstrated a potential analgesic and anti-inflammatory effect. Considering that not all patients respond to traditional drugs therapy to RA, the aim of this study was to evaluate the peripheral effect of 15d-PGJ_2 in the inflammatory process induced by RA into the TMJ of rats and its action mechanisms. For this study, male Wistar rats were used and the antigen-induced arthritis (AIA) was generated in rats with methylated bovine serum albumin (mBSA) diluted in complete Freund's adjuvant. RA-induced TMJ hypernociception in rats was assessed by measuring the behavioral nociceptive responses, such as rubbing the orofacial region and flinching the head, induced by the injections of low dose of formalin (0.5%) into TMJ. After behavioral experiments, animals were terminally anesthetized and periarticular tissues were removed for analysis of the inflammatory cytokines TNF-α, IL-1β, IL-6, IL-18 and IL-12; anti-inflammatory cytokine IL-10; inflammatory chemokines KC and CINC-1; protein kinases PKA and PKCε; decay accelerating factor (CD55); leukocytes migration, plasma-protein extravasation and histopathological analysis. The intra-articular injection of mBSA, but not phosphate buffered saline (PBS, control), in immunized rats induced dose-and time-dependent behavioral nociceptive responses in which the peak of behavioral nociceptive responses were obtained by using 10 ug/TMJ of mBSA after 24 h. Pretreatment with intra-TMJ injection of 15d-PGJ_2 (30, 100 and 300 ng/TMJ) prevented the RA-induced inflammatory hypernociception. TMJ tissues analysis demonstrated that peripheral treatment of 15d-PGJ_2 was able to attenuate RA-induced inflammation by: (1) reduced the release of inflammatory cytokines TNF-α, IL-1β, IL-6, IL-18, IL-12 and inflammatory chemokines KC and CINC-1; (2) increased the expression of anti-inflammatory cytokine IL-10; (3) reduced the

expression of protein kinases PKA and PKCε; (4) increased expression of decay accelerating factor (CD55); (5) decreased the RA-induced leukocytes migration and plasma-protein extravasation ($p<0,05$: ANOVA, Tukey Test). In addition the histopathological analysis showed that 15d-PGJ_2 reduced the inflammatory parameters induced by RA. These findings suggest that 15d-PGJ_2 may be considered a new perspective for the treatment of the inflammatory hypernociception induced by AR in TMJ of rats.

Key Words: Rheumatoid Arthritis. Temporomandibular joint. Inflammation. Pain. 15d-PGJ_2.

RESUMO

O processo inflamatório induzido pela Artrite Reumatoide (AR) na articulação temporomandibular (ATM) resulta em uma dor persistente causando estresse em muitos pacientes. A 15-desoxi-$^{\Delta 12,14}$-prostaglandina J_2 (15d-PGJ_2) vem demonstrando um potente efeito analgésico e anti-inflamatório quando administrado na ATM de ratos. Sendo assim, considerando que muitos pacientes com AR não respondem à conduta terapêutica tradicional, o objetivo deste estudo foi avaliar o efeito periférico da 15d-PGJ_2 no processo inflamatório induzido pela AR na ATM de ratos assim como o mecanismo envolvido. Para este estudo foram utilizados ratos Wistar que desenvolveram uma artrite induzida por antígeno (AIA) através de albumina bovina metilada (mBSA) diluída em Adjuvante de Freund Completo. A AR induziu uma hipernocicepção inflamatória na ATM de ratos avaliada por comportamentos nociceptivos, tais como, coçar a região orofacial e levantar reflexamente a cabeça, em resposta à injeção intra-articular de uma dose sublimiar de formalina 0,5 %. Após a avaliação dos comportamentos nociceptivos os animais foram mortos por anestesia e os tecidos periarticulares removidos para análises das citocinas inflamatórias TNF-α, IL-1β, IL-6, IL-18 e IL-12; citocina anti-inflamatória IL-10; quimiocinas inflamatórias KC e CINC-1; proteoquinases PKA e PKCε; fator de aceleração de decaimento (CD55); migração leucocitária, extravasamento plasmático e análise histológica. A injeção intra-articular de mBSA, mas não de tampão fosfato salina (PBS; grupo controle), nos animais imunizados, induziu comportamentos nociceptivos dose- e tempo-dependentes onde o pico dos comportamentos nociceptivos foi obtido utilizando 10 µg/ATM de mBSA depois de 24 h. O pré-tratamento com uma injeção intra-articular de15d-PGJ_2 (30, 100 e 300 ng/ATM) impediu a hipernocicepção induzida pela AR. Análises dos tecidos periarituclares demonstraram que o tratamento periférico com 15d-PGJ_2 foi capaz de reduzir o processo inflamatório induzido pela AR através: (1) da redução da liberação das citocinas inflamatórias TNF-α, IL-1β, IL-6, IL-18,

IL-12 e quimiocinas inflamatórias KC e CINC-1; (2) do aumento da expressão da citocina anti-inflamatória IL-10; (3) da redução da expressão das proteinoquinases PKA e PKCε; (4) do aumento da expressão do fator de aceleração de decaimento (CD55); e (5) da diminuição da migração de leucócitos e extravasamento plasmático ($p<0,05$: ANOVA, Teste Tukey). Complementando estes resultados, as análises histológicas demonstraram que a 15d-PGJ_2 reduziu os parâmetros inflamatórios induzidos pela AR. Sendo assim, os resultados do presente estudo sugerem que a 15d-PGJ_2 pode ser considerada uma nova perspectiva para o tratamento da hipernocicepção inflamatória induzida pela AR na ATM em ratos.

Palavras-chaves: Artrite Reumatoide. Articulação temporomandibular. Inflamação. Dor. 15d-PGJ_2.

SUMÁRIO

Dedico esta tese aos meus amados pais, ***Magnólia e Deusdete*** *e a meu querido irmão,* ***Gustavo****, pelo apoio incondicional, amor e por serem o alicerce da minha vida.*

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"De nada vale o brilho da inteligência, se o coração permanece às escuras".

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LISTA DE ABREVIATURAS E SIGLAS

15d-PGJ$_2$	15-desoxi-Δ12,14 prostaglandina J2
AA	Ácido Araquidônico
AR	Artrite Reumatoide
ATM	Articulação Temporomanbibular
CFA	Adjuvante Completo de Freund
CINC-1	Citocina Induzida por Neutrófilo quimioatraente
COX	Ciclooxigenase
COX-1	Ciclooxigenase 1
COX-2	Ciclooxigenase 2
DTMs	Distúrbios temporomandibulares
EDTA	Ácido etilenodiaminotetracético
ERK	Quinase reguladora sinal extracelular
ICAM	Molécula de adesão intercelular
IFA	Adjuvante Incompleto de Freund
IL-1	Interleucina 1
IL-15	Interleucina 15
IL-18	Interleucina 18
IL-1β	Interleucina 1 beta

IL-6	Interleucina 6
IL-8	Interleucina-8
KC	Quimiocina derivada de queratinócito
LTB_4	Leucotrieno B4
mBSA	Proteína albumina bovina sérica metilada
MCP-1	Proteína quimiotática de monócito -1
MMP-9	Matrix Metaloproteinase 9
NF-κB	Fator nuclear-κB
NO	Óxido nítrico
PBS	Tampão Fosfato Salina
PG	Prostaglandina
PGD_2	Prostaglandina D_2
PGE_2	Prostaglandina E_2
$PGF_{2\alpha}$	Prostaglandina $F_{2\alpha}$
PGH_2	Prostaglandina H_2
PGI_2	Prostaciclina
PKA	Proteoquinase A
PKC	Proteoquinase C
PPAR	Receptor de ativação de proliferação de peroxissomo
PPAR-γ	Receptor de ativação de proliferação de peroxissomo gama

PPAR-α	Receptor de ativação de proliferação de peroxissomo alfa
PPAR-β/δ	Receptor de ativação de proliferação de peroxissomo beta/delta
RNAm	Ácido ribonucleico
RPM	Rotações por minuto
s.c.	Subcutânea
TGF-β	Fator de transformação do crescimento beta
TNF-α	Fator de necrose tumoral alfa
TXA_2	Tromboxano A_2

INTRODUÇÃO

A Artrite Reumatoide (AR) é uma poliartrite autoimune, caracterizada por hiperplasia sinovial e destruição das articulações, levando à dor, à perda da função articular e concomitante redução da qualidade de vida (Firestein, 2005; Németh & Mocsai, 2012). Considerando a natureza crônica e debilitante da doença, a AR é responsável por um ônus socioeconômico significativo com custos de recursos humanos, econômicos e de saúde (Dunlop *et al.*, 2003; Campbell, Lowe e Sleeman, 2011; Mclnnes & Schett, 2011).

Embora a etiologia da AR permaneça elusiva, fatores de susceptibilidade são evidentes envolvendo uma complexa interação entre genótipo, fatores ambientais, ação hormonal, desordens autoimunes e quadros infecciosos (Scott *et al.*, 2010; Mclnnes & Schett, 2011).

Tipicamente a doença inicia em pequenas articulações, principalmente interfalangianas proximais, metacarpofalangianas e metatarsofalangianas, evoluindo para articulações maiores como punhos, cotovelos, ombros, tornozelos, joelhos e quadris. Sendo uma articulação sinovial, a articulação temporomandibular (ATM) está sujeita às mesmas doenças que afetam outras articulações sinoviais, incluindo AR (Aliko *et al.*, 2011). A ATM é uma articulação sinovial bilateral composta por um côndilo mandibular e sua correspondente cavidade temporal (fossa glenoide e eminência articular), com a presença de um disco articular. A ATM e suas estruturas associadas desempenham um papel fundamental na orientação dos movimentos mandibulares e distribuição das tensões produzidas pelas tarefas diárias como a mastigação, deglutição e fala (Murphy *et al.*, 2013).

O acometimento da ATM nos pacientes com AR tem uma prevalência variável de 65-92,9% (Lin *et al.*, 2007; Aliko *et al.*, 2011) e os sintomas mais comumente relatados incluem dor na região da ATM, sensibilidade dos músculos mastigatórios, ruídos articulares e função da articulação limitada (Goupille *et al.*,

1993;. Lin *et al*., 2007). A magnitude do envolvimento da ATM está relacionado à severidade da AR. Os níveis do fator reumatoide, sedimentação de eritrócitos, proteína C-reativa e TNF-α estão correlacionados com a severidade das disfunções temporomandibulares decorrentes da AR (Celiker *et al*., 1995; Yoshida *et al*., 1998; Liu *et al*., 2003).

Embora sua prevalência seja alta, as disfunções temporomandibulares na AR são frequentemente ignoradas pelos reumatologistas ou pelos próprios pacientes, uma vez que, os tratamentos são centrados em outras articulações, além das limitações das técnicas de diagnóstico (Arabshahi & Cron, 2006). É importante ressaltar que a severidade das disfunções temporomandibulares na AR podem levar a sequelas graves, sendo assim o diagnóstico precoce assim como o manejo apropriado se faz necessário.

A patogênese da AR é caracterizada pela proliferação de células sinoviais e fibrose, formação de *pannus* e erosão da cartilagem e osso (Di Paola & Cuzzocrea, 2008). A sinovite ocorre quando os leucócitos se infiltram no compartimento sinovial. A migração dos leucócitos é possível pelo aumento da expressão de moléculas de adesão (integrinas e selectinas) e quimiocinas no endotélio dos microvasos sinoviais. Na AR, uma variedade de células efetoras inatas, incluindo macrófagos, mastócitos e *natural killers* encontram-se na membrana sinovial, enquanto que os neutrófilos residem principalmente no fluido sinovial, em contraste com a natureza acelular do fluido sinovial normal. Os macrófagos são efetores centrais na sinovite e atuam na liberação de citocinas inflamatórias, tais como o Fator de Necrose Tumoral alfa (TNF-α) e Interleucinas (IL) 1, 6, 12, 15, 18 e 23; na produção de prostanoides e enzimas degradadoras da matriz, na fagocitose e apresentação de antígeno (Mclnnes & Schett, 2011). Esta rede interdependente de citocinas, especialmente TNF-α e IL-1β, prostanoides e enzimas proteolíticas medeiam muitos dos processos imunológicos associados à patogênese da AR (Di Paola & Cuzzocrea , 2008; Sachs *et al.,* 2011).

O TNF-α é reconhecido como mediador de uma ampla variedade de funções efetoras relevantes para a patogênese da AR, incluindo a sua capacidade de induzir a liberação de outras (igualmente) citocinas pró-inflamatórias, , conduzindo ao acúmulo de leucócitos, osteoclastos e ativação de condrócitos, promovendo a destruição articular e sensibilização dos nociceptores (Brennan & McInnes, 2008). Em ratos, o TNF-α induz a hipernocicepção por duas vias independentes e paralelas: (1) O TNF-α induz a liberação da IL-1β e IL-6 resultando na liberação prostanoides; e (2) o TNF-α induz a liberação IL-8/CINC-1 resultando na liberação de aminas simpáticas (Cunha *et al.*, 2005; Verri *et al*., 2006).

A IL-6 desempenha um papel na imunidade adaptativa, estimulando a diferenciação das células B e diferenciação dos linfócitos T em células T auxiliares (Th1). Além disso, a IL-6 promove a sinovite pela indução da neovascularização, proliferação do *pannus*, resultando na infiltração de células inflamatórias e hiperplasia sinovial e induz a reabsorção óssea e degeneração da cartilagem articular (Md Yusof & Emery, 2013).

Tem sido demonstrado que as citocinas IL-18 e IL-12 também induzem a hipernocicepção inflamatória e possuem papel relevante na inflamação articular. A IL-12 é o principal estimulador da produção de interferon-gama (IFN-γ) e do desenvolvimento de células Th1, além disso, promove a ativação de células *natural killer* e liberação de citocinas (Asquith & McInnes, 2007; Pope & Shahrara, 2013). A IL-18 é capaz de induzir e sustentar a resposta articular de células Th1 e a liberação de múltiplas citocinas e quimiocinas, em particular o TNF-α. Ademais, em sinergia com a IL-12 e IL-15, induz a produção de IFN-γ (Verri *et al*., 2006). Corroborando com esses dados, um estudo utilizando modelo de artrite induzida por colágeno, evidenciou que a IL-18 ativa a produção de TNF-α, o qual induz a síntese de Leucotrieno B4 (LTB_4), que por sua vez atrai neutrófilos para o sítio inflamatório onde estes podem contribuir para a resposta inflamatória aguda e crônica (Canetti *et al*., 2003).

