JULIANA MAIA TEIXEIRA

INVOLVEMENT OF P2X3 AND P2X7 PURINERGIC RECEPTORS IN INFLAMMATORY ARTICULAR HYPERALGESIA IN THE KNEE JOINT OF RATS AND THE STUDY OF THE PERIPHERAL MECHANISMS INVOLVED

PARTICIPAÇÃO DOS RECEPTORES PURINÉRGICOS P2X3 E P2X7 NA HIPERALGESIA INFLAMATÓRIA ARTICULAR EM JOELHO DE RATOS E O ESTUDO DOS MECANISMOS PERIFÉRICOS ENVOLVIDOS

CAMPINAS
2014

Universidade Estadual de Campinas

Instituto de Biologia

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Thesis presented to the Institute of Biology of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor in Functional and Molecular Biology, in Physiology area.

Tese apresentada ao Instituto de Biologia da Universidade Estadual de Campinas como parte dos requisitos exigidos para obtenção do título de Doutora em Biologia Funcional e Molecular, na área de Fisiologia.

Orientadora: Profa. Dra. Claudia Herrera Tambeli

Este exemplar corresponde à versão final da tese defendida pela aluna Juliana Maia Teixeira e orientada pela Profa. Dra. Claudia Herrera Tambeli.

Claudia

CAMPINAS

2014

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca do Instituto de Biologia
Ana Marria Rabetti - CRB 8/2471

T235i

Teixeira, Juliana Maia, 1984-
Involvement of P2X3 and P2X7 purinergic receptors in inflammatory articular hyperalgesia in the knee joint of rats and the study of the peripheral mechanisms involved / Juliana Maia Teixeira. – Campinas, SP : [s.n.], 2014.

Orientador: Cláudia Herrera Tambeli.
Tese (doutorado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. Hiperalgesia. 2. Receptores purinérgicos. 3. Condrócitos. 4. Citocinas. 5. Neutrófilos. I. Tambeli, Cláudia Herrera,1969-. II. Universidade Estadual de Campinas. Instituto de Biologia. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: Participação dos receptores purinérgicos P2X3 e P2X7 na hiperalgesia inflamatória articular em joelho de ratos e o estudo dos mecanismos periféricos envolvidos
Palavras-chave em inglês:
Hyperalgesia
Purinergic receptors
Chondrocytes
Cytokines
Neutrophils
Área de concentração: Fisiologia
Titulação: Doutora em Biologia Funcional e Molecular
Banca examinadora:
Cláudia Herrera Tambeli [Orientador]
Gisele Picolo
Jozi Godoy Figueiredo
Marcelo Nicolás Muscará
Anderson Luiz Ferreira
Data de defesa: 27-05-2014
Programa de Pós-Graduação: Biologia Funcional e Molecular

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Campinas, 27 de maio de 2014

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ABSTRACT

Osteoarthritis (OA) is a degenerative and progressive disease, characterized by cartilage breakdown which covers the bone ends and by synovial membrane inflammation, causing disability, joint swelling and pain. The relief of severe pain is the main goal of the acute treatment, but little is known about the mechanisms involved in the development of pain in OA. It has been demonstrated the role of ATP (adenosine 5'-triphosphate) in processes of hyperalgesia through activation of purinergic receptors P2X3, P2X2/3 and P2X7. Therefore, the aims of this study were: (1) to investigate the role of P2X3, P2X2/3 and P2X7 receptors in articular hyperalgesia in the knee joint arthritis model in males and estrus females rats and, if so, whether there are sex differences in the effect induced by the selective P2X3, P2X2/3 and P2X7 receptors antagonists. (2) to test the hypothesis that the carrageenan-induced articular inflammation increases the expression of P2X3 receptor in chondrocytes of articular cartilage of the knee joint. (3) to verify whether the mechanism by which the P2X3, P2X2/3 and P2X7 receptors activation contributes to articular hyperalgesia depends on previous pro-inflammatory cytokines release and neutrophil migration. (4) to investigate whether the P2X3, P2X2/3 and P2X7 receptors activation induces articular hyperalgesia in the rat's knee joint which depends on release of inflammatory mediators. (5) to verify whether the activation of P2X3, P2X2/3 and P2X7 receptors contributes to the articular hyperalgesia induced by the inflammatory mediators bradykinin, pro-inflammatory cytokines, PGE_2 and dopamine. For the aims 1, 4 and 5, the articular hyperalgesia was quantified by the rat knee joint Incapacitation Test. The immunofluorescence method was used for the aim 2. For aims 3 and 4, the ELISA and MPO immunoenzymatic assays were used. The results demonstrate that P2X3, P2X2/3 and P2X7 receptors activation by endogenous ATP is essential for the development of carrageenan-induced articular hyperalgesia in the knee joint of

male and estrus female rats, which are more sensitive than males to anti-hyperalgesic and anti-inflammatory effects induced by the P2X7 receptor antagonist. During carrageenan-induced joint inflammation occurs an increased of P2X3 receptors expression in chondrocytes of the articular cartilage. The essential role played by P2X3, P2X2/3 and P2X7 receptors in the development of articular hyperalgesia is mediated by an indirect sensitization of the primary afferent nociceptors dependent on the previous pro-inflammatory cytokines release and neutrophil migration. Moreover, the P2X3, P2X2/3 and P2X7 receptors activation induces articular hyperalgesia which depends on bradykinin, sympathomimetic amines, prostaglandins and pro-inflammatory cytokines release. Finally, the articular hyperalgesia induced by inflammatory mediators bradykinin, PGE_2 and dopamine depends on the P2X3 and P2X2/3 receptors activation, while the P2X7 receptor activation contributes to the bradykinin- and dopamine- induced articular hyperalgesia. In conclusion, our results suggest that P2X3, P2X2/3 and P2X7 receptors are interesting pharmacological targets for the treatment of inflammatory joint diseases such as osteoarthritis. In particular, selective P2X7 receptor antagonists can be used to reduce inflammation and pain in the knee joint, especially in women.

Keywords: Articular hyperalgesia, P2X3 and P2X7 purinergic receptors, Chondrocytes, Pro-inflammatory cytokines, Neutrophil migration.

RESUMO

A osteoartrite (OA) é uma doença degenerativa e progressiva, caracterizada pela degradação da cartilagem que reveste as extremidades ósseas e inflamação da membrana sinovial, causando incapacidade física, inchaço articular e dor. Embora o alívio da dor severa seja o principal objetivo no tratamento agudo, pouco se sabe sobre os mecanismos envolvidos no desenvolvimento da dor na OA. Estudos demonstram a participação do ATP (adenosina 5'-trifosfato) em processos de hiperalgesia através da ativação dos receptores purinérgicos P2X3, P2X2/3 e P2X7. Portanto, os objetivos deste estudo foram: (1) investigar a participação dos receptores P2X3, P2X2/3 e P2X7 na hiperalgesia articular em modelo de artrite na articulação do joelho de ratos machos e fêmeas em estro e se há diferenças sexuais no efeito induzido pelos antagonistas de receptores P2X3, P2X2/3 e P2X7. (2) testar a hipótese de que a inflamação articular induzida pela carragenina aumenta a expressão do receptor P2X3 nos condrócitos da cartilagem articular da articulação do joelho de ratos. (3) verificar se o mecanismo pelo qual a ativação dos receptores P2X3, P2X2/3 e P2X7 contribui para a hiperalgesia articular depende da liberação prévia de citocinas pró-inflamatórias e da migração de neutrófilos. (4) investigar se a ativação dos receptores P2X3, P2X2/3 e P2X7 induz hiperalgesia na articulação do joelho de ratos dependente da liberação de mediadores inflamatórios. (5) testar a hipótese de que a ativação dos receptores P2X3, P2X2/3 e P2X7 contribui para a hiperalgesia articular induzida pelos mediadores inflamatórios: bradicinina, citocinas pró-inflamatórias, PGE_2 e dopamina. Para os objetivos 1, 4 e 5, a hiperalgesia articular foi quantificada através do teste de Incapacitação Articular. Para o objetivo 2, foi utilizado o ensaio de imunofluorescência. Para os objetivos 3 e 4 foram utilizados os ensaios imuno-enzimáticos ELISA e MPO. Os resultados demonstram que a ativação dos receptores P2X3, P2X2/3 e P2X7 pelo ATP endógeno é essencial para o

desenvolvimento da hiperalgesia articular induzida pela carragenina na articulação do joelho de ratos machos e fêmeas em estro, que são mais sensíveis do que os machos aos efeitos anti-hiperalgésicos e anti-inflamatórios induzidos pelo antagonista de receptor P2X7. Durante a inflamação articular induzida pela carragenina ocorre um aumento na expressão dos receptores P2X3 nos condrócitos da cartilagem articular. O papel dos receptores P2X3, P2X2/3 e P2X7 na hiperalgesia articular é mediado pela sensibilização indireta dos nociceptores aferentes primários, dependente da liberação prévia de citocinas pró-inflamatórias e da migração de neutrófilos. Além disso, a ativação dos receptores P2X3, P2X2/3 e P2X7 induz hiperalgesia articular dependente da liberação de bradicinina, aminas simpatomiméticas, prostaglandinas e citocinas pró-inflamatórias. Finalmente, a hiperalgesia articular induzida pelos mediadores inflamatórios bradicinina, PGE_2 e dopamina depende da ativação de receptores P2X3 e P2X2/3, enquanto que a ativação de receptor P2X7 contribui para a hiperalgesia articular induzida pela bradicinina e dopamina. Concluindo, os resultados apresentados sugerem que os receptores P2X3, P2X2/3 e P2X7 são alvos farmacológicos interessantes para o tratamento das doenças inflamatórias articulares como a osteoartrite. Particularmente em relação ao receptor P2X7, antagonistas seletivos podem ser usados para reduzir a dor e inflamação no joelho, especialmente em mulheres.

Palavras-chave: Hiperalgesia articular, Receptores purinérgicos P2X3 e P2X7, Condrócitos, Citocinas pró-inflamatórias, Migração de neutrófilo.

SUMÁRIO

DEDICATÓRIAS

*À **Deus**, pelo dom da vida, por não me deixar perder a fé nos momentos de dificuldade e por sempre iluminar meu caminho;*

*Aos meus pais, **Luiz e Vilma**, pelo amor incondicional e exemplo de pessoas, pela dedicação incansável e por serem o alicerce da minha vida;*

*Ao meu querido marido **Neto**, amor da minha vida, por ser meu grande incentivador, por ter me amparado todos os dias no período em que passamos distantes durante meu estágio no exterior, por sempre ser meu companheiro na concretização dos meus e dos nossos sonhos e por todo amor, apoio, carinho, cumplicidade e felicidade.*

*Aos meus irmãos **Fabrício e Júnior**, por estarem sempre torcendo por mim e pela amizade, carinho e admiração.*

AGRADECIMENTOS

À **Universidade Estadual de Campinas** por meio do seu Magnífico Reitor, Prof. Dr. José Tadeu Jorge.

Ao **Instituto de Biologia** da Universidade Estadual de Campinas, na pessoa de sua Diretora, Profa. Dra. Shirlei Pimentel. À **Coordenadoria dos Cursos de Pós-Graduação do Instituto de Biologia da** Universidade Estadual de Campinas, na pessoa de sua coordenadora Profa. Dra. Sandra Carmello-Guerreiro. À **Coordenadoria do Programa de Pós-Graduação em Biologia Funcional e Molecular** do Instituto de Biologia da Universidade Estadual de Campinas, na pessoa de seu coordenador Prof. Dr. Carlos Amílcar Parada.