Por outro lado, a IL-10 foi a primeira citocina antinociceptiva descrita e é produzida por diversos tipos de células, tais como linfócitos, monócitos, macrófagos e mastócitos. Tem sido demonstrado que a IL-10 inibe a produção de citocinas pró-inflamatórias, incluindo a IL-1β e TNF-α, e a proliferação de células T *in vitro* (Isomäki & Punnonen, 1997). A IL-10 também pode inverter a degradação da cartilagem mediada por células mononucleares estimuladas por antígeno em pacientes com AR (van Roon *et al*., 1996).

Os eicosanoides (prostanoides, leucotrienos e lipoxinas) são metabólitos biologicamente ativos derivados do ácido araquidônico (AA). As prostaglandinas são produzidas pela ação da enzima ciclooxigenase utilizando-se do ácido araquidônico como substrato. Em condições fisiológicas o ácido araquidônico encontra-se esterificado em fosfolipídios de membrana sendo mobilizados durante o processo inflamatório pela fosfolipase A_2 que é ativada por estímulos químicos, mecânicos e produtos bacterianos. A cicloogenase (COX) (a COX-1 constitutivamente e a COX-2 induzida) catalisa a formação de prostaglandina H_2 (PGH_2), a qual é convertida por células específicas (várias sintases específicas para cada prostanoide) em produtos biologicamente ativos incluindo prostaglandinas (PG) E_2, $F_{2\alpha}$, I_2, D_2 e tromboxano A_2 (TXA_2)(Funk, 2001).

Gilroy *et al*. (1999) demonstraram que durante o início do processo inflamatório, no qual predominantemente encontramos polimorfonucleares, a COX-2 sintetiza a PGE_2, a qual possui um papel pró-inflamatório. Entretanto, após esta fase inicial, ocorre uma inversão de predominância celular na qual os mononucleares se destacam. Nesta segunda fase, aparentemente, a COX-2 participa da resolução da fase aguda da inflamação pela alternância do padrão de prostaglandina sintetizada, passando a estimular a produção de PG da família J.

Tem sido dada grande importância no papel da 15d-PGJ_2 na regulação do processo inflamatório (Jiang *et al*., 1998; Ricote *et al*., 1998a; Ricote *et al*., 1999; Willoughby *et al*., 2000; Clark *et al*., 2000), devido à possibilidade de intervenção farmacológica ser aplicada na regulação da produção de citocinas. A 15d-PGJ_2, que é um dos derivados do metabolismo da PGD_2, é um ligante natural

do receptor de ativação de proliferação de peroxissomo-γ (PPAR-γ) (Forman *et al.,* 1995; Ricote *et al.,* 1998b), o que sugere que esta molécula possui um importante papel na regulação da reação inflamatória *in vivo*.

O PPAR pertence à subfamília de receptores nucleares e consiste de três diferentes isoformas as quais são codificados por diferentes genes além de possuírem diferentes promotores. São eles: PPAR-α, PPAR-β/δ e PPAR-γ (Zhu *et al*., 1995). As três isoformas possuem padrões distintos em relação à distribuição celular e de tecidos. Inicialmente, sabia-se que o PPAR-γ era expresso em adipócitos e hepatócitos, porém atualmente, sabe-se que o PPAR-γ é encontrado em macrófagos/monócitos, miócitos, fibroblastos e células precursoras de medula (Braissant *et al.,* 1996).

Acredita-se que o PPAR-γ em estado inativado esteja conjugado com proteínas corepressoras, localizado no citoplasma ao invés do núcleo celular (Bishop-bailey & Hla, 1999). A ligação da 15d-PGJ_2 ao PPAR-γ , induz a dissociação do PPAR de seus repressores, e permite a interação com coativadores, o que resulta na translocação deste do citoplasma para o núcleo (Zhu *et al*., 1997). O resultado desta translocação é a ativação da expressão ou repressão de uma variedade de genes (Negishi & Katoh, 2002). Entretanto, a 15d-PGJ_2 aparentemente possui a capacidade de produzir efeitos de maneira independente ao PPAR (Chawla *et al*., 2001). Alguns destes efeitos podem ser mediados por interações covalentes entre a 15d-PGJ_2 e proteínas intracelulares. Duas moléculas já foram identificadas como mediadoras da 15d-PGJ_2 de maneira independente do PPAR: o Fator nuclear-κB (NF-κB) e a via de sinalização da quinase reguladora de sinal extracelular (ERK) (Scher & Pillinger, 2005).

Kawahito *et al*. (2000), observaram que após a administração intraperitoneal de ligantes de PPAR-γ, como a 15d-PGJ_2, a artrite induzida por adjuvante em ratos foi suprimida, além de reduzida a formação de *pannus* e o infiltrado inflamatório. Estudos com monócito/macrófago demonstraram que a 15d-PGJ_2 pode ser a responsável pela inibição de genes que codifica IL-1β, TNF-α,

iNOS e metaloproteinase 2 (Jiang *et al.,* 1998; Ricote *et al*., 1998a). Estas observações vislumbram a possibilidade da 15d-PGJ_2 ser um potencial composto terapêutico para o tratamento de doenças inflamatórias. Entretanto, outros estudos não conseguiram demonstrar o efeito inibitório da 15d-PGJ_2 com relação a expressão de TNF-α e IL-6 em culturas de monócitos/macrófagos (Thieringer *et al*., 2000). Em estudo realizado utilizando o rosiglitazone, um agonista sintético do PPAR-γ, os autores não conseguiram reduzir a secreção de IL-8 após estimulação com LPS em linhagem celular monocítica, mas observaram a diminuição da expressão de MMP-9. Além disso, a 15d-PGJ_2 induziu a expressão do gene de IL-8 e suprimiu o MCP-1 (Zhang *et al*, 2001).

A 15d-PGJ_2 apresenta um potente efeito antinociceptivo e anti-inflamatório na ATM (Pena-dos-santos *et al*., 2009) por ativação dos receptores PPAR-γ. Esses resultados sugerem que o efeito antinociceptivo da 15d-PGJ_2 na ATM é também mediado pelos receptores opioides kappa e delta com ativação da via intracelular L-Arginina/ON/cGMP/K^+_{ATP}. As propriedades farmacológicas da administração periférica da 15d-PGJ_2 descritas, sugerem o uso em potencial deste agonista de receptores PPAR-γ nas condições inflamatórias da ATM.

Considerando o exposto acima, o desenvolvimento do presente estudo demonstra, em dois capítulos, o efeito analgésico e anti-inflamatório da 15d-PGJ_2 no processo inflamatório induzido pela AR na ATM de ratos.

CAPÍTULO 1: The indirect antinociceptive mechanism of 15d-PGJ_2 on rheumatoid arthritis-induced TMJ inflammatory pain in rats.

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The indirect antinociceptive mechanism of 15d-PGJ_2 on rheumatoid arthritis-induced TMJ inflammatory pain in rats.

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Conflicts of interest

None declared.

Abstract

Background

Inflammation of the temporomandibular joint (TMJ) induced by rheumatoid arthritis (RA) have resulted in persistent pain and caused distress to many patients. Considering that not all patients respond to traditional drugs therapy to RA and it has demonstrated that 15-deoxy-$^{\Delta 12,14}$-prostaglandin J_2 (15d-PGJ_2) into TMJ has a potential peripheral antinociceptive effect, the aim of this study was to evaluate the peripheral effect of 15d-PGJ_2 in RA-induced TMJ inflammatory hypernociception.

Methods

Antigen-induced arthritis (AIA) was generated in rats with methylated bovine serum albumin (mBSA). RA-induced TMJ hypernociception was assessed by measuring the behavioural nociceptive responses. After behavioural experiments, the animals were terminally anaesthetized and periarticular tissues were removed and homogenized. The supernatants were used to evaluate the levels of tumour necrosis factor (TNF)-α, interleukin (IL)-1β and keratinocyte-derived chemokine (KC) by enzyme-linked immunosorbent assay as well the expression of PKCε and PKA by western blotting analysis.

Results

The intra-articular injection of mBSA, but not phosphate buffered saline (control), in immunized rats induced dose- and time-dependent behavioural nociceptive responses in which the peak of nociceptive responses were obtained by using 10 µg/TMJ of mBSA after 24 h. Pretreatment with 15d-PGJ_2 (30, 100 and

300 ng/TMJ) inhibited the RA-induced TMJ inflammatory hypernociception. In addition, 15d-PGJ_2 reduced the RA-induced release of TNF-α, IL-1β and KC ($p < 0.05$) as well the expression of PKA and PKCε ($p < 0.05$).

Conclusions

In the present study, we demonstrated that 15d-PGJ_2 was able to reduce the RA-induced TMJ inflammatory hypernociception by an indirect mechanism. This antinociceptive effect is in part due to decrease of TNF-α, IL-1β and KC levels and PKA/PKCε expression in the TMJ.

1. Background

Rheumatoid arthritis (RA) is a chronic and progressive inflammatory disorder characterized by synovitis and severe joint destruction (Di Paola and Cuzzocrea, 2008). Being a synovial joint, the temporomandibular joint (TMJ) is subject to the same disorders affecting other synovial joints, including RA (Aliko et al., 2011). There was a high prevalence of temporomandibular disorders in RA patients from 67 to 92.9% (Lin et al., 2007; Twilt et al., 2008; Aliko et al., 2011) in which the most common clinical findings are pain in the TMJ area, tenderness of the masticatory muscles, joint sounds and limited joint function (Goupille et al., 1993; Lin et al., 2007).

The pathogenesis of RA is a complex process, involving synovial cell proliferation and fibrosis, *pannus* formation and cartilage and bone erosion. This process is mediated by an interdependent network of cytokines, prostanoids and proteolytic

enzymes. Pro-inflammatory cytokines, such as interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) are central mediators in RA (Brennan and McInnes, 2008; Di Paola and Cuzzocrea, 2008; Sachs et al., 2011). Particularly, it is well established that cytokines, such as TNF-α, IL-1β, IL-6 and the chemokines IL-8, chemokine-induced neutrophil chemoattractant-1 and keratinocyte-derived chemokine (KC), trigger the release of prostanoids or sympathetic amines that act directly on the nociceptors to cause hypernociception (Verri et al., 2006). Drug therapy for RA rests on two principal approaches: symptomatic treatment with non-steroidal anti-inflammatory drugs and disease-modifying anti-rheumatic drugs (Di Paola and Cuzzocrea, 2008). However, a proportion of patients exhibit only partial responses to such agents, and the rates of long-term remission achieved remain too low (Hueber et al., 2010).

Peroxissome proliferators-activated receptor-γ (PPAR-γ) is a ligand-activated transcription factor of nuclear hormone receptor superfamily (Escher and Wahli, 2000). PPAR ligands represent a promising therapeutic strategy for inflammatory diseases (Cuzzocrea et al., 2003; Shan et al., 2004; Kaplan et al., 2005; Chima et al., 2008; Napimoga et al., 2008a,b; Pena-dos-Santos et al., 2009; Alves et al., 2011), and in particular, PPAR-γ agonists are extremely neuroprotective in animal models of acute central nervous system injury (Sundararajan et al.,2005; Zhao et al., 2005, 2006; Collino et al., 2006; Pereira et al., 2006; McTigue et al., 2007; Park et al., 2007; Tureyen et al., 2007; Hyong et al., 2008). Considering the neuroprotective effect of PPAR-γ agonists,

previously, we demonstrated that 15-deoxy-$^{\Delta 12,14}$-prostaglandin J_2(15d-PGJ_2), a natural ligand for PPAR-γ (Schoonjans et al., 1997; Ricote et al., 1998), has a potential peripheral antinociceptive effect in the TMJ via PPAR-γ with the co-participation of κ/δ opioid receptors (Pena-dos-Santos et al., 2009). Inflammation of the TMJ induced by RA have often resulted in persistent pain and caused distress to many patients. Considering that not all patients respond to traditional drugs therapy to RA and our previous data showing that the administration of 15d-PGJ_2 into the TMJ has antinociceptive effect, in this study, we evaluated the peripheral effect of 15d-PGJ_2 in RA-induced TMJ inflammatory pain in rats as well as its mechanisms of action.

What's already known about this topic?

- Inflammation of the temporomandibular joint (TMJ) induced by rheumatoid arthritis (RA) result in pain. A proportion of patients exhibit partial responses to conventional therapy for RA.

What does this study add?

- The 15-deoxy-$^{\Delta 12,14}$-prostaglandin J_2 (15d-PGJ_2) induces peripheral antinociceptive effect in the RA-induced TMJ pain and this antinociceptive effect involves inhibition of tumour necrosis factor-α, interleukin-1β and keratinocyte-derived chemokine (KC) levels. In addition, 15d-PGJ_2 decrease Protein Kinase A (PKA) and protein kinase C (PKC)ε expression of the RA-induced TMJ.

2. Methods

2.1 Animals

This study was carried out with male Wistar rats obtained from the Multidisciplinary Center for Biological Research (CEMIB) at the University of Campinas (Campinas, Sao Paulo, Brazil) (150–250 g) maintained in a temperature-controlled room (23° ± 1 °C) with a 12-hour light–dark cycle. All experiments were conducted in accordance to the International Association for the Study of Pain guidelines on using laboratory animals for investigations of experimental pain in conscious animals (Zimmermann, 1983). All animal experimental procedures and protocols were approved by the Committee on Animal Research of the University of Uberaba (no. 059/2009). The animals suffering and number per group were kept at a minimum and each animal was used once.

2.2 Induction of experimental arthritis

The protocol used to induce the experimental arthritis was modified from previous work (Vieira et al., 2009). Briefly, male Wistar rats were sensitized with 500 µg of methylated bovine serum albumin (mBSA) (Sigma-Aldrich, St. Louis, MO, USA) in 200 µL of an emulsion containing 100 µL phosphate buffered saline (PBS) and 100 µL Freund's complete adjuvant (CFA) (Sigma-Aldrich) administered by subcutaneous injection in the back. Booster injections of mBSA dissolved in Freund's incomplete adjuvant (IFA) (Sigma-Aldrich) were given 7 and 14 days after the first immunization in different sites in the back of the rat. Non-immunized rats

received similar injections but without the antigen (mBSA). Twenty-one days after the initial injection, TMJ-arthritis was induced in the immunized animals by intra-articular injection of mBSA (1, 3 or 10 µg/cavity) dissolved in 10 µL of PBS. Non-immunized and immunized rats were challenged with mBSA or with PBS.