Ao **Departamento de Biologia Estrutural e Funcional** do Instituto de Biologia da Universidade Estadual de Campinas, na pessoa de chefe do departamento Prof. Dr. Alexandre Leite Rodrigues de Oliveira.

À minha orientadora **Profa. Dra. Cláudia Herrera Tambeli**, agradeço muito as oportunidades e a confiança depositada em meu trabalho ao longo desses nove anos. Obrigada pela orientação, incentivo, respeito e amizade. Seu exemplo de dedicação e competência é muito importante para o meu amadurecimento científico.

Ao **Prof. Dr. Carlos Amílcar Parada**, pelo apoio, ideias, entusiasmo e discussões científicas, fundamentais para a realização deste trabalho;

To **Professor Kathleen A. Sluka** for welcome me in her laboratory with all cordiality. Thank you for sharing a little piece of your scientific experience and make possible such a grateful experience;

To all the technicians and students of **Neurobiology of Pain Laboratory, University of Iowa**, specially Lynn, Sandra, Jing, Ann, Weiyi, Nick and Eric for the scientific support, patience and friendship. You all really made my stay in Iowa City better;

Aos membros da banca examinadora do exame de Qualificação, **Prof. Dr. Anderson Luiz Ferreira**, **Profa. Dra. Elenice de Moraes Ferrari** e **Profa. Dra. Dora Grassi Kassisse**, pelas considerações e sugestões;

Aos membros da banca examinadora de defesa de Tese, **Prof. Dr. Marcelo Nicolás Muscará**, **Profa. Dra. Gisele Picolo**, **Profa. Dra. Jozi Godoy Figueiredo**, **Prof. Dr. Anderson Luiz Ferreira, Prof. Dr. César Renato Sartori**, **Profa. Dra. Vanessa Olzon Zambelli** e **Prof. Dr. Edson Antunes**, por aceitarem avaliar esse trabalho e por me atenderem prontamente com atenção;

À querida **Profa. Dra. Maria Cláudia G. de Oliveira Fusaro**, agradeço pela amizade, apoio e todos os ensinamentos no início da minha carreira;

Às queridas amigas **Dionéia Araldi**, **Elayne Veira Dias**, **Jalile Garcia**, **Maria Carolina Athiê**, **Lilian Calili** e **Lilian Wünsch**, por compartilharem inúmeros momentos divertidos e também as angústias, pelas conversas, ajuda, carinho e muitas risadas;

Aos antigos e novos colegas de laboratório **André Vieira**, **Ivan Bonet**, **Filipe Prado**, **Amanda Neves**, **Rafaella Ignarro**, **Paulinha Marião**, **Gilson Gonçalves**, **Luis Manzo**, **Kiko Pagliusi**, **Felipe de Faria**, **Catarine Nishijima**, pela ajuda e pelos ótimos momentos de convívio;

Aos amigos da FOP: **Nádia Moreira**, **Karla Helena T. Chavez**, **Gustavo Obando**, **Patrícia Lima**, **Rafaela Costa**, **Andrea Sanches**, **Luana Fischer**, **Juliana Napimoga**, **Letícia Fanton**, **Ana Paula Botelho, Priscila Kawashita, Cristina Macedo** e **Monaliza Lamana**, pelos ensinamentos fundamentais e por todo o carinho e companheirismo;

À querida amiga **Franciane Bobinski**, por ter sido minha irmã durante minha passagem por Iowa City, dividindo muitos momentos felizes e os tristes também, quando a saudade da família e do Brasil apertava o coração;

Aos amigos Brazucas em Iowa City: **Lucindo Quintans**, **Ana Paula Monteiro**, **Renan Guedes**, **Hersília Santos**, **Evelin Rodrigues**, **Aline Hilzendeger** e **Bruno Uzeda**, por toda ajuda, apoio e por diminuírem minha saudade de casa;

À minha aluna de iniciação científica **Samantha Camargo**, pelas colaborações, conversas e por participar da minha formação como docente;

Às queridas amigas **Bruna de Araújo Lima** e **Renata Proença Ferreira**, pela torcida a cada vitória e pela amizade ao longo desses onze anos;

À **Andréia Ap. Vigilato dos Santos**, secretária do Programa de Pós-Graduação em Biologia Funcional e Molecular e à **Alexandra Ferraz**, secretária do Departamento Biologia Estrutural e Funcional, agradeço pela ajuda, atenção e gentileza dispensada durante esses anos de convívio como aluna de pós-graduação;

À **Fundação de Amparo à Pesquisa do Estado de São Paulo** (**FAPESP)**, pela bolsa de estudos concedida (2009/16854-3) e pelo auxílio à pesquisa (2010/05381-4), essenciais para a realização deste trabalho;

Ao **CEMIB**, por fornecer os animais de pesquisa utilizados neste trabalho, aos quais devo eterno reconhecimento e respeito;

Aos meus familiares: avós, tios, primos, sogra, cunhados e sobrinhos, por estarem sempre presentes, me incentivando e torcendo por mim;

À todos meus amigos "fora do ambiente acadêmico", que são fundamentais na minha vida, por torcerem sempre por mim, pelo orgulho e admiração. Obrigada pela torcida!

Meu agradecimento a todas as pessoas que participaram e contribuíram, direta ou indiretamente, para realização deste trabalho e também a todos que passaram por minha vida durante este período. Muito Obrigada!

"Os que desprezam os pequenos acontecimentos nunca farão grandes descobertas. Pequenos momentos mudam grandes rotas".

Augusto Cury

I. INTRODUÇÃO

I.I. Dor e a Osteoartrite

A dor é um fenômeno complexo que pode ser definido como uma percepção desagradável de uma sensação nociceptiva e que envolve uma série de aspectos tanto cognitivos quanto sensoriais. Essa definição envolve dois componentes: a percepção e a nocicepção. Percepção dolorosa é uma função integrativa modulada por condições motivacionais, emocionais e psicológicas, bem como pela história pregressa individual (MERSKEY & BOGDUK, 1994). Nocicepção (do latim *nocere*, "ferir"), ou sensação nociceptiva, resulta da ativação de uma população específica de neurônios aferentes primários, denominados de nociceptores, que são amplamente distribuídos por todo o organismo, presentes na pele, membranas, mucosas, vísceras, músculos, tendões, articulações e vasos sanguíneos (JULIUS & BASBAUM, 2001; ALMEIDA *et al.*, 2004; WILLIS, 2007; GOLD & GEBHART, 2010). Os nociceptores detectam seletivamente estímulos capazes de comprometer a integridade física do organismo e transmitem a informação nociceptiva para o sistema nervoso central (MILLAN, 1999; JULIUS & BASBAUM, 2001; WILLIS, 2007).

Em determinadas condições, quando os estímulos nocivos são intensos e resultam em lesão tecidual, a dor é acompanhada por fenômenos paralelos, como a hiperalgesia. Este fenômeno é resultado da sensibilização das fibras neuronais sensoriais responsáveis pela ativação do sistema nociceptivo. Essa sensibilização dos nociceptores, caracterizada eletrofisiologicamente pela diminuição do limiar de excitabilidade necessário para ativar o nociceptor (RIEDEL & NEECK, 2001), ocorre por ação de mediadores inflamatórios que são produzidos durante e no final do processo inflamatório (HUANG *et al.*, 2006; VERRI *et al.*,

2006). Estes mediadores inflamatórios atuam em seus respectivos receptores, induzindo como resultado final, alterações metabólicas que facilitarão a produção de potenciais de ação pelos nociceptores (FERREIRA *et al.*, 1978; NAKAMURA & FERREIRA, 1987).

A osteoartrite (OA) é uma doença degenerativa e progressiva, na qual a cartilagem que reveste as extremidades ósseas se deteriora, causando diferentes graus de incapacidade, inchaço articular, dor e inflamação (ROCHA *et al*., 1999; HUNTER, 2009). Pode afetar toda a estrutura articular, incluindo a membrana sinovial, a cartilagem articular, o osso subcondral, o menisco e os músculos periarticulares (FELSON, 2006) e os fatores de risco incluem a obesidade, o envelhecimento, lesão articular e fatores mecânicos e metabólicos (FELSON, 2003). Esta artropatia, que corresponde à forma mais comum de artrite, pode ocorrer em qualquer articulação do corpo, mas sua ocorrência é mais comum nos joelhos, nos quadris e nas mãos (HUNTER & ECKSTEIN, 2009) e tem sido relatado que as mulheres têm maior prevalência de OA na articulação do joelho do que os homens (NEVITT & FELSON, 1996; FELSON & NEVITT, 1998; SRIKANTH *et al.*, 2005, FELSON, 2006).

Tem sido sugerido que a inflamação articular em casos de OA é o resultado, pelo menos em parte, de uma interação cíclica entre a cartilagem danificada e a membrana sinovial inflamada. Estudos demonstraram uma relação estreita entre a expressão de citocinas pró-inflamatórias e os sintomas da OA (FERNANDES *et al.*, 2002; STANNUS *et al*., 2010). Os produtos da degradação da cartilagem são fagocitados pelas células sinoviais, resultando em uma sinóvia inflamada, que por sua vez produz mediadores e citocinas pró-inflamatórias. Isto resulta em um aumento da liberação de enzimas proteolíticas, que por sua vez colaboram com a degradação da cartilagem (SELLAM & BERENBAUM, 2010).

A dor e a rigidez nas articulações com consequente limitação dos movimentos, redução da participação em atividades laborais e baixa qualidade de vida são os principais problemas enfrentados por pacientes com artropatias inflamatórias (YELIN *et al.*, 2007) e embora o alívio da dor severa seja frequentemente o principal objetivo no tratamento agudo dessas condições, os tratamentos analgésicos atuais têm ou eficácia incompleta, ou efeitos adversos potencialmente graves, limitando as opções de tratamento para os pacientes com OA (ROCHA *et al.*, 1999; BERENBAUM, 2011). Os processos periféricos envolvidos na dor em condições como a OA são pouco conhecidos, desta forma uma melhor compreensão desses mecanismos pode permitir avanços no desenvolvimento de terapias locais para o tratamento dos sintomas da OA (HUNTER, 2009).

I.II. Modelos experimentais para o estudo da artrite

Os aspectos da fisiopatologia da artrite do joelho tais como dor e inflamação associadas com a destruição periarticular, podem ser estudados através de modelos pré-clínicos pela injeção de agentes inflamatórios na articulação do joelho de roedores (TONUSSI & FERREIRA, 1992; ROCHA *et al.*, 1999). O teste de incapacitação articular (IA) para ratos foi desenvolvido para avaliar indiretamente a dor articular do joelho através da perturbação da marcha do animal, definida como a incapacidade de um animal (rato) para deambular normalmente, depois de submetido à injeção intra-articular de um agente inflamatório (TONUSSI & FERREIRA, 1992).

A carragenina é um mucopolissacarídeo sulfatado extraído de algas marinhas vermelhas, denominadas *Chondrus*, comumente usado como um agente inflamatório por provocar uma reação inflamatória local aguda (GARDNER, 1960), resultando em edema e hiperalgesia, que são os sintomas mais frequentes das artropatias em seres humanos. Vários tipos de carragenina

podem ser encontrados de acordo com seu teor de sulfato e sua configuração estrutural: kappa (κ), iota (ι) e lambda (λ), sendo o último o mais eficiente como irritante por ser mais rico em resíduos sulfatados (DI ROSA *et al.*, 1971).