2.3 Testing procedure for TMJ hypernociception

Testing sessions took place during light phase (between 9:00 a.m. and 5:00 p.m.) in a quiet room maintained at 23 °C. Each animal was manipulated for 7 days to be habituated to the experimental manipulation. After this period, the animal was placed in a test chamber (30 × 30 × 30 cm mirrored wood chamber with a glass at the front side) for 15 min habituation period to minimize stress. The animals were briefly anaesthetized by inhalation of halothane to allow the TMJ injection, which was performed with a 30-gauge needle connected to a 50-µl Hamilton syringe (Roveroni et al., 2001). Each animal regained consciousness approximately 30 s after discontinuing the anaesthetic and was returned to the test chamber for counting nociceptive responses. The nociceptive response score was defined as the cumulative total number of seconds that the animal spent rubbing the orofacial region asymmetrically with the ipsilateral fore or hind paw plus the number of head flinches counted during the observation period as described previously. Since head flinches followed a uniform pattern of 1 s of duration, each flinch was expressed as 1 s. Results are expressed as the duration time of nociceptive behaviour (Roveroni et al., 2001; Clemente et al., 2004). A different investigator performed each test,

prepared the solution and administered the TMJ injections. All animals received a final volume of 30 µl into TMJ. All experiments were conducted in a double-blind fashion in which the person who injected the solutions was different of the one who made the behavioural assessment.

2.4 Experimental design

2.4.1 RA-induced chemical hypernociception in the TMJ

RA-induced TMJ hypernociception was assessed by measuring behavioural nociceptive responses induced by intra-articular injection of a low dose of formalin (0.5%) into the TMJ. At day 21 after RA induction, the immunized rats were challenged by an intra-articular injection of 10 µg of mBSA. Twenty-four hours later, 15d-PGJ_2 (15 µL/TMJ) was injected, and after 15 min, an additional injection of formalin (15 µL/TMJ) was administered. Immediately after the formalin injection, the behavioural nociceptive response was evaluated for a 45 min observation period as described above. The behavioural nociceptive response measured was used as a quantitative measurement of RA-induced TMJ hypernociception. Formalin solution was prepared from commercially stock formalin (an aqueous solution of 37% of formaldehyde – Sigma-Aldrich) and further diluted in 0.9% NaCl. The experimental design is summarized in Fig. 1.

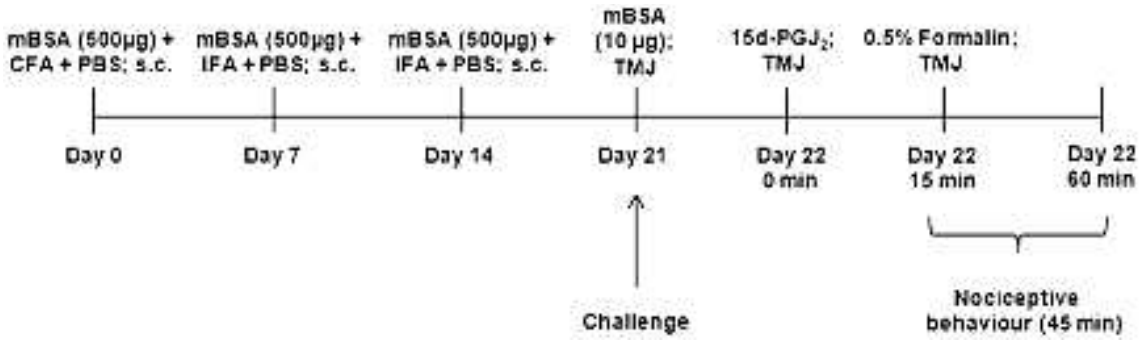

Figure 1. Experimental design of RA-induced TMJ inflammatory hypernociception.

2.4.2 Effect of the 15d-PGJ_2 on TMJ-arthritis hypernociception

The rats were pretreated (15 min) with an intra-TMJ injection of 15d-PGJ_2 (Calbiochem, San Diego, CA, USA) (30, 100 or 300 ng; $n = 6$; 15 µl/TMJ) followed by ipsilateral intra-TMJ injection of 0.5% formalin in a final volume of 30 µl. Behavioural nociception response was evaluated for a 45-min observation period as described above.

2.5 Protein extraction from TMJ periarticular tissue

The TMJ tissue was removed after the 45-min behavioural nociceptive, frozen with liquid nitrogen crushed in a mortar and pestle then solubilized in 500 µl of the appropriate buffer containing protease inhibitors (Sigma-Aldrich) followed by a centrifugation of 10 min/10,000 g. The total amount of extracted proteins was colorimetric measured using the micro bicinchoninic acid (BCA) protein assay kit (Thermo, Rockford, IL, USA). The supernatants were stored at −70 °C until further analysis.

2.5.1 Effect of the 15d-PGJ_2 on RA-induced released of cytokines TNF-α, IL-1β and chemokine KC in the TMJ tissue

Levels of IL-1β, TNF-α (RD Systems, Minneapolis, MN, USA) and KC (Peprotech, Rocky Hill, NJ, USA) were quantified in the rats' TMJ tissues by enzyme-linked immunossorbend assay (ELISA). Briefly, the supernatant obtained from the TMJ periarticular tissue was used for ELISA assay, with the results expressed as picograms per milligram of tissue.

2.5.2 Effect of the 15d-PGJ_2 on RA-induced expression of PKA and PKCε in the TMJ

PKA and PKCε expressions were quantified in the rats' TMJ tissues by using the supernatant obtained from the TMJ periarticular tissue. For western blotting analysis, aliquots containing 80 µg of total protein were boiled in loading Laemmli buffer (Bio-Rad, Hercules, CA, USA), thereafter; each aliquot was loaded onto a 10% polyacrylamide gel. After electrophoresis separation, proteins were transferred to a nitrocellulose membrane (Bio-Rad). Membrane was blocked in Tris-Buffered Saline and Tween 20 (TBST; 20 mM Tris-HCL, 150 mM NaCl and 0.1% Tween 20) containing 5% non-fat dry milk overnight at 4 °C, followed by incubation with PKA (sc-48412), PKCε (sc-214) or α-tubulin (sc-5286) (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at room temperature, rinsed six times with TBST and then incubated for 1 h with the respective IgG peroxidase conjugate (1:1000; Sigma-Aldrich). Membrane was visualized using

chemiluminescent reagents (ECL, Thermo Fisher Scientific, Rockford, IL, USA) for 60 s then exposed to X-ray film (Kodak, Windsor, CO, USA) in a dark room. Films were scanned into Image Quant 5.2 for analysis (GE Healthcare, Piscataway, NJ, USA). Banding specificity was determined by omission of primary antibody from the western blot protocol. To compensate for any differences in the amount of loaded protein, the intensity of the PKA and PKCε bands were divided by the intensity of α-tubulin bands for each sample.

2.5 Statistical analysis

To determine if there were significant differences ($p < 0.05$) among treatment groups, the data were analysed using one-way analysis of variance (ANOVA) as appropriate. If there was a significant among-subjects main effect of treatment group following one-way ANOVA, post hoc contrasts, using the Tukey test, were performed to determine the basis of the significant difference. Data are presented in figures as means ± standard deviation.

3. Results

3.1 RA-induced TMJ hypernociception

To demonstrate RA-induced TMJ hypernociception in rats, we applied a low dose of formalin into TMJ challenged by an intra-articular injection of mBSA or PBS as previously described. Under this condition, the intra-articular injection of mBSA, but not PBS (control), in the immunized rats induced dose- and time-dependent nociceptive behavioural response (Fig. 2A and B, respectively). The mBSA

challenge induced a significant nociceptive behavioural response at 12 h, which peaked at 24 h ($p < 0.05$) and declined 48 h after challenge (Fig. 2B). According to this experiment, we established the dose of the mBSA challenge at 10 µg/TMJ and the higher nociceptive behavioural response after 24 h of the challenge. These parameters were used in the next experiments.

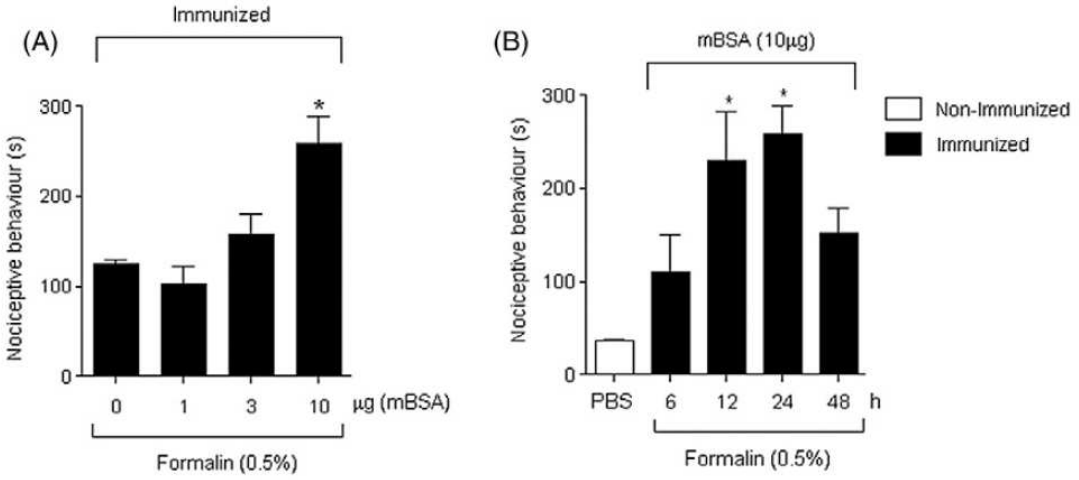

Figure 2. Rheumatoid arthritis-induced TMJ hypernociception. (A) The nociceptive behavioural response induced by an intra-articular injection of formalin (0.5%) previously challenged with an intra-articular injection of mBSA (10 but not 1 and 3 µg) of immunized rats was significantly greater than that no-immunized rats + intra-articular injection of PBS (column 1). The symbol (*) indicates the statistical difference ($p < 0.05$, ANOVA, Tukey test) than PBS (control) group. (B) The nociceptive behavioural response induced by an intra-articular injection of formalin (0.5%) previously challenged with an intra-articular injection of mBSA (10 µg) of immunized reaching the peak at 24 h after the mBSA injection. Results are expressed as mean ± SD of six animals per group. The symbol (*) indicates the significant highest response ($p < 0.05$, ANOVA, Tukey test).

3.2 15 d-PGJ_2 inhibits RA-induced TMJ hypernociception

Pretreatment with an intra-articular injection of 15d-PGJ_2 (30, 100 or 300 ng/TMJ) inhibited the RA-induced TMJ hypernociception. These findings indicate that 15d-PGJ_2 was able to reduce the inflammatory episode evoked by RA into TMJ that contributes for hypernociception progress. Important, nociceptive behaviour was observed in all animals that receive an intra-articular injection of low concentration of formalin (0.5%); however, formalin-induced nociception (0.5%) of immunized animals challenged with mBSA (column 5; 10 µg/TMJ 24 h before) was significantly higher than that other groups ($p < 0.05$). The statistical analysis has shown no difference among groups that were treated with different concentrations of 15d-PGJ_2 (columns 6, 7 and 8; Fig. 3). According to these results, which is in agreement with our previous study while showing that 15d-PGJ_2 (100 ng/TMJ) has a potential antinociceptive effect in the TMJ (Pena-dos-Santos et al., 2009), we established for the next experiments 100 ng/TMJ as the ideal concentration of 15d-PGJ_2.

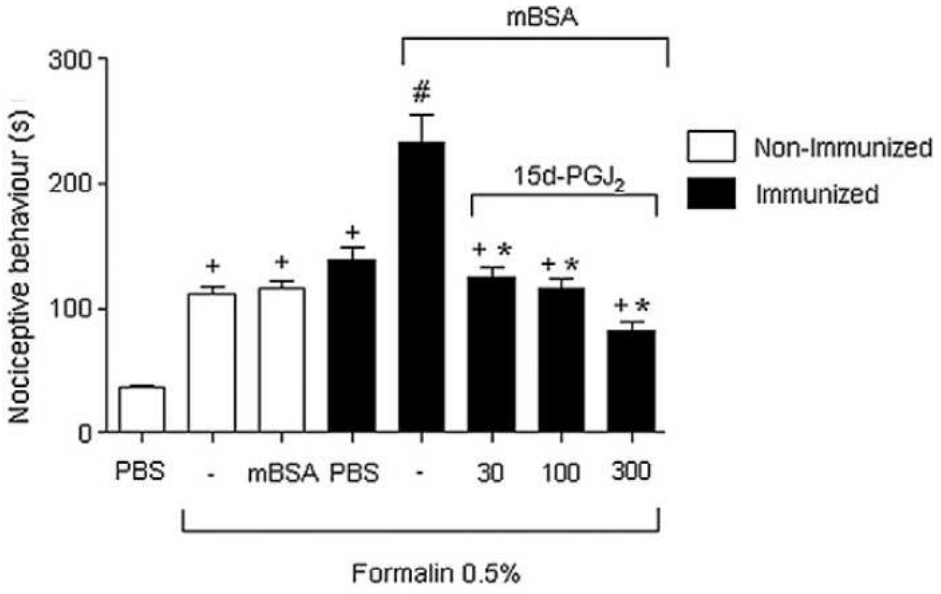

Figure 3. 15d-PGJ_2 inhibits RA-induced TMJ hypernociception. (A) Pretreatment (15 min) with 15d-PGJ_2 (30, 100 or 300 ng/TMJ) significantly reduced RA-induced TMJ hypernociception. Statistical analysis demonstrated no difference among different concentrations of 15d-PGJ_2 treatment. No statistical difference was observed in the nociceptive behavioural response induced by an intra-articular injection of formalin (0.5%) among no-immunized group, no-immunized + intra-articular injection of mBSA (10 µg) and immunized + intra-articular injection of PBS. The symbol (+) indicates a significantly higher nociceptive behaviour response ($p < 0.05$, ANOVA, Tukey test) than that no-immunized rats + intra-articular injection of PBS (column 1). Results are expressed as mean ± SD of six animals per group. The symbol (#) indicates a significantly higher nociceptive behaviour response ($p < 0.05$, ANOVA, Tukey test) than other groups. The symbol (*) indicates a significantly lower nociceptive behaviour than that immunized rats + intra-articular injection of mBSA group (column 5; $p < 0.05$, ANOVA, Tukey test).

3.3 15 d-PGJ$_2$ reduced TNF-α, IL-1β and KC levels in RA-induced in the TMJ

Data show that pretreatment (15 min) with 15d-PGJ$_2$ significantly reduces the amount of TNF-α; (Fig. 4A), IL-1β (Fig. 4B) and KC (Fig. 4C) induced by mBSA challenge in immunized rats.