A injeção intra-articular de carragenina em ratos é um modelo experimental amplamente utilizado para estudar a hiperalgesia e inflamação nas articulações (SLUKA *et al.*, 1997; SOLANO & HERRERO, 1999; LAWAND *et al.*, 2000; MIN *et al.*, 2001; OLIVEIRA *et al.*, 2005; RODRIGUES *et al.*, 2006; TEIXEIRA *et al.*, 2010a; VALENTI *et al.*, 2010; GOMIS *et al.*, 2013). A carragenina induz hiperalgesia mediada pela liberação de dois mediadores inflamatórios finais, as prostaglandinas e as aminas simpatomiméticas, que sensibilizam diretamente os nociceptores aferentes primários (GOLD *et al.*, 1996; KHASAR *et al.*, 1999; RUSH & WAXMAN, 2004). A produção desses mediadores finais depende da liberação prévia de uma cascata de citocinas, que envolve inicialmente a formação da bradicinina, que por sua vez, induz à liberação da citocina pró-inflamatória fator de necrose tumoral α (TNFα) (FERREIRA *et al.*, 1993a). Esta citocina desencadeia a liberação de duas vias distintas de citocinas, uma mediada pela interleucina 1β (IL-1β) e interleucina 6 (IL-6) que estimulam a síntese da ciclooxigenase-2 (COX-2) convertendo o ácido araquidônico em prostaglandinas (PGE) e outra mediada pela interleucina 8 (IL-8, em humanos) ou CINC-1 (em ratos) que estimula a produção de aminas simpatomiméticas (CUNHA *et al.*, 1991; CUNHA *et al.*, 1992; LORENZETTI *et al.*, 2002).

I.III. O ATP como mediador inflamatório

Além dos mediadores inflamatórios citados, estudos demonstraram o papel importante do ATP (adenosina 5'-trifosfato) como mediador da hiperalgesia inflamatória (WU *et al.*, 2004;

MCGARAUGHTY & JARVIS, 2005; OLIVEIRA *et al.*, 2005; WANG *et al.*, 2007; TEIXEIRA *et al.*, 2010a e b; PRADO *et al*., 2013).

O ATP está presente em todas as células do corpo (MCCLESKEY & GOLD, 1999), uma vez que é a fonte de energia essencial das células. Experimentos demonstraram que algumas fibras nervosas sensoriais liberam ATP (HOLTON, 1959) e essa descoberta levou à proposição do termo "neurônios purinérgicos" (BURNSTOCK, 1972). Esses achados foram muito importantes, pois evidenciaram o papel extracelular do ATP, que até então era somente conhecido por sua função intracelular. Atualmente existem inúmeras evidências da ação do ATP extracelular como molécula sinalizadora em diversos processos fisiológicos e patológicos (BURNSTOCK & SAWYNOK, 2010; BURNSTOCK, 2013).

No meio extracelular o ATP exerce suas funções por meio da ativação de receptores conhecidos como purinérgicos, que são distinguidos em dois tipos, nomeados de P1 e P2, os quais medeiam as funções fisiológicas da adenosina e do ATP, respectivamente (ABBRACCHIO & BURNSTOCK, 1998). Em meados da década de 1990, vários estudos demonstraram a diversificada distribuição desses receptores nos tecidos de mamíferos e em 1994, estudos que evidenciaram as diferenças estruturais e propriedades eletrofisiológicas dos receptores P2 levaram a proposição de um novo sistema de divisão dos receptores P2 em duas grandes famílias: Receptores P2Y: metabotrópicos, acoplados à proteína G e Receptores P2X: ionotrópicos ligante-dependentes (FREDHOLM *et al.*, 1994; HYNIE, 1995).

I.IV. Os receptores purinérgicos P2X3 e P2X7

Dados obtidos em nosso laboratório demonstram a participação do ATP endógeno no desenvolvimento da hiperalgesia inflamatória induzida pela carragenina na articulação

temporomandibular (OLIVEIRA *et al.*, 2005; TEIXEIRA *et al.*, 2010a) e no tecido subcutâneo da pata de ratos através da ativação de receptores purinérgicos (OLIVEIRA *et al.*, 2009; TEIXEIRA *et al.*, 2010b). Durante o processo inflamatório, o ATP é liberado das células lesadas, tais como macrófagos, plaquetas, neutrófilos, bem como células mortas (DUBYAK & EL-MOATASSIM, 1993, BEIGI *et al*., 1999, SIKORA *et al*., 1999, CAMPWALA & FOUNTAIN, 2013) para o meio extracelular e contribui com o desenvolvimento da hiperalgesia inflamatória via ativação dos receptores purinérgicos P2X.

Especificamente, os receptores purinérgicos P2X são canais ionotrópicos ativados pelo ATP, cuja ativação induz despolarização da membrana celular através do aumento da permeabilidade ao Na^+, K^+ e Ca^{2+} (DUBYAK & EL-MOATASSIM, 1993; VALERA *et al.*, 1994). Dentre os sete subtipos de receptores purinérgicos P2X (P2X1 - P2X7) (BUELL *et al.*, 1996), os subtipos P2X3 e P2X7 têm sido muito estudados por estarem envolvidos em processos de hiperalgesia e inflamação em vários tecidos, como por exemplo, na pele (DELL'ANTONIO *et al.*, 2002a e b; MCGARAUGHTY *et al.*, 2003; WU *et al.*, 2004; CHESSELL *et al.*, 2005; FULGENZI *et al.*, 2005; MCGARAUGHTY *et al.*, 2007; OLIVEIRA *et al.*, 2009; TEIXEIRA *et al.*, 2010b; PRADO *et al*., 2013), no tecido muscular (HORI *et al*., 2010), em vísceras (FULGENZI *et al.*, 2008; BURNSTOCK, 2012), na polpa dental (RENTON et al., 2003), além de tecidos articulares como a articulação temporomandibular (ATM) e articulação do joelho de ratos (SHINODA *et al.*, 2005; SEINO *et al.*, 2006; BROOM *et al.*, 2008; TEIXEIRA *et al.*, 2010a).

Os receptores P2X3 e P2X2/3 estão localizados nos terminais sensoriais aferentes periféricos e centrais de fibras C amielínicas e de fibras Aδ mielínicas, predominantemente na subpopulação não peptidérgica dos nociceptores, onde medeiam a neurotransmissão sensorial

(BURNSTOCK & KNIGHT, 2004; GEVER *et al.*, 2006). A expressão desses receptores em células não neuronais é pouco explorada, mas foi demonstrado que queratinócitos da epiderme possuem RNA mensageiro para o receptor P2X3 (INOUE *et al.*, 2005) e que células endoteliais do timo (GLASS *et al.*, 2000), células uroteliais (SUN & CHAI, 2004) e condrócitos (VARANI *et al*., 2008) expressam o receptor purinérgico P2X3.

Em condições normais, os condrócitos são responsáveis por manter um equilíbrio dinâmico entre a síntese e degradação da matriz extracelular, fornecendo funcionalidade mecânica à articulação (VARANI *et al.*, 2008). Em doenças degenerativas como a OA, o metabolismo dos condrócitos é instável devido à produção excessiva de citocinas pró-inflamatórias e de enzimas que degradam a matriz extracelular pela membrana sinovial inflamada. Consequentemente, os condrócitos respondem a essas condições inflamatórias, participando das atividades catabólicas que levam à degradação da matriz cartilaginosa, incluindo o aumento da liberação de óxido nítrico (NO) e de mediadores inflamatórios como a PGE_2 (de MATTEI *et al.*, 2002; VARANI *et al*., 2008; LEE *et al.*, 2013). Ainda, estudos *in vitro* têm demonstrado que a presença de IL-1β (GUERNE *et al*., 1990; LOTZ *et al*., 1992; AIDA *et al*., 2006) ou do fluído sinovial de pacientes com OA (HOFF *et al*., 2013) estimulam a produção de citocinas pró-inflamatórias pelos condrócitos.

Sabe-se que o ATP extracelular é frequentemente encontrado no líquido sinovial de pacientes com artropatias (RYAN *et al*., 1991; KUMAHASHI *et al*., 2011) e tem sido sugerido que os nucleotídeos extracelulares, sinalizando através de receptores purinérgicos, podem desempenhar um papel importante na regulação do metabolismo da cartilagem (HOEBERTZ *et al.*, 2003). Além disso, foi demonstrado que o ATP e o difosfato de adenosina (ADP) estimulam a produção de PGE em cultura de condrócitos (CASWELL *et al*., 1991), que pode ser aumentada

na presença das citocinas pró-inflamatórias IL-1β , IL-1α e TNF-α (CASWELL *et al*., 1992; LEONG *et al*., 1993; KOOLPE *et al*., 1999).

Trabalhos desenvolvidos em nosso laboratório demonstraram que o ATP extracelular, através da ativação dos receptores P2X3 e P2X2/3, é essencial para o desenvolvimento da hiperalgesia mecânica induzida pela carragenina no tecido subcutâneo da pata de ratos (OLIVEIRA *et al.*, 2009) e para a hiperalgesia induzida pela carragenina na ATM de ratos (TEIXEIRA *et al.*, 2010a). Um estudo mostrou que receptores P2X3 são expressos nas fibras nociceptivas aferentes que inervam o tecido articular dos joelhos de ratos (DOWD *et al.*, 1998). Além disso, foi sugerido que a ativação do receptor P2X3 está envolvida na hipersensibilidade mecânica induzida por adjuvante completo de Freund (CFA) da articulação do joelho de ratos (SEINO *et al.*, 2006).

Em relação aos receptores P2X7, no tecido periférico eles são seletivamente expressos em células de origem hematopoiéticas, incluindo mastócitos, linfócitos, eritrócitos, fibroblastos, monócitos, macrófagos periféricos e sinoviócitos do tipo B (SURPRENANT *et al.*, 1996; COLLO *et al.*, 1997; MANCINO *et al.*, 2001; CAPORALI *et al*., 2008). No SNC, receptores P2X7 funcionais estão localizados na micróglia, astrócitos e em células de Schwann (COLLO *et al.*, 1997; SIM *et al.*, 2004). Nos gânglios das raízes dorsais, os receptores P2X7 parecem ser seletivamente localizados em células da glia (ZHANG *et al.*, 2005).

Sabe-se que o receptor P2X7 apresenta um papel importante no desenvolvimento dos processos inflamatórios por modular a produção de mediadores inflamatórios (LISTER *et al.*, 2007). Durante a inflamação induzida por CFA ou lipopolissacarídeos (LPS), o receptor P2X7 contribui com a liberação de citocinas pró-inflamatórias (IL-1β, IL-6, IL-18 e TNFα) relacionadas à produção de mediadores inflamatórios finais (CHESSELL *et al.*, 2005; FERRARI

et al., 2006; HONORE *et al.*, 2006; LISTER *et al.*, 2007; MINGAM *et al.*, 2008) e durante a inflamação induzida pela carragenina, os receptores P2X7 contribuem com a liberação das quimiocinas pró-inflamatórias: proteína quimiotática de monócitos-1 (MCP-1), proteína induzida por Interferon gama-10 (IP-10) e IL-8, relacionadas à migração e ativação de células inflamatórias (FULGENZI *et al.*, 2005).

Estudos prévios demonstraram que a ativação do receptor P2X7 em células estimuladas com LPS, como por exemplo, macrófagos, micróglia e célula de Schwann, promove a conversão da pró-IL-1β na forma madura IL-1β (através da ativação da caspase-1, enzima conversora da IL-1β, THORNBERRY, 1997) e sua liberação para o meio extracelular (SANZ & DI VIRGILIO, 2000; SOLLE *et al*., 2001; COLOMAR *et al*., 2003; KAHLERBERG & DUBYAK, 2004). Além disso, foi demonstrado que o efeito anti-hiperalgésico do bloqueio do receptor P2X7 na hiperalgesia induzida pelo CFA é mediado pela modulação da atividade da IL-1β (HONORE *et al*., 2009).