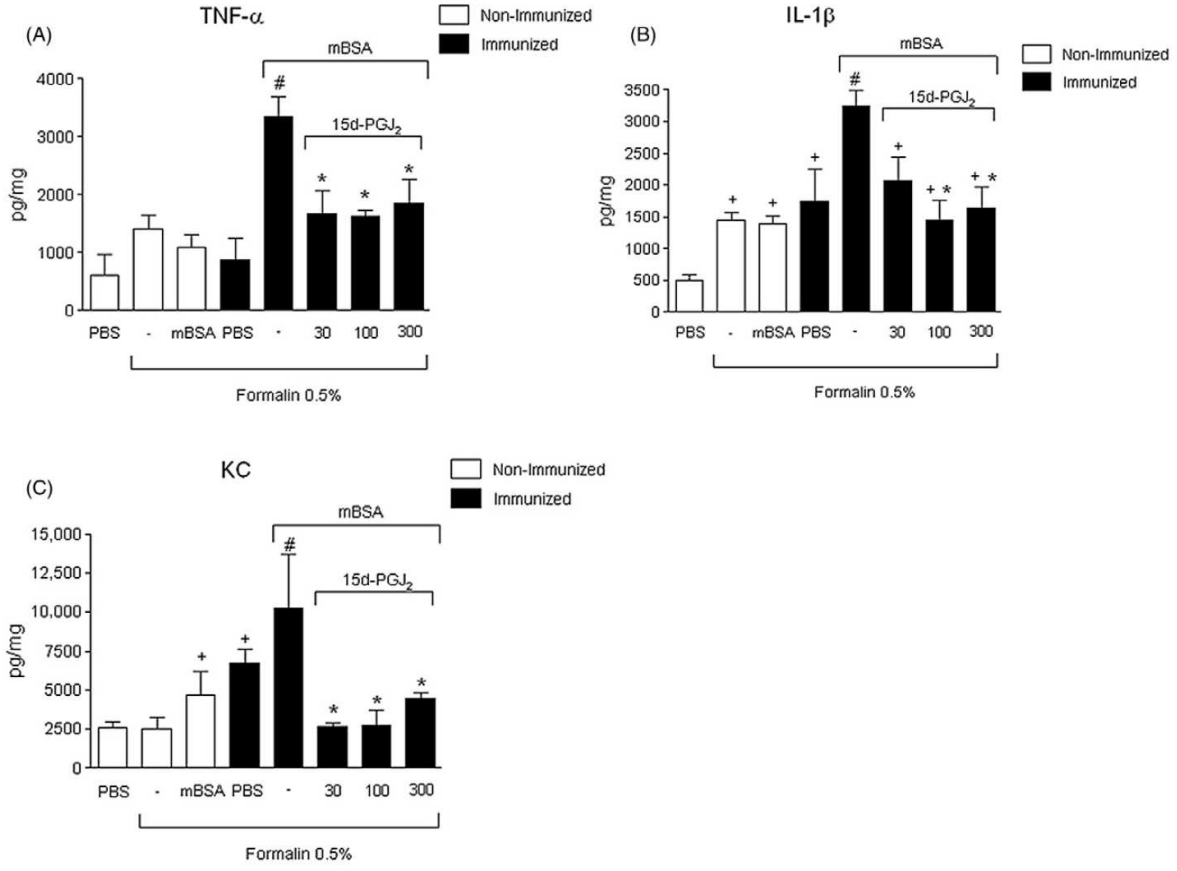

Figure 4. 15d-PGJ$_2$ reduces TNF-α, IL-1β and KC levels in RA-induced in the TMJ. (A) RA-induced in the TMJ significantly increase the release of TNF-α. Pretreatment (15 min) with an intra-articular injection of 15d-PGJ$_2$ (30, 100 or 300 ng/TMJ) significantly reduced the release of TNF-α in RA-induced in the TMJ ($p < 0.05$, ANOVA, Tukey test). Statistical analysis demonstrated no difference among groups that received intra-articular injection of 15d-PGJ$_2$30, 100 or 300 ng/TMJ. No significant difference was observed among non-immunized groups and immunized rats + intra-articular injection of PBS. (B) RA-induced in the TMJ significantly increase the release of IL-1β. Pretreatment (15 min) with an intra-articular injection of 15d-PGJ$_2$ (100 and 300 but not 30 ng/TMJ) significantly reduced the release of IL-1β in RA-induced in the TMJ plus intra-articular injection of

0.5% formalin ($p < 0.05$, ANOVA, Tukey test). Statistical analysis demonstrated no difference between groups that receive intra-articular injection of 15d-PGJ_2 at 100 or 300 ng/TMJ. (C) RA-induced in the TMJ significantly increase the release of KC. Pretreatment (15 min) with an intra-articular injection of 15d-PGJ_2 (30, 100 or 300 ng/TMJ) significantly reduced the release of KC in RA-induced in the TMJ ($p < 0.05$, ANOVA, Tukey test). Statistical analysis demonstrated no difference between groups that receive intra-articular injection of 15d-PGJ_2 30, 100 or 300 ng/TMJ. The symbol (#) indicates a significantly higher level of TNF-α, IL-1β or KC ($p < 0.05$, ANOVA, Tukey test) than that other groups. The symbol (+) indicates a significantly higher level of TNF-α, IL-1β or KC ($p < 0.05$, ANOVA, Tukey test) than that no-immunized rats + intra-articular injection of PBS (column 1).The symbol (*) indicates statistical significance ($p < 0.05$, ANOVA, Tukey test) compared to immunized rats + intra-articular injection of mBSA + intra-articular injection of 0.5% formalin group. Results are expressed as mean ± SD of six animals per group.

3.4 15 d-PGJ_2 inhibits PKA and PKCε expression in RA-induced in the TMJ

Western blotting analysis demonstrated a significantly higher expression of protein kinases PKA and PKCε induced by mBSA challenge in immunized rats. Pretreatment (15 min) with 15d-PGJ_2 (100 ng/TMJ) in RA induced in the TMJ was able to statistically reduce the expression of PKA (Fig. 5) as well as the expression of PKCε (Fig. 6).

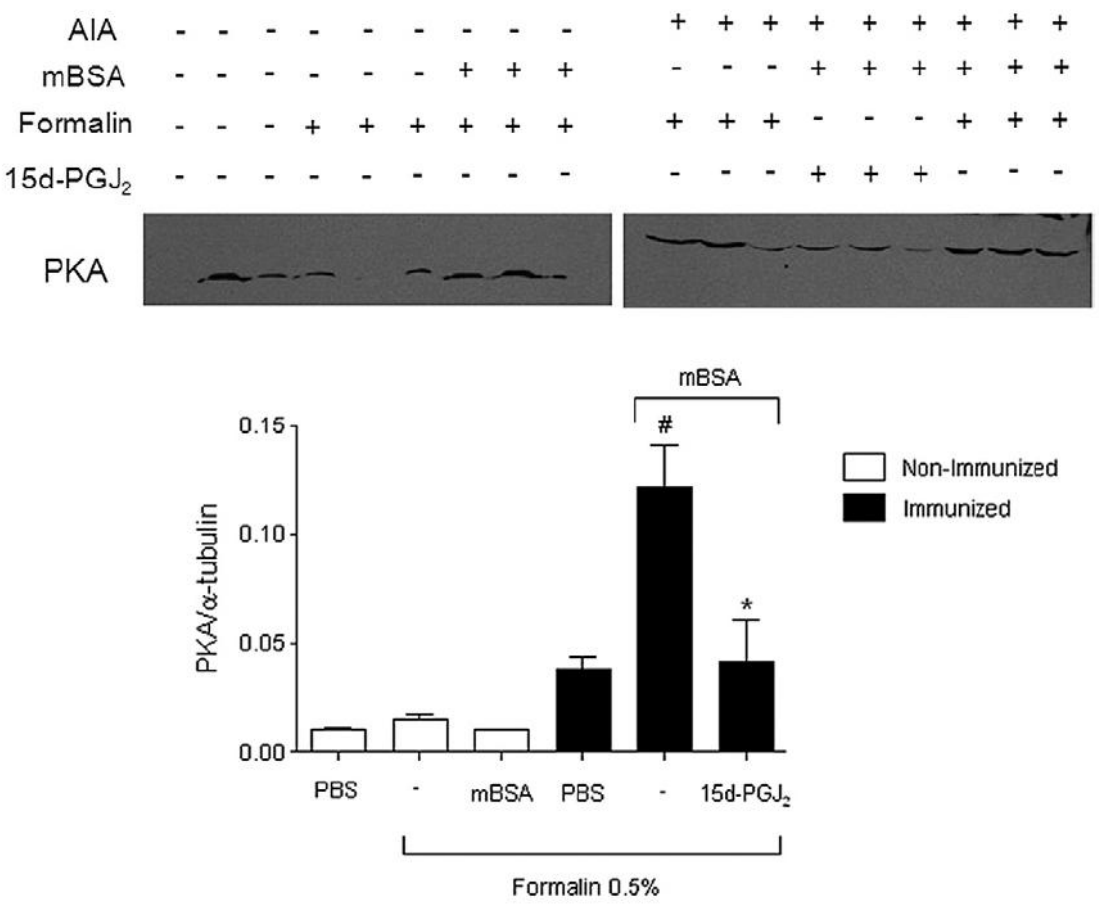

Figure 5. 15d-PGJ_2 inhibits RA-induced TMJ PKA expression. RA-induced in the TMJ significantly increase the expression of PKA ($p < 0.05$, ANOVA, Tukey test). Pretreatment (15 min) with an intra-articular injection of 15d-PGJ_2 (100 ng/TMJ) significantly reduce RA-induced TMJ PKA expression. Results are expressed as mean ± S.D of 6 animals per group. The symbol (#) indicates a significantly higher expression of PKA ($p < 0.05$, ANOVA, Tukey test) than that other groups. The symbol (*) indicates a significantly lower expression of PKA than that immunized rats + intra-articular injection of mBSA + intra-articular injection of 0.5% formalin group ($p < 0.05$, Tukey test).

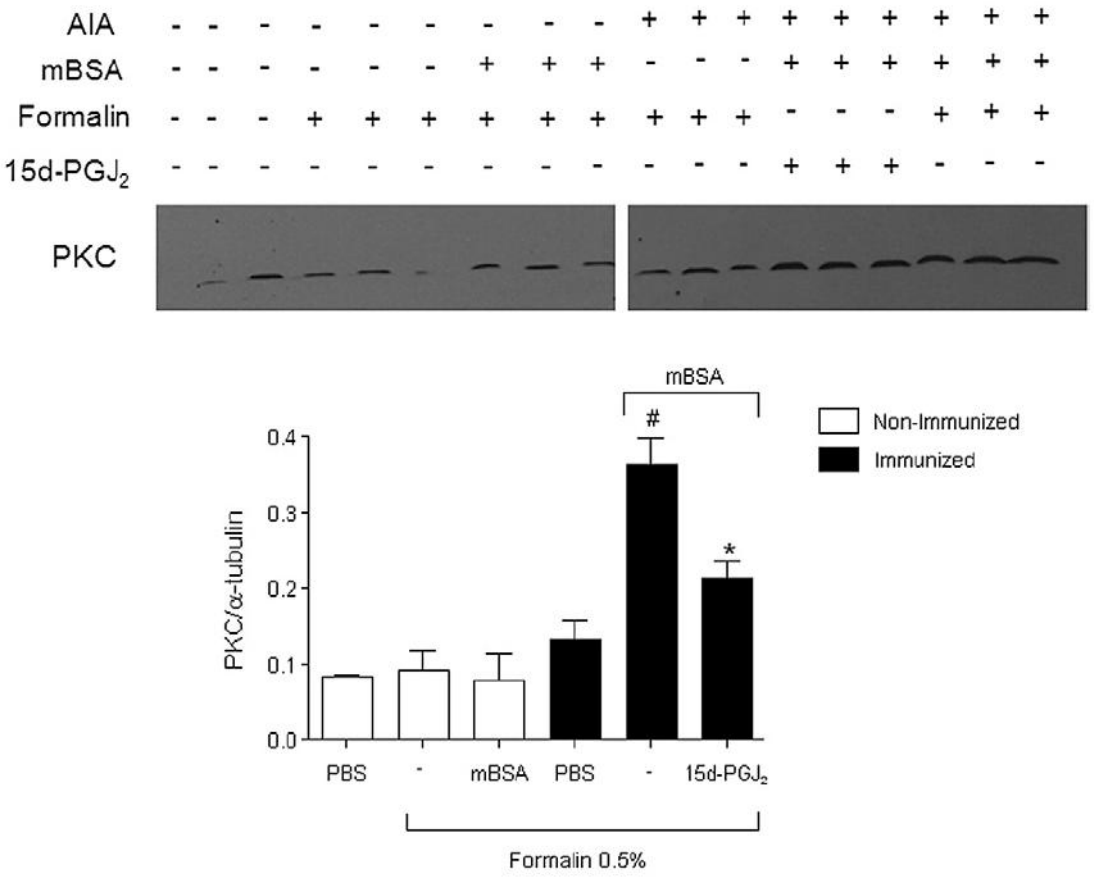

Figure 6. 15d-PGJ_2 inhibits RA-induced TMJ PKCε expression. RA-induced in the TMJ significantly increase the expression of PKCε ($p < 0.05$, ANOVA, Tukey test). Pretreatment (15 min) with an intra-articular injection of 15d-PGJ_2(100 ng/TMJ) significantly reduce RA-induced TMJ PKCε expression. Results are expressed as mean ± S.D of 6 animals per group. The symbol (#) indicates a significantly higher expression of PKCε ($p < 0.05$, ANOVA, Tukey test) than that other groups. The symbol (*) indicates a significantly lower expression of PKCε than that immunized rats + intra-articular mBSA + 0.5% formalin group.

4. Conclusions

Arthritis refers to a group of rheumatic diseases and other conditions that are characterized as a chronic and progressive inflammatory disorder that can cause pain, stiffness and swelling in the joint. In the present study, we employed a behavioural model that allows investigating the RA-induced TMJ inflammatory hypernociception following antigen challenge and the pharmacological activity of 15d-PGJ_2in this process. To evaluated RA-induced TMJ inflammatory hypernociception, it was applied a low dose of formalin into the TMJ challenged by the injection of antigen (mBSA) in immunized rats. Under this condition, the behavioural nociceptive response was used as a quantitative measurement of RA-induced TMJ hypernociception. The intra-articular injection of antigen (mBSA) induced a dose- and time-dependent hypernociception response, which peaked at 24 h after the challenge and decline thereafter 48 h later. This response was an antigen-specific immune reaction because the same injection of mBSA did not induce hypernociception in non-immunized rats. Furthermore, the 15d-PGJ_2 treatment was able to reduce the RA hypernociception due to decrease of endogenous release of cytokines TNF-α and IL-1β and the chemokine KC as well as by down-modulating the PKA and PKCε expression.

We used a model of delayed-type hypersensitivity (DTH) with mBSA as antigen. DTH is an inflammatory reaction mediated by effector memory T lymphocytes that infiltrate the site of injection of an antigen against, which the immune system has been primed (Hadden,1994). Corroborating our data regarding

the inflammatory reaction to antigen challenge, it is well accepted that activation of immune system leads to the release of pro-inflammatory cytokines and chemokines (Brennan and McInnes, 2008; Di Paola and Cuzzocrea, 2008; Grespan et al., 2008), which, in turn, leads to the release of a series of final inflammatory mediators that were responsible for the sensitization of nociceptors, a common denominator of inflammatory pain and represents a functional up-regulation of nociceptors that leads to a state known as hypernociception (Cunha et al., 2005; Verri et al., 2006). Particularly, in the present study, it was demonstrated that RA-induced TMJ evoke the release of cytokines TNF-α and IL-1β, and the chemokine KC. Thus, it is possible to suggest that RA-induced TMJ inflammatory hypernociception is due to the release of these inflammatory cytokines and chemokines.

The cytokine TNF-α is recognized as mediating a wide variety of effector functions relevant to the pathogenesis of RA, including its ability to induce a production of other (equally) pro-inflammatory cytokines, including IL-1β and IL-6, leading to leukocyte accumulation, osteoclast and chondrocyte activation, promoting articular destruction and nociceptor sensitization (Brennan and McInnes, 2008). Interestingly, blockade of TNF-α partially decreased IL-1β production, and blockade of IL-1β partially decreased TNF-α production, suggesting that these cytokines facilitate the production of each other in the context of antigen-induced arthritis (Sachs et al., 2011). Furthermore, it is well established that TNF-α induces hypernociception in rats via two independent and

parallel pathways: TNF-α-induced hypernociception via IL-1β/IL-6 by prostanoids and TNF-α-induced hypernociception via IL-8/KC by sympathetic amines (Cunha et al., 2005; Verri et al., 2006). Thus, it has been shown that the inflammatory state of the TMJ results in the release of several pro-inflammatory cytokines, in particular TNF-α and ILs (Kopp, 2001; Vernal et al., 2008), which contribute to inflammatory TMJ hypernociception by the release of inflammatory mediator such as prostaglandin E_2, serotonin (5-HT) and sympathomimetic amines (Kopp, 2001; Rodrigues et al., 2006). The results of the present investigation show that peripheral application of 15d-PGJ_2 into TMJ was able to reduce RA-induced endogenous release of TNF-α, IL-1β and KC, and consequently, the RA-induced hypernociception. Thus, considering that the TNF-α is one of the most important cytokine in the hypernociceptive pathway, and our results in which the pretreatment into TMJ with 15d-PGJ_2 was able to inhibit the RA-induced hypernociception concomitantly to a reduction of TNF-α, IL1-β and KC release, it is possible to suggest that the peripheral antinociceptive effect of 15d-PGJ_2 in TMJ inflammatory pain conditions involve an indirect mechanism mediated by the inhibition of TNF-α-induced hypernociception pathways.

It is well known that 15d-PGJ_2 is a natural ligand for PPAR-γ (Schoonjans et al., 1997; Ricote et al., 1998). Several *in vitro* studies demonstrated that pharmacological activation of PPAR-γ by 15d-PGJ_2 produces anti-inflammatory effects, such as repression of the expression of several inflammatory response genes in activated macrophages including the genes encoding TNF-α, gelatinase B

and cyclooxygenase-2 (Jiang et al., 1998; Ricote et al., 1998) as well as the inhibition of neutrophil recruitment (Napimoga et al., 2008a). Interesting, there are some data demonstrating that endogenous 15d-PGJ_2 is generated at concentrations sufficient to promote inflammatory resolution (Gilroy et al., 2004; Díez-Dacal and Pérez-Sala, 2010). Thus, altogether, these findings arise the possibility of using 15d-PGJ_2 as novel treatment protocols for acute and chronic inflammatory diseases that involve activated macrophages and neutrophils, such as atherosclerosis and rheumatoid arthritis. Otherwise, we have previously provided evidence that activation of PPAR-γ into TMJ by 15d-PGJ_2 induced a peripheral antinociceptive effect with the co-participation of κ/δ opioid receptors mediated by the activation of the intracellular L-arginine-NO/cGMP/K^+_{ATP} on primary nociceptive neurons from TMJ (Pena-dos-Santos et al., 2009). Thus, taken together, these findings suggest that 15d-PGJ_2-antinociceptive activity is driven by two different mechanisms: direct mechanism that involves activation of κ/δ opioid receptors following the activation of the intracellular L-Arginine-NO/cGMP/K^+_{ATP} on primary nociceptive neurons from TMJ (Pena-dos-Santos et al., 2009) and indirect mechanism that involves inhibition of TNF-α-induced hypernociception intracellular cascade (as demonstrated by our present findings).

Complaining these results, it is well established that inflammatory mediators induced phosphorylation of protein kinases that contribute to the lowering of the nociceptor threshold. In particular, it has been suggested that prostanoids such as PGE_2 (Sachs et al., 2009; Villarreal et al., 2009) and sympathetic amines induce

sensitization of the nociceptors in part by the activation of cAMP-dependent PKA and PKCε (Gold et al., 1998; Khasar et al., 1999a,b). Recent data from literature showed that both PKA and PKCε participate in acute mechanical hypernociception downstream from PGE_2 receptor and suggest that PKA may activate PKCε (Sachs et al., 2009). Thus, we tested the hypothesis that RA-induced TMJ inflammatory hypernociception also has been related to a higher expression of PKA and PKCε. In this way, our results demonstrated that RA-induced TMJ hypernociception increases the expression of both PKA and PKCε. Furthermore, the 15d-PGJ_2 treatment also significantly reduced the expression of PKA and PKCε in RA-induced hypernociception.

Experimentally, the peripheral pharmacological control of inflammatory pain is based on two main strategies. The first is the use of drugs that prevent the nociceptor sensitization, and second, drugs that prevent the development of hypernociception (Napimoga et al., 2008b). In the present study, we provided evidence that peripheral administration of 15d-PGJ_2 was able to prevent the nociceptor sensitization by inhibiting cytokines and chemokines release; this datum associated with the previously described ability of the 15d-PGJ_2 in inhibiting the development of hypernociception by the activation of the intracellular L-arginine-NO/cGMP/K^+_{ATP} pathway on primary nociceptive neurons from TMJ leads us to suggest that the peripheral application of 15d-PGJ_2 into TMJ seems to be a great option for this compound to improve disease outcomes.

Author contributions

Mariana da S. Quinteiro: oversaw the overall execution of the project, performed the experiments, contributed to the experimental design and the interpretation of the results

Karen M. Perozzo: performed the experiments, contributed to the experimental design and the interpretation of the results

Dr. Marcelo H. Napimoga: conceived and designed the experiments, analysed the data, wrote the manuscript and provided financial support.

Dr. Juliana T. Clemente Napimoga: conceived and designed the experiments, analysed the data, wrote the manuscript and provided financial support.

All authors discussed the results and commented on the manuscript.

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CAPÍTULO 2: 15d-PGJ_2 ameliorates the inflammatory process on rheumatoid arthritis-induced into rats' temporomandibular joint.

O presente artigo foi submetido para o periódico "European Journal of Pharmacology" (Anexo IV).

15d-PGJ_2 ameliorates the inflammatory process on rheumatoid arthritis-induced into rats' temporomandibular joint.

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Abstract

The aim of this study was to evaluate the peripheral effect of 15d-PGJ_2 in Rheumatoid Arthritis-induced inflammation in the temporomandibular joint (TMJ) of rats. Antigen-induced arthritis (AIA) was generated in rats with methylated bovine serum albumin (mBSA) diluted in complete Freund's adjuvant. Pretreatment with an intra-articular injection of 15d-PGJ_2 (100 ng/TMJ) before mBSA intra-articular injection (10 µg/TMJ) (challenge) in immunized rats significantly reduced the RA-induced inflammation. The results demonstrated that 15d-PGJ_2 was able to inhibit plasma extravasation, leukocyte migration and the release of inflammatory cytokines IL-6, IL-12, IL-18 and the chemokine CINC-1 in the TMJ tissues. In addition, 15d-PGJ_2 was able to increase the expression of the anti-adhesive molecule CD55 and the anti-inflammatory cytokine IL-10. Taken together, it is possible suggest that 15d-PGJ_2 inhibit leukocyte infiltration and subsequently inflammatory process, through a shift in the balance of the pro- and anti-adhesive properties. Thus, 15d-PGJ_2 might be used as a potential anti-inflammatory drug to treat RA inflammation of the temporomandibular joint.

Key words: Temporomandibular joints, inflammation, PGJ_2, hypernociception

1 INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune polyarthritis with synovial hyperplasia and joint destruction, leading to pain, loss of joint function and concomitant reduction in the quality of life (Németh and Mócsai, 2012). Being a synovial joint, the temporomandibular joint (TMJ) is subject to the same disorders affecting other synovial joints, including RA (Aliko et al. 2011). TMJ involvement in RA has high prevalence, from 65 to 92.9% (Aliko et al. 2011; Lin et al. 2007) and the most commonly reported symptoms include pain in the TMJ area, tenderness of the masticatory muscles, joint sounds and limited joint function (Goupille et al. 1993; Lin et al. 2007).

In the RA, leukocyte migration is enabled by endothelial activation in synovial microvessels, which increases the expression of adhesion molecules and chemokines. A variety of innate effector cells, including macrophages, mast cells and natural killer cells, are found in the synovial membrane, whereas neutrophils reside mainly in synovial fluid. Macrophages are central effectors of synovitis acting through release cytokines, such as TNF-α and interleukin-1, 6, 12, 15, 18, and 23, production of prostanoids and matrix-degrading enzymes, phagocytosis, and antigen presentation (McInnes and Schett, 2011). This interdependent network of cytokines, particularly TNF-α and IL-1β, prostanoids and proteolytic enzymes mediates many of the immune processes associated with the pathogenesis of RA (Di Paola and Cuzzocrea, 2008; Sachs et al. 2011).

The keystone of RA therapeutics has been conventional Disease-modifying antirheumatic drug (DMARDs), comprising a group of agents such as methotrexate, sulphasalazine, hydroxychloroquine and azathioprine. The precise mechanisms of action of these compounds remain elusive and, importantly, their introduction was not directed by a rationalization of target biology related to RA pathogenesis. Moreover, conventional DMARDs do not specifically target immune cells (O'Shea et al. 2013). Given the complex molecular pathogenesis and highly heterogeneous clinical picture of RA, there is an urgent need to dissect its multifactorial nature and to propose new strategies for preventive, early and curative treatments.

The 15-deoxy-$^{\Delta 12,14}$-prostaglandin J_2 (15d-PGJ_2), a cyclopentenone-type prostaglandin with a wide spectrum of physiological activities is one of the terminal products of the cyclooxygenase-2 (COX-2) pathway. 15d-PGJ_2 was initially discovered as a potent ligand for peroxisome proliferator-activated receptor γ (PPAR-γ), a member of the nuclear receptor superfamily and a ligand-activated transcription factor with pleiotropic effects on adipocyte differentiation, glucose homeostasis, lipid metabolism, growth, and inflammation (Surh et al. 2011). Our group has previously demonstrated that peripheral administration of 15d-PGJ_2 was able to prevent the nociceptor sensitization into the TMJ by two different mechanisms: direct mechanism that involves activation of κ/δ opioid receptors following the activation of the intracellular L-Arginine-NO/cGMP/K^+_{ATP} on primary nociceptive neurons from TMJ (Pena-dos-Santos et al. 2009) and indirect

mechanism that involves inhibition of TNF-α-induced hypernociception intracellular cascade (Quinteiro et al. 2012). It is currently known that the temporomandibular disorder, including the RA-affected, has an important inflammatory component as well as painful state, thus the aim of this study was to evaluate the peripheral effect of 15d-PGJ_2 in RA-induced TMJ inflammation in rats as well its mechanisms.

2 METHODS

2.1 Animals

Male Wistar rats (*Rattus norvegicus),* weighing about 150–250 g, obtained from the Multidisciplinary Center for Biological Research (CEMIB) at the State University of Campinas (Campinas, São Paulo, Brazil), were housed in temperature-controlled rooms (23 ± 1°C) with a 12/12 h light–dark cycle (lights on at 06:00 a.m.), with access to water and food *ad libitum*. All experiments were conducted in accordance to the guidelines of National Council for Control of Animal Experimentation (CONCEA) and International Association for the Study of Pain (IASP) in conscious animals (Zimmermann, 1983) and with the approval of the Ethics Committee on Animal Research of the State University of Campinas (CEUA/UNICAMP n°. 2949-1). The animals suffering and number per group were kept at a minimum and each animal was used once.

2.2 Induction of experimental arthritis

The protocol used to induce the experimental arthritis was described previously (Quinteiro et al. 2012). Briefly, male Wistar rats were sensitized with 500

µg of methylated bovine serum albumin (mBSA) (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 200 µL of an emulsion containing 100 µL phosphate buffered saline (PBS) and 100 µL Freund's complete adjuvant (CFA) (Sigma-Aldrich, St. Louis, MO, USA) administered by subcutaneous injection in the back. Booster injections of mBSA dissolved in Freund's incomplete adjuvant (IFA) (Sigma-Aldrich, St. Louis, MO, USA) were given 7 and 14 days after the first immunization in different sites in the back of the rat. Twenty-one days after the initial injection, TMJ-arthritis was induced in the immunized animals by intra-articular injection of mBSA (10 µg/ TMJ) dissolved in 15 µL of PBS (challenge). Non-immunized rats (control group) were treated by an intra-articular injection of PBS.

2.3 Experimental design

In a previously work (Quinteiro et al., 2012) we demonstrated that RA induced a higher nociceptive behavioral response 24 h after intra-TMJ injection of mBSA (challenge, 10 µg/TMJ) in immunized rats, and the intra-TMJ injection of 15d-PGJ_2, after this period, was able to inhibit the RA-induced hypernociception into TMJ. Considering these results, in the present work it was evaluated the development of RA-induced inflammation into TMJ in different sets of inflammatory process and the ability of the 15d-PGJ_2 to prevent this process. For that, it was necessary a modification of the original experimental protocol (Quinteiro et al., 2012) and for the present work, animals receive the intra-TMJ injection of 15d-

PGJ_2 prior the intra-TMJ injection of mBSA (challenge, 10 µg/TMJ) in immunized rats (Figure 1),

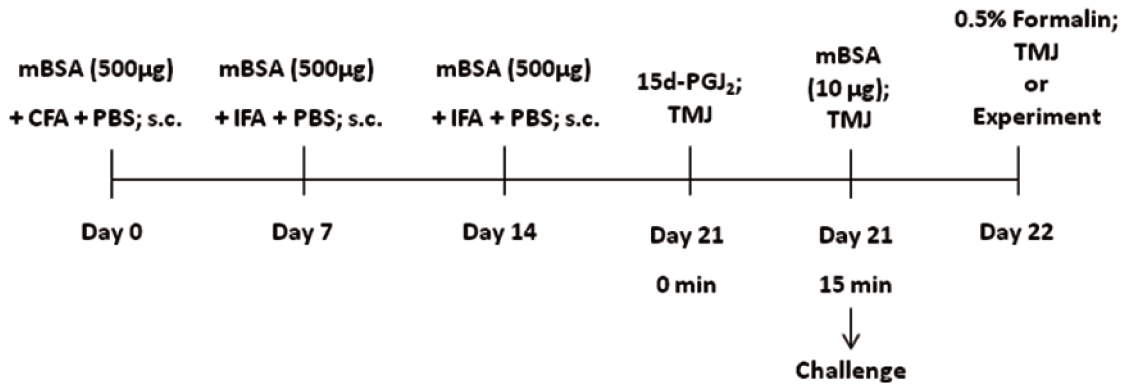

Figure 1: Experimental design of the effect of 15d-PGJ_2 on RA-induced TMJ hypernociception.