Dados obtidos em nosso laboratório demonstraram que a ativação do receptor P2X7 pelo ATP endógeno é essencial para o desenvolvimento da hiperalgesia mecânica induzida pela carragenina no tecido subcutâneo da pata de ratos, além de contribuir com a liberação das citocinas pró-inflamatórias TNFα e IL-6 e da quimiocina CINC-1 no mesmo tecido (TEIXEIRA *et al.*, 2010b). Estudos anteriores mostraram que os receptores P2X7 também participam em processos de artrite, pois a severidade da artrite induzida por anticorpo monoclonal anti colágeno é atenuada em camundongos *knockout* para receptores P2X7 quando comparados aos animais normais (LABASI *et al.*, 2002) e o bloqueio do receptor P2X7 previne a ocorrência de danos histopatológicos e o aparecimento de sinais e sintomas da artrite induzida por anti colágeno em ratos (BROOM *et al.*, 2008).

Considerando que a articulação do joelho é uma das mais afetadas pelas artropatias inflamatórias e que estudos demonstraram que os receptores P2X3 e P2X7 são importantes em processos de artrite, o papel desses receptores e os mecanismos periféricos envolvidos na hiperalgesia inflamatória induzida pela carragenina na articulação do joelho ainda não eram conhecidos. Além disso, estudos têm relatado diferenças sexuais no efeito analgésico de drogas, por exemplo, na ação de agonistas kappa opióides (CLEMENTE *et al*., 2004; CLEMENTE-NAPIMOGA *et al*., 2009) e de antagonistas β-adrenérgicos (FAVARO-MOREIRA *et al*., 2012) na nocicepção da ATM entre ratos machos e fêmeas, onde as fêmeas com baixos níveis de hormônios ovarianos são mais responsivas à ação analgésica dessas drogas. Por outro lado, ratos machos são mais sensíveis aos efeitos antinociceptivos da morfina do que as fêmeas (CICERO *et al*., 1996; CAI *et al*., 2001).

Desta forma, o objetivo geral deste trabalho foi investigar a participação dos receptores purinérgicos P2X3, P2X2/3 e P2X7 na hiperalgesia inflamatória articular em modelo de artrite na articulação do joelho de ratos machos e fêmeas e os mecanismos periféricos envolvidos. Os objetivos específicos foram:

(1) Testar a hipótese de que a ativação dos receptores P2X3 e P2X2/3 através do ATP endógeno contribui para a hiperalgesia articular induzida pela carragenina na articulação do joelho de ratos machos e fêmeas em estro (níveis baixos de hormônios ovarianos) de forma dependente do sexo. Verificar se a inflamação articular induzida pela carragenina aumenta a expressão de receptor P2X3 nos condrócitos da cartilagem articular do joelho de ratos. Investigar se a contribuição dos receptores P2X3 e P2X2/3 para a hiperalgesia articular induzida pela carragenina ocorre através da liberação prévia de citocinas pró-inflamatórias e/ou migração de neutrófilos.

(2) Investigar se o ATP endógeno, através da ativação do receptor P2X7, contribui para a hiperalgesia articular induzida pela carragenina na articulação do joelho de ratos machos e fêmeas em estro e, em caso afirmativo, se essa contribuição depende da liberação prévia de citocinas pró-inflamatórias e/ou migração de neutrófilos. Testar a hipótese de que o efeito induzido pelo bloqueio do receptor P2X7 na articulação do joelho de ratos machos e fêmeas em estro é dependente do sexo.

(3) Verificar se a ativação dos receptores P2X3 e P2X2/3 na articulação do joelho de ratos induz hiperalgesia articular dependente da liberação de mediadores inflamatórios envolvidos na hiperalgesia, como a bradicinina, prostaglandinas, aminas simpatomiméticas, citocinas pró-inflamatórias e migração de neutrófilos.

(4) Verificar se a ativação do receptor P2X7 na articulação do joelho de ratos induz hiperalgesia articular dependente da liberação de mediadores inflamatórios envolvidos na hiperalgesia, como a bradicinina, prostaglandinas, aminas simpatomiméticas, citocinas pró-inflamatórias e migração de neutrófilos.

(5) Investigar se a ativação dos receptores P2X3, P2X2/3 e P2X7 contribui para a hiperalgesia articular induzida pelos mediadores inflamatórios pertencentes à cascata inflamatório da carragenina: bradicinina, TNF-α, IL-1β, IL-6, CINC-1, PGE_2 a dopamina.

O presente estudo está apresentado em formato alternativo, conforme deliberação da Comissão Central de Pós-graduação (CCPG) da Universidade Estadual de Campinas (UNICAMP) nº 002/2013.

II. CAPÍTULO 01

P2X3 and P2X2/3 receptors play a crucial role in hyperalgesia development through inflammatory mechanisms in the knee joint experimental synovitis

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Abstract

Osteoarthritis (OA) is a degenerative and progressive disease, characterized by cartilage breakdown which covers the bone ends and by synovial membrane inflammation, causing different degrees of disability, joint swelling and pain. The P2X3 and P2X2/3 receptors contribute to the development of inflammatory hyperalgesia, participate in arthritis processes in the knee joint and are expressed in chondrocytes and nociceptive afferent fibers innervating the knee joint. In this study, we hypothesized that P2X3 and P2X2/3 receptors activation by endogenous ATP induces articular hyperalgesia in the knee joint of males and females rats through an indirect sensitization of primary afferent nociceptors dependent on the previous release of pro-inflammatory cytokines and/or on neutrophil migration. We found that the blockade of articular P2X3 and P2X2/3 receptors attenuates the carrageenan-induced hyperalgesia in the knee joint of male and estrus female rats in a similar manner. The carrageenan-induced knee joint inflammation increased the expression of the P2X3 receptor in the chondrocytes of the articular cartilage. Further, the blockade of articular P2X3 and P2X2/3 receptors significantly reduced the increase of concentration of TNF-α, IL-6 and CINC-1 and the neutrophil migration induced by carrageenan. These findings indicate that P2X3 and P2X2/3 receptors activation by endogenous ATP is essential to hyperalgesia development in the knee joint through an indirect sensitization of primary afferent nociceptors dependent on the previous release of pro-inflammatory cytokines and/or on neutrophil migration.