2.3.1 Effect of the 15d-PGJ_2 on RA-induced inflammatory hypernociception into TMJ of rats.

RA-induced TMJ inflammatory hypernociception was assessed by measuring behavioural nociceptive responses induced by intra-articular injection of a low dose of formalin (0.5%) into the TMJ 6, 12, 24 and 48 h after challenge in immunized rats. To test the effect of 15d-PGJ_2 on TMJ-arthritis hypernociception, the rats were pretreated (15 min) with an intra-TMJ injection of 15d-PGJ_2 (Calbiochem, San Diego, CA, USA) (100 ng/ TMJ; Quinteiro et al. 2012) followed by challenge. Twenty-four hours later, an additional injection of formalin was administered. Immediately after the formalin injection, the behavioral nociceptive response was evaluated for a 45 min observation period. As previously described (Clemente et al. 2004; Roveroni et al. 2001) to evaluate the behavioral nociceptive response the animals were briefly anaesthetized by inhalation of isoflurane to allow

the TMJ injection, which was performed with 30-gauge needle connected to 50-µl Hamilton syringe (Roveroni et al. 2001). Each animal regained consciousness approximately 30 s after discontinuing the anaesthetic and was returned to the test chamber for counting nociceptive responses. The nociceptive response score was defined as the cumulative total number of seconds that the animal spent rubbing the orofacial region asymmetrically with the ipsilateral fore or hind paw plus the number of head flinches counted during the observation period as described previously. Since head flinches followed a uniform pattern of 1 s of duration, each flinch was expressed as 1 s. Results are expressed as the duration time of nociceptive behaviour (Clemente et al. 2004; Roveroni et al. 2001). All experiments were conducted in a double-blind manner, in which the person who prepared and injected the solutions was different from the one who made the behavioral assessment. Formalin solution was prepared from commercially stock formalin (an aqueous solution of 37 % of formaldehyde – Sigma Aldrich, St. Louis, MO, USA) and further diluted in 0.9 % NaCl.

2.3.2 Leukocytes and Protein extraction from TMJ periarticular tissues

After behavioral experiments, the rats were killed and the articular cavity was washed with 10 µL with PBS containing 1mM EDTA for leukocytes migration analysis. The TMJ periarticular tissues were also removed and homogenized in 500 µl of the appropriate buffer containing protease inhibitors (Ripa Lysis Buffer, Santa Cruz, Biotechnology, Dallas, Texas, USA) followed by a centrifugation of 10

min/10.000 rpm/4°C. The total amount of extracted proteins was colorimetric measured using the BCA protein assay kit (Thermo Scientific, Rockford, IL, USA). The supernatants were stored at -20°C until further analysis.

2.3.3 Effect of 15d-PGJ_2 of leukocyte migration on RA-induced in TMJ

Total leukocyte counts were performed in a Neubauer chamber diluting the exudate in Türk solution (1:2) and expressed as number of cells x 10^4/cavity. The differential leukocyte counts, was performed by preparing smears in a cytocentrifugue, which were stained with fast Panotic kit, and for differentiated cells (100 cells total), an optical microscope (1000 x increase) was utilized. The result of each cell type was calculated using the percentage of those cells and the total number of cells obtained in the total count (Dal Secco et al. 2006).

2.3.4 Effect of the 15d-PGJ_2 on RA-induced released of cytokines IL-6, IL-12, IL-18, IL-10 and chemokine CINC-1 in the TMJ tissue

Levels of IL-6, IL-10 , CINC-1 (R&D Systems, Mineapolis, MN, USA), IL-12 (Uscn Life Science Inc., Wuhan, PR, China) and IL-18 (Boster Immunoleader, Fremont, CA) were quantified in the rats TMJ tissues by enzyme-linked immunossorbent assay (ELISA) using protocols supplied by the manufacturers. Briefly, 96-well plate for ELISA were incubated overnight at 4°C with 100 µl of capture antibody. Then the plate was washed and blocked for 1 hour at room temperature with 300 µl of reagent diluent. Fifty microliters standard and samples

were added on the plate and incubated 2 hours at room temperature. Then, the plate was washed and incubated for 2 hour with 100 µl of detection antibody. After this, the plate was washed again and incubated for 20 min. with Streptavidin-HRP solution. After a new wash, the TMB one-step substrate reagent was added to each well. The reaction was stopped with the Stop Solution and read immediately in a spectrophotometer (Epoch, Biotek, Winooski, VT, USA) at 490 nm. The results were expressed as picograms per milligram of tissue.

2.3.5 Effect of the 15d-PGJ_2 on RA-induced expression of intercellular adhesion molecule (ICAM) and decay-accelerating factor (CD55) in the TMJ

The ICAM and CD55 expression were quantified by using the supernatants obtained from the TMJ periarticular tissue as described above. For western blotting analysis, equal amounts of protein (80 µg) from the TMJ periarticular tissue were separated by electrophoresis into a 10% polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). A molecular mass standard (Bio-Rad, Hercules, CA, USA) was run in parallel to estimate molecular mass. Membranes were blocked overnight at 4°C in TBST (20 mM Tris-HCl [pH 7.5], 500 mM NaCl, and 0.1% Tween 20) containing 5% of nonfat dried milk. After blocking, the membranes were incubated with anti-ICAM (1:10.000; Abcam, Cambridge, MA, USA), anti-CD55 (1:1.000; Santa Cruz Biotechnology, Santa Cruz, CA,USA) or GAPDH (1:10.000; Novus Biologicals, Littleton, CO, USA), used as control, diluted in TBST containing 5% of nonfat dried milk at room temperature

for two hours. The membranes were then incubated with appropriate secondary antibody conjugated with peroxidase (1:10.000; Sigma-Aldrich, St. Louis, MO, USA) diluted in TBST containing 5% of nonfat dried milk at room temperature for 60 min. Finally, the bands recognized by the specific antibody were visualized using a chemiluminescence-based ECL system (Amersham Biosciences, Piscataway, NJ, USA) and exposed to an X-ray film (Eastman Kodak, Rochester, NY, USA) for 30 seconds. A computer-based imaging system (ImageJ; National Institutes of Health, Bethesda, MD, USA) was used to measure the optical density of the bands.

2.3.6 Effect of 15d-PGJ_2 in a plasma extravasation on RA-induced in TMJ

In another set of experiments, immediately after TMJ treatments the animals were anesthetized with an intraperitoneal injection of α-cloralose (50 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) and urethane (100 mg/kg; Sigma-Aldrich, St. Louis, MO, USA), and the Evan's Blue dye (1%, 50 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) (Torres-Chávez et al. 2012) was injected into the penile vein and 45 min later TMJ inflammation was assessed by the extravasation of Evan's Blue dye bound to plasma protein (Fiorentino et al. 1999; Haas et al. 1992). Each rat was then killed under deep anesthesia and perfused with saline. Joint tissues were dissected, weighed and stored at -20 °C. Evans' Blue dye was extracted by immersing the joint tissue in 1 ml of formamide (Sigma-Aldrich, St. Louis, MO, USA) at 60 °C for 24 h. The samples absorbance was then determined in a

spectrophotometer (Epoch, Biotek, Winooski, VT, USA) at 620 nm, and the Evan's Blue dye concentration determined by comparison to a standard curve of known amounts of Evan's Blue dye in extraction solution, which was assessed within the same assay. The amount of Evan's Blue (micrograms) was then calculated per gram weight of tissue.

2.3.7 Histological analysis of TMJ tissues

Animals of a new set of experiments, immediately after TMJ treatments were killed under deep anesthesia and perfused with saline. The head of animal was removed and fixed in 10% buffered neutral formalin for 48h and decalcified in a solution of ethlenediaminetetraacetic acid (EDTA) 10% for 3 months. After that, TMJs were removed in block, decalcified samples were briefly washed in running tap water, dehydrated and embedded in paraffin wax. Each sample was sliced into 6 µm sections in transversal directions and staining with hematoxylin-eosin. The sections were analyzed qualitatively according to inflammatory cell infiltrate intensity by an examiner blinded to the experimental conditions.

2.3.8 Statistical analysis

To determine if there were significant differences ($p < 0.05$) among treatment groups, the data were analysed using one-way analysis of variance (ANOVA) as appropriate. If there was a significant among subjects main effect of treatment group following one-way ANOVA, post hoc contrasts, using the Tukey

test, were performed to determine the basis of the significant difference. Data are presented in figures as means ± standard deviation.

3 RESULTS

3.1 15d-PGJ_2 inhibits RA-induced TMJ inflammatory hypernociception

Corroborated our first study, the results of the present work demonstrated that pretreatment with an intra-articular injection of 15d-PGJ_2 (100 ng/TMJ) before mBSA intra-articular injection (10 µg/TMJ) (challenge) in immunized rats significantly reduced the RA-induced hypernociception (Figure 2). These findings suggest that 15d-PGJ_2 was able to avoid the inflammatory episode evoked by RA into TMJ that contributes for hypernociception progress. To test this hypothesis in the next experiments it was evaluated the effect of 15d-PGJ_2 in different sets of inflammatory process in RA-induced TMJ.

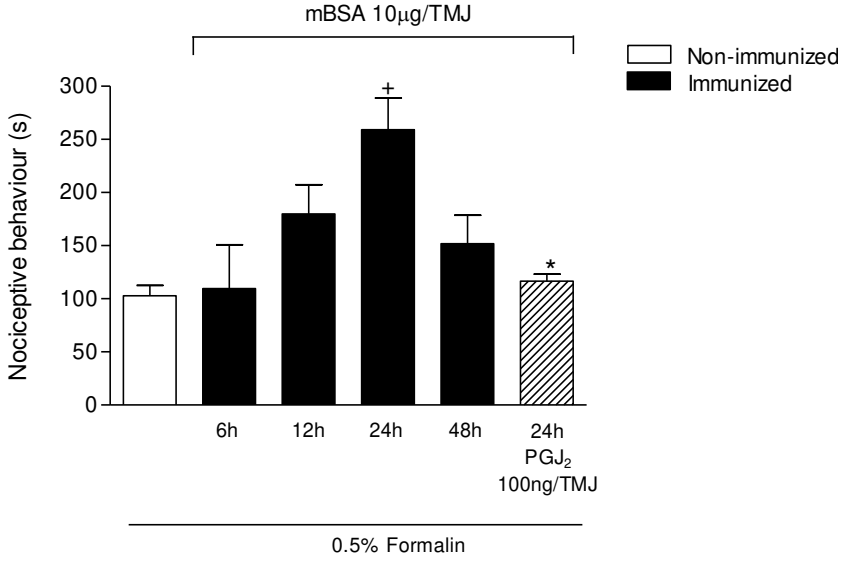

Figure 2: 15d-PGJ_2 inhibits RA-induced TMJ hypernociception. Pretreatment (15 min) with 15d-PGJ_2 (100 ng/TMJ) significantly reduced RA-induced TMJ hypernociception. The nociceptive behavioural response induced by an intra-articular injection of formalin (0.5%) previously challenged with an intra-articular injection of mBSA (10 µg) of immunized rats reaching the peak at 24 h after mBSA injection. The symbol (+) indicates nociceptive behavioural response significantly higher than that control group ($p<0.05$: ANOVA, Tukey test). The symbol (*) indicates nociceptive behavioural response significantly lower than that immunized + mBSA (24 hours) group (column 4) ($p<0.05$: ANOVA, Tukey test). For all experiments results are expressed as mean ± SD of 4-6 animals per group.

3.2 15d-PGJ_2 reduced plasma extravasation and leukocyte migration on RA-induced in TMJ.

Rheumatoid arthritis induced in the rats' TMJ evoked a significantly protein plasma extravasation and leukocyte migration in the TMJ tissues 24 hours after mBSA challenge in immunized rats ($p<0.05$: ANOVA, Tukey test) (Figure 3A e 3B). These exacerbated leukocytes exudate is characterized by lymphocytes (Figure 3C), neutrophils (Figure 3D) and mast cells (Figure 3E). Pretreatment with an intra-articular injection of 15d-PGJ_2 (100 ng/ TMJ) 15 min prior to mBSA challenge in immunized rats inhibit this inflammatory infiltrate in RA-induced TMJ. The number of macrophage cells was significantly higher in the TMJ tissues 48 hours after intra-articular mBSA challenge in immunized rats ($p<0.05$) and the treatment with an intra-articular injection of 15d-PGJ_2 did not affect this influx (Figure 3F). Histological sections confirm these results (Figure 4) showing an important inflammatory influx in the mBSA challenge in immunized rats mainly after 24h (Figure 4D) which was abrogated in the 15d-PGJ_2-treated (100 ng/ TMJ) animals (Figure 4F).

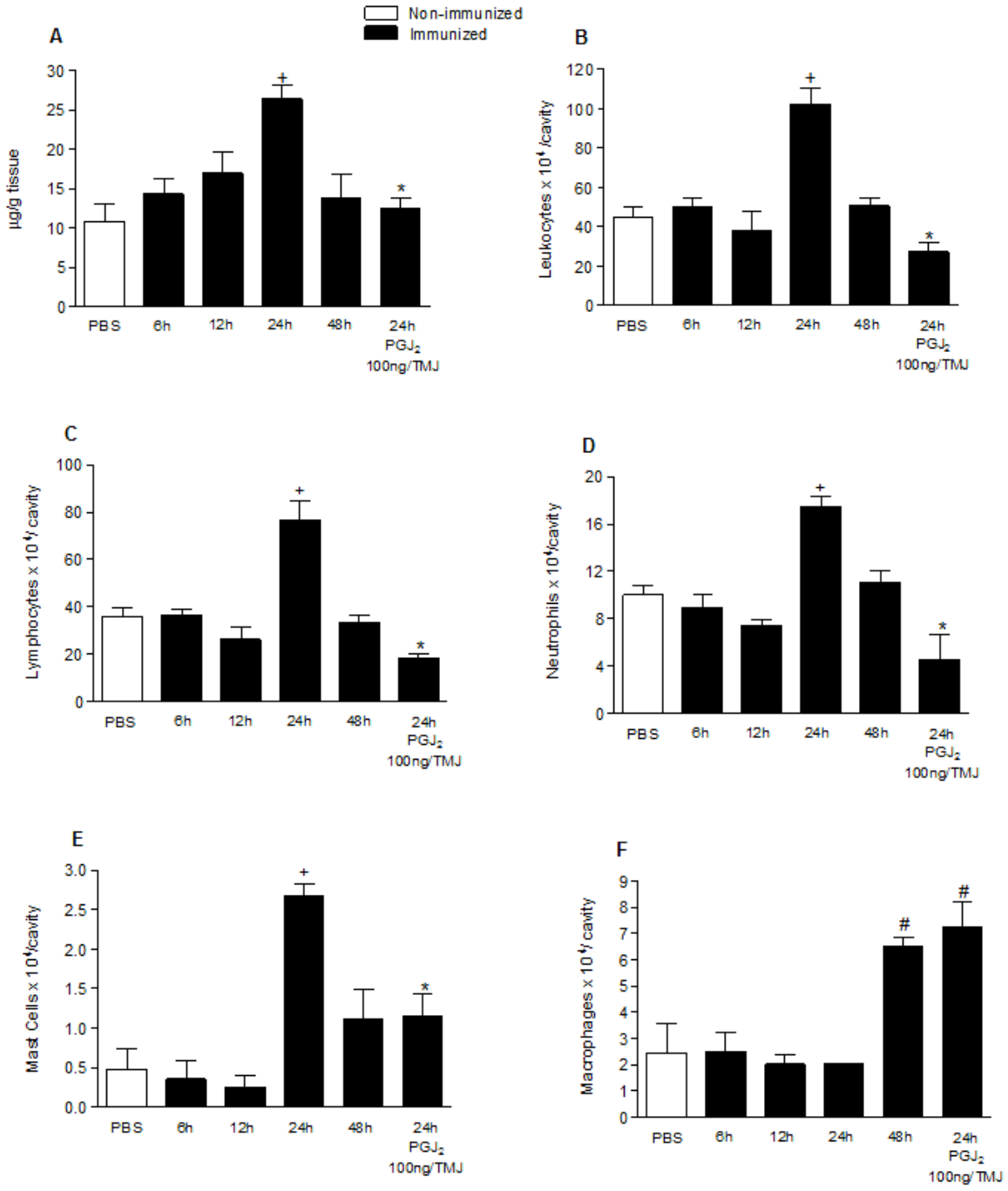

Figure 3: 15d-PGJ$_2$ reduced plasma extravasation and leukocyte migration on RA-induced in TMJ. (A) and (B): Pretreatment with 15d-PGJ$_2$ (100 ng/TMJ) significantly reduced protein plasma extravasation and leukocyte migration on RA-induced in TMJ. The protein plasma extravasation and leukocyte migration induced by RA in the TMJ reached the peak at 24 h after mBSA injection in

immunized rats. The symbol (+) indicates values significantly higher than that control group ($p<0.05$: ANOVA, Tukey test). The symbol (*) indicates values significantly lower than that immunized + mBSA 24 hours group (column 4) ($p<0.05$: ANOVA, Tukey test). **(C), (D) and (E):** The differential counts of inflammatory cells demonstrated that pretreatment of 15d-PGJ_2 (100 ng/ATM) significantly reduced lymphocytes (C), neutrophils (D) and mast cells (E) migration. The symbol (+) indicates values significantly higher than that control group ($p<0.05$: ANOVA, Tukey test). The symbol (*) indicates values significantly lower than that immunized + mBSA (24 hours) group (column 4). **(F)** Pretreatment of 15d-PGJ_2 (100 ng/ATM) no affect macrophage migration. The symbol (#) indicates values significantly higher than that control group ($p<0.05$: ANOVA, Tukey test). There no difference between immunized + mBSA (48 hours) group (column 5) and 15d-PGJ_2 group (column 6) ($p>0.05$: ANOVA, Tukey test).

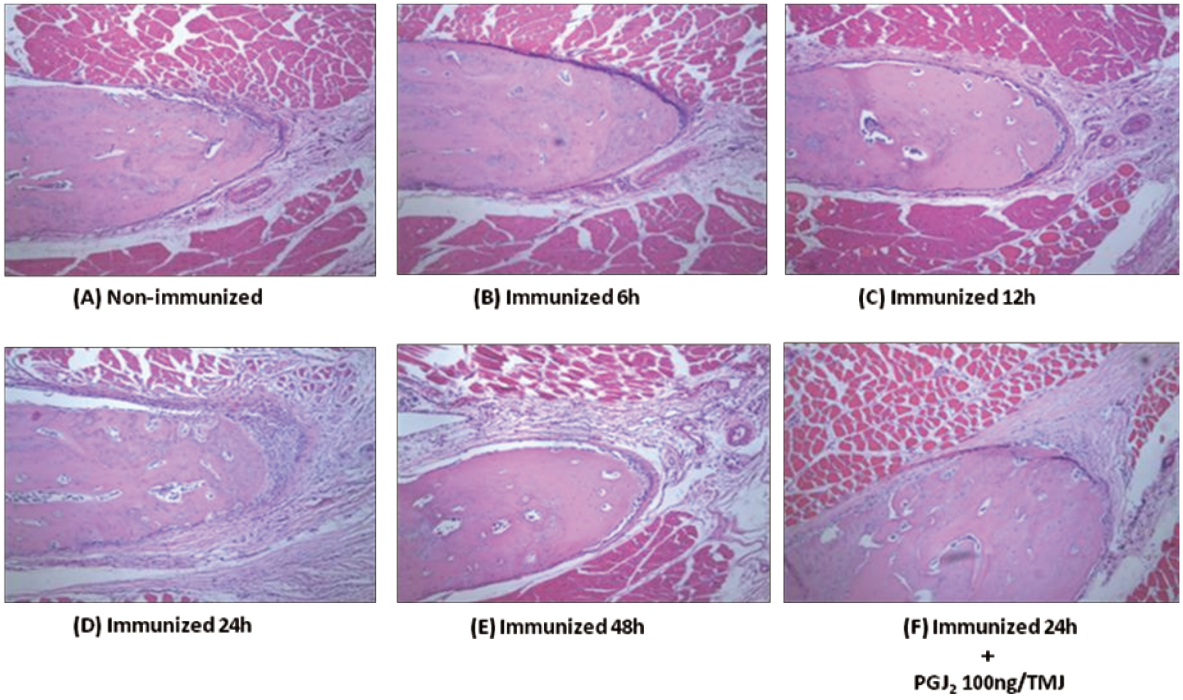

Figure 4: 15d-PGJ_2 reduced inflammatory *pannus* in RA-induced TMJ. Histological sections (HE, 20x). The Immunized + mBSA (24 hours) group demonstrated exacerbated leukocytes exudate around collagen fibers.

3.3 15d-PGJ_2 reduced the release of inflammatory cytokines on RA-induced in the TMJ

Rheumatoid arthritis induced in the rats' TMJ significantly increased the release of inflammatory cytokines IL-6 (Figure 5A), IL-12 (Figure 5C) IL-18 (Figure 5D) and the chemokine CINC-1 (Figure 5B) in the TMJ tissues 24 hours after mBSA challenge in immunized rats ($p<0.05$; ANOVA, Tukey test). Intra-articular injection of 15d-PGJ_2 significantly reduced the release of inflammatory cytokines IL-6 (Figure 5A), IL-12 (Figure 5C), IL-18 (Figure 5D) and the chemokine CINC-1 (Figure 5B) in immunized rats challenge with mBSA (24 hours) group ($p<0.05$; ANOVA, Tukey test). The release of the anti-inflammatory cytokine IL-10 (Figure 5E) was significantly higher in the TMJ tissue 24 hours after intra-articular mBSA challenge in immunized rats ($p<0.05$) and the pretreatment with an intra-articular injection of 15d-PGJ_2 potentialized this release ($p<0.05$).

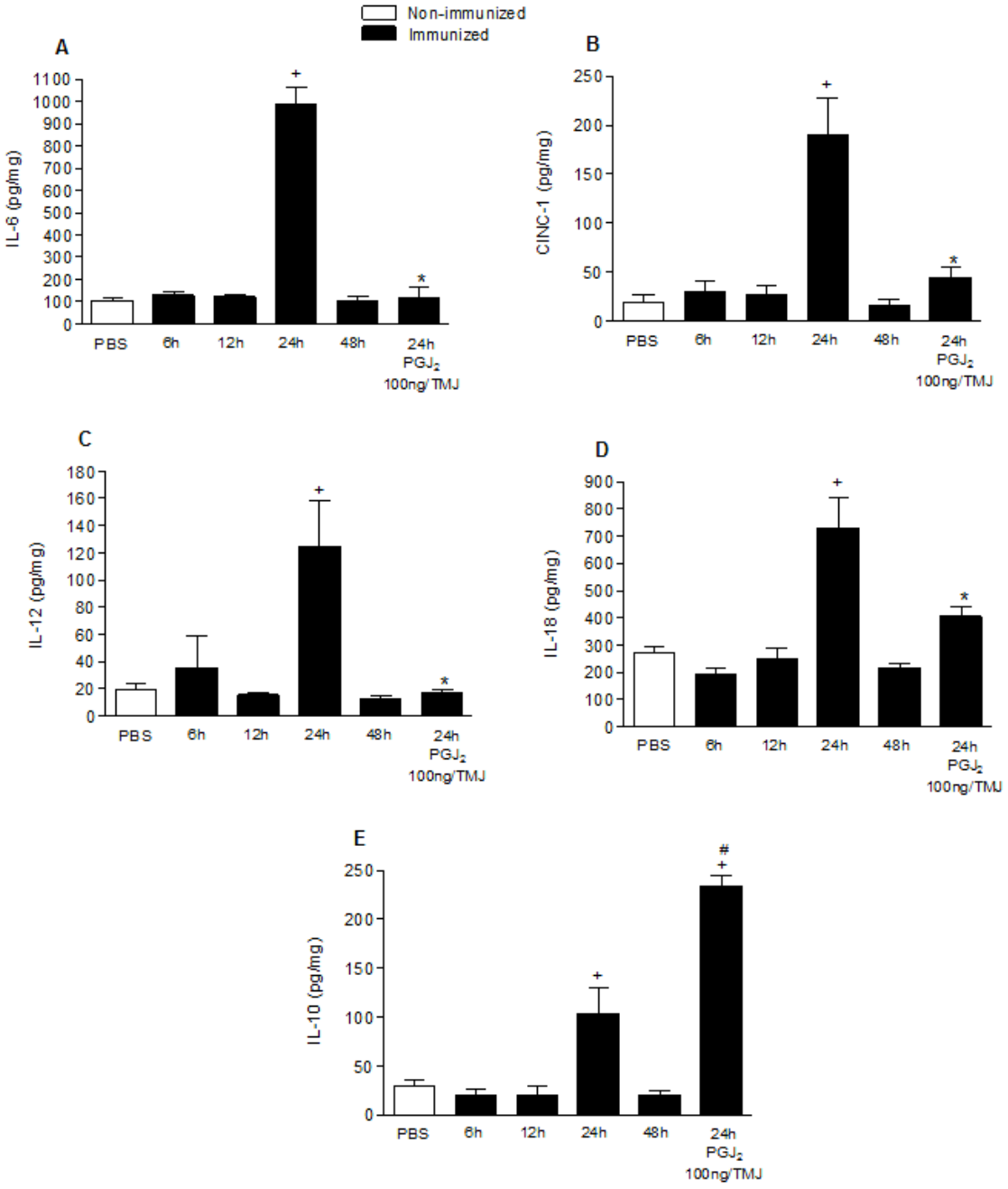

Figure 5: 15d-PGJ$_2$ reduced the release of inflammatory cytokines on RA-induced in the TMJ. Pretreatment with 15d-PGJ$_2$ (100 ng/TMJ) significantly reduced the release of IL-6 **(A)**, CINC-1 **(B),** IL-12 **(C)** and IL-18 **(D)** induced by RA induced in the TMJ of rats. The release of cytokines IL-6, IL-12 and IL-18 and the chemokine CINC-1 induced by RA in the TMJ, reaching the peak at 24 h after mBSA injection in immunized rats. **(E)** Pretreatment with 15d-PGJ$_2$ (100 ng/ATM) significantly

increased the release of the anti-inflammatory cytokine IL-10. The symbol (+) indicates values significantly higher than that control group ($p<0.05$: ANOVA, Tukey test). The symbol (*) indicate values significantly lower than that immunized + mBSA (24 hours) group (column 4) ($p<0.05$: ANOVA, Tukey test). The symbol (#) indicates values significantly higher than that immunized + mBSA (24 hours) group (column 4) ($p<0.05$: ANOVA, Tukey test).

3.4 15d-PGJ_2 up-regulates the expression of the decay-accelerating factor (CD55) in TMJ tissue

Western blotting analysis demonstrated that pretreatment with an intra-articular injection of 15d-PGJ_2 (100 ng/ TMJ) 15 min prior to mBSA challenge in immunized rats significantly increased the expression of CD55 ($p<0.05$; ANOVA, Tukey test) (Figure 6B). On the other hand, there was no difference among groups in the expression of ICAM-1 (Figure 6A).

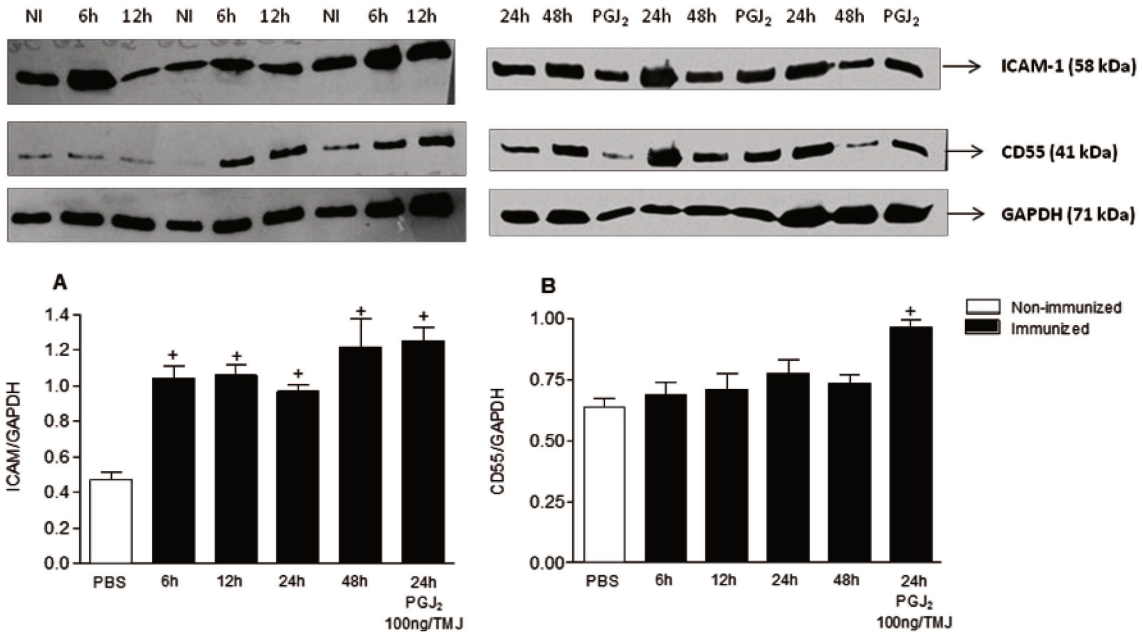

Figure 6: 15d-PGJ_2 up-regulated the expression of the decay-accelerating factor (CD55) in TMJ tissue. (A) There no difference among groups in the expression of ICAM ($p>0.05$: ANOVA, Tukey test). **(B)** Pretreatment with 15d-PGJ_2 (100 ng/ATM) significantly increases the expression of CD55 in the TMJ. The symbol (+) indicates values significantly higher than other groups ($p<0.05$: ANOVA, Tukey test).

4 DISCUSSION

In the present study it was demonstrated that the pretreatment with an intra-articular injection of 15d-PGJ_2 (100 ng/ TMJ) 15 min prior to mBSA challenge in immunized rats inhibit the development of inflammatory process in RA-induced into temporomandibular joint (TMJ). This effect was due to inhibition of cytokines and chemokine release associated to a reduced inflammatory cells infiltrate in the TMJ tissue.

Rheumatoid arthritis is an autoimmune systemic disease that affects the connective tissue characterized by chronic synovitis accompanied by destruction of cartilage and osseous alterations (Scott et al. 2010) including the TMJ. The local pathology of RA is hallmarked by immunocompetent cells accumulate and expansion of resident stromal and vascular cells and the relevant role for cytokine networks. Crosstalk between these elements generates an aggressive environment contributing to tissue damage and perpetuation of the disease process, and also plays an essential role in the development of inflammatory pain as well as other inflammatory events. The first cytokines described as participating in the development of inflammatory and/or neuropathic pain were Interleukin (IL)-1β, TNF-α, IL-6, and the chemokines IL-8, chemokine-induced neutrophil

chemoattractant-1 (CINC-1) and keratinocyte-derived chemokine (KC). Recently, it has been demonstrated that IL-18 and IL-12 also induce inflammatory hypernociception (Verri Jr et al. 2006).

As demonstrated in the current work IL-6, IL-12, and IL-18 and CINC-1 were increased in the TMJ of RA-induced animals and the injection of 15d-PGJ_2 prior to challenge have decreased the release of these cytokines. High levels of IL-6 were detected in the joint cartilage and synovium from the joint tissue in patients with degenerative lumbar spinal disorders (Igarashi et al. 2004) and also produce a dose-and time dependent mechanical hypernociception in rats, which was inhibited by indomethacin, antiserum anti-IL-1β or IL-1ra, suggesting that IL-6 induces the production of IL-1β in cutaneous tissue of rats (Cunha et al. 1992; Cunha et al. 2000). Interestingly, IL-6 knockout mice exhibit reduced thermal and mechanical inflammatory hypernociception in response to injection of carrageenan in the peripheral tissue (Xu et al. 1997). IL-12 administration stimulates the onset and exacerbates the severity of collagen-induced arthritis and anti-murine IL-12 treatment ameliorates the onset of collagen-induced arthritis (Joosten et al. 1997). Furthermore, IL-18 mRNA and expressed protein are present at significant levels in the rheumatoid arthritis synovium of humans as well as in experimentally collagen-induced arthritis (Plater-Zyberk et al. 2001). IL-18 administration promoted neutrophil accumulation *in vivo*, an important event involved in the pathogenesis of tissue lesions in arthritis (Leung et al. 2001). This milieu of cytokines is crucial to mediate inflammatory and neuropathic pain, linking the inflammatory stimuli and

the release of the final mediators (as prostaglandins, sympathetic amines) ultimately responsible for the nociceptor sensitization. Thus, this data may also support the increased inflammatory infiltrate observed in the immunized rats challenge with mBSA. Moreover, the administration of 15d-PGJ_2 was able to decrease these cytokines and consequently the inflammatory cells infiltration.

On the other hand, IL-10 was the first anti-hypernociceptive cytokine described, and is produced by various cell types such as lymphocytes, monocytes, macrophages and mast cells. It has been shown that IL-10 inhibits pro-inflammatory cytokines production and inhibits the production of several cytokines, including interleukin-1β and TNF-α, and the proliferation of T cells *in vitro* (Isomäki and Punnonen, 1997). Interleukin-10 can also reverse the cartilage degradation mediated by antigen-stimulated mononuclear cells from patients with rheumatoid arthritis (van Roon et al. 1996). In our study we have demonstrated that the immunized rats challenge with mBSA had an increase in the IL-10 release after 24h. This scenario may be a physiological response in order to control the inflammatory process that is occurring, although it is unsatisfactory to control it. In agreement, it has been demonstrated interleukin-10 in the synovial fluid of patients with rheumatoid arthritis, but it is suggested that the amount is insufficient to suppress inflammation (Katsikis et al. 1994). Importantly, the administration of 15d-PGJ_2 strongly increases the release of this anti-inflammatory cytokine suggesting an important pathway to control the inflammatory process into the RA-affected TMJ.

It is important to point out that besides all other inflammatory cells, macrophages were the only cell that the administration of 15d-PGJ_2 did not inhibit the influx. This is interesting since macrophage is one of the most important cells to participate in the resolution of inflammation which involves the removal of inflammatory cells from the damage tissue. Besides, macrophages are also an important source of endogenous opioids (Stein et al. 2003) and in previous study our group has demonstrated that increasing the number of peripheral macrophages by previous administration of thioglycollate in the rat paw enhances the antinociceptive effect of 15d-PGJ_2. This result suggests that the naloxone-sensitive antinociceptive effect of 15d-PGJ_2 probably depends on paw skin macrophages (Napimoga et al. 2008). Indeed, there is evidence that opioid-containing macrophages are involved in the endogenous control of inflammatory pain (Brack et al. 2004).

For immigration of circulating leukocytes into tissues, transmigration through the vascular endothelial layer involves two independently regulated events: binding to vessel endothelium, followed by diapedesis. For this purpose, cell arrest is mediated by activation of adhesion receptors on the moving cell, followed by attachment to counter-receptors on other cells or endothelial cells, leading to an immobilized cell (Mackay, 2008). Thereafter, adhesion and transmigration are mediated by the CD11/CD18 complex on the leukocyte, which interacts with its ligands, such as intercellular adhesion molecule-1 (ICAM-1), present mostly on endothelial cells (Mayadas and Cullere, 2005). On the other hand, the molecule

called decay accelerating factor (DAF, also termed CD55) is an anti-adhesive molecule that promotes the clearance of epithelial-bound leukocytes. According to the current study, the use of 15d-PGJ_2 increased the expression of the CD55 molecule in the TMJ tissue. The observed induction of CD55 associated to decreased pro-inflammatory cytokines may explain the dynamics of leukocyte adhesion observed, through a shift in the balance of the pro- and anti-adhesive properties.

In conclusion, peripheral release of 15d-PGJ_2 in periarticular tissues modulated chemotaxis, cytokine and chemokine release induced by RA. Thus, 15d-PGJ_2 could be considered as a new therapeutic perspective to control the induction and progression of RA-induced inflammatory conditions in the TMJ.

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CONCLUSÃO

O presente estudo demonstrou que o tratamento periférico com a 15d-PGJ_2 foi capaz de reduzir o processo inflamatório e a hipernocicepção induzidos pela artrite reumatoide na ATM de ratos, através: (1) da inibição da liberação das citocinas e quimiocinas inflamatórias TNF-α, IL-1β, IL-6, IL-18, IL-12, KC e CINC-1; (2) do aumento da expressão da citocina anti-inflamatória IL-10; (3) da inibição da expressão das proteinoquinases PKA e PKCε; (4) do aumento da expressão do fator de aceleração de decaimento (CD55); (5) da inibição da migração de leucócitos e extravasamento plasmático. Sendo assim, considerando o potencial efeito anti-inflamatório da 15d-PGJ_2 demonstrado neste estudo, a 15d-PGJ_2 pode ser considerada uma nova perspectiva terapêutica para controlar a progressão das condições inflamatórias induzidas pela artrite reumatoide na articulação temporomandibular.

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(*) De acordo com as normas UNICAMP/FOP, baseadas na padronização do Internacional Committe of Medical Journal Editors. Abreviatura dos periódicos em conformidade com o Medline.

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ANEXO 1

UNIUBE Educação e Responsabilidade Social — Comitê de Ética em Experimentação Animal

Ofício CEEA–072/2009 — Uberaba, 19 de junho de 2009

Ilma. Profª.
Juliana Trindade Clemente Napimoga

Assunto: Encaminha parecer nº 059/2009, sobre o protocolo de pesquisa "*Avaliação do efeito periférico da 15d-PGJ2 na artrite reumatóide induzida experimentalmente na articulação temporomandibular de ratos* " – Processo 059/2009.

Prezada Senhora.

Em resposta a sua solicitação, informo que o protocolo acima referido foi submetido à avaliação do CEEA-UNIUBE na reunião do dia 19/06/2009, sendo **aprovado.**

Atenciosamente,

Profa. Joely F. Figueiredo Billar
Vice-Coordenadora do CEEA-UNIUBE

Campus Aeroporto – Av. Nenê Sabino, 1801, Bloco R – B. Universitário – 38055-500 – Uberaba, MG – Fone: (34) 3319-8819 – Fax: (34) 3314-8910
e-mail: cep@uniube.br

ANEXO 2

UNICAMP

CEUA/Unicamp

Comissão de Ética no Uso de Animais
CEUA/Unicamp

C E R T I F I C A D O

Certificamos que o projeto **"Avaliação do efeito periférico da 15d-PGJ2 no processo inflamatório induzido pela artrite reumatoide na articulação temporomandibular de ratos"** (protocolo nº **2949-1**), sob a responsabilidade de **Profa. Dra. Juliana Trindade Clemente Napimoga / Mariana da Silva Quinteiro**, está de acordo com os **Princípios Éticos na Experimentação Animal** adotados pela **Sociedade Brasileira de Ciência em Animais de Laboratório (SBCAL)** e com a legislação vigente, **LEI Nº 11.794, DE 8 DE OUTUBRO DE 2008**, que estabelece procedimentos para o uso científico de animais, e o **DECRETO Nº 6.899, DE 15 DE JULHO DE 2009**.

O projeto foi aprovado pela Comissão de Ética no Uso de Animais da Universidade Estadual de Campinas - CEUA/UNICAMP - em **10 de dezembro de 2012**.

Campinas, 10 de dezembro de 2012.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

Fátima Alonso
Secretária Executiva

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ANEXO 3

EJP
European Journal of Pain

ORIGINAL ARTICLE

The indirect antinociceptive mechanism of 15d-PGJ_2 on rheumatoid arthritis-induced TMJ inflammatory pain in rats

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Conflicts of interest
None declared.

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Abstract

Background: Inflammation of the temporomandibular joint (TMJ) induced by rheumatoid arthritis (RA) have resulted in persistent pain and caused distress to many patients. Considering that not all patients respond to traditional drugs therapy to RA and it has demonstrated that 15-deoxy-$^{\Delta 12,14}$-prostaglandin J_2 (15d-PGJ_2) into TMJ has a potential peripheral antinociceptive effect, the aim of this study was to evaluate the peripheral effect of 15d-PGJ_2 in RA-induced TMJ inflammatory hypernociception.
Methods: Antigen-induced arthritis (AIA) was generated in rats with methylated bovine serum albumin (mBSA). RA-induced TMJ hypernociception was assessed by measuring the behavioural nociceptive responses. After behavioural experiments, the animals were terminally anaesthetized and periarticular tissues were removed and homogenized. The supernatants were used to evaluate the levels of tumour necrosis factor (TNF)-α, interleukin (IL)-1β and keratinocyte-derived chemokine (KC) by enzyme-linked immunosorbent assay as well the expression of PKCε and PKA by western blotting analysis.
Results: The intra-articular injection of mBSA, but not phosphate buffered saline (control), in immunized rats induced dose- and time-dependent behavioural nociceptive responses in which the peak of nociceptive responses were obtained by using 10 μg/TMJ of mBSA after 24 h. Pretreatment with 15d-PGJ_2 (30, 100 and 300 ng/TMJ) inhibited the RA-induced TMJ inflammatory hypernociception. In addition, 15d-PGJ_2 reduced the RA-induced release of TNF-α, IL-1β and KC ($p < 0.05$) as well the expression of PKA and PKCε ($p < 0.05$).
Conclusions: In the present study, we demonstrated that 15d-PGJ_2 was able to reduce the RA-induced TMJ inflammatory hypernociception by an indirect mechanism. This antinociceptive effect is in part due to decrease of TNF-α, IL-1β and KC levels and PKA/PKCε expression in the TMJ.

1. Background

Rheumatoid arthritis (RA) is a chronic and progressive inflammatory disorder characterized by synovitis and severe joint destruction (Di Paola and Cuzzocrea, 2008). Being a synovial joint, the temporomandibular joint (TMJ) is subject to the same disorders affecting other synovial joints, including RA (Aliko et al., 2011). There was a high prevalence of temporomandibular disorders in RA patients from 67 to 92.9% (Lin et al., 2007; Twilt et al., 2008; Aliko et al., 2011) in which the most common clinical findings are pain in the TMJ area, tenderness of the masticatory muscles, joint sounds and limited joint function (Goupille et al., 1993; Lin et al., 2007).

ANEXO 4

---------- Forwarded message ----------
From: **The European Journal of Pharmacology** <ejp-office@pharm.uu.nl>
Date: 2014-02-07 16:16 GMT-02:00
Subject: Submission Confirmation for 15d-PGJ2 ameliorates the inflammatory process on rheumatoid arthritis-induced into rats' temporomandibular joint
To: juliana.napimoga@fop.unicamp.br, jnapimoga.unicamp@gmail.com

Dear Juliana,

Your submission entitled "15d-PGJ2 ameliorates the inflammatory process on rheumatoid arthritis-induced into rats' temporomandibular joint" has been received by journal European Journal of Pharmacology

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

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

Thank you for submitting your work to this journal.

Kind regards,

European Journal of Pharmacology