Key words: articular hyperalgesia, P2X3 and P2X2/3 receptors, knee joint, chondrocytes, pro-inflammatory cytokines, neutrophil migration.

Introduction

Osteoarthritis (OA) is the most common form of arthritis. It is a progressive and degenerative disease with a higher prevalence in women than in men (Nevitt and Felson, 1996, Srikanth et al., 2005, Felson, 2006), characterized by deterioration of the cartilage covering the bone ends and by inflammation of the synovial membrane, causing limitation of motion, different degree of disability, synovitis and inflammatory pain (Rocha et al., 1999, Hunter, 2009, Sellam and Berenbaum, 2010). A better understanding of the peripheral processes linking inflammatory pain with OA is necessary for the improvement of the analgesic treatments of OA that have either incomplete efficacy, or potentially severe adverse events (Berenbaum, 2011).

Among the seven P2X purinergic receptor subtypes (P2X1-P2X7) (Buell et al., 1996), the P2X3 and P2X2/3 subtypes have been implicated in processes of pain and hyperalgesia in the articular tissues of the knee and temporomandibular joint (TMJ) of rats (Shinoda et al., 2005, Seino et al., 2006, Teixeira et al., 2010). The P2X3 and P2X2/3 receptors are localized on peripheral and central terminals of unmyelinated C-fiber and thinly myelinated Aδ sensory afferents, predominantly in the non-peptidergic subpopulation of nociceptors, where they mediate sensory neurotransmission (Bradbury et al., 1998, Burnstock and Knight, 2004, Gever et al., 2006). The expression of these receptors in non-neuronal cells has been weakly explored, but it has demonstrated that human epidermal keratinocytes express P2X3 receptors RNAm (Inoue et al., 2005) and that endothelial cells of thymus (Glass et al., 2000), urothelial cells (Sun and Chai, 2004) and chondrocytes (Varani et al., 2008) express P2X3 receptors. Although P2X3 and P2X2/3 are expressed on chondrocytes, it is not known whether their expression on these cells is increased during inflammation.

Studies have been reported that the involvement of some receptors in pain and analgesia processes is sex-dependent, for example females with lower levels of ovarian hormones are more responsive to some analgesic drugs than males rats (Clemente et al., 2004, Clemente-Napimoga et al., 2009, Favaro-Moreira et al., 2012). However, it is not known whether the involvement of P2X3 and P2X2/3 receptors in the carrageenan-induced articular hyperalgesia in the knee joint differs between male and estrus female rats (low levels of ovarian hormones).

In this study we used the carrageenan-induced knee joint inflammation model in rats (Tonussi and Ferreira, 1992, Ekundi-Valentim et al., 2010, Valenti et al., 2010, Gomis et al., 2013) to test the hypothesis that (I) the activation of P2X3 and P2X2/3 receptors by endogenous ATP contributes to carrageenan-induced articular hyperalgesia in the knee joint of males and estrus females (low levels of ovarian hormones) in a sex-dependent manner. (II) the carrageenan-induced articular inflammation increases the expression of P2X3 receptor in the chondrocytes of articular cartilage of the knee joint. (III) the contribution of P2X3 and P2X2/3 receptors activation to carrageenan-induced articular hyperalgesia occurs through an indirect sensitization of the primary afferent nociceptors dependent on the previous release of pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and chemokine-induced chemoattractant-1 (CINC-1, analog to IL-8 in rats) and/or on the migration of neutrophils to the inflamed knee joint.

Materials and Methods

Animals

Male and female Wistar rats (200-250g) obtained from the Multidisciplinary Center for Biological Research (CEMIB - UNICAMP, SP, Brazil) and from Harlan Laboratories (Madison, WI, USA), were used in this study. The animals were housed in plastic cages with soft bedding (five/cage) on a 12:12 light cycle (lights on at 06:00 A.M.) with food and water available *ad libitum*. They were maintained in a temperature-controlled room (±23°C) and handled for at least one week prior to the experiments. Each animal was used once and the number of animals per group was kept to a minimum. Experimental protocols were approved by the Committee on Animal Research of the University of Campinas (protocol number: 2049-1) and by the Animal Care and Use Committee at the University of Iowa and were conformed to IASP guidelines for the study of the pain in animals (Zimmermann, 1983).

General Procedures

Testing sessions took place during light phase (between 09:00 AM and 5:00 PM) in a quiet room maintained at 23ºC (Rosland, 1991). During the tests the animals had no access to water or food. The animals were habituated for 1 hour prior to the experiment to minimize stress.

Drugs administration

Drugs or their vehicle were intra-articularly administrated in the right rat's knee joint by a 26-gauge needle that was connected to a catheter of polyethylene and also to a Hamilton syringe. The volume of injection was 50 μL.

Carrageenan-induced knee joint inflammation (synovitis)

Under brief inhalation of isoflurane anesthesia, rats were subjected to intra-articular (i.a.) injection of λ-carrageenan dissolved in 25μL sterile 0.9% saline solution into their right knee joints (Tonussi and Ferreira, 1992, Ekundi-Valentim et al., 2010). The injection site was shaved and treated with an antiseptic solution of iodine alcohol. The other drugs were also injected into the knee joint in the same manner that the carrageenan and the control animals received vehicle or sterile 0.9% saline solution.

Drugs and doses

The following drugs were used: λ-carrageenan (Cg; 300 μg/knee, i.a., Tonussi and Ferreira, 1992, De-Melo et al., 1998, Tonussi and Ferreira, 1999, Ekundi-Valentim et al., 2010) and 5-([(3-Phenoxybenzyl) [(1S)-1,2,3,4-tetrahydro-1-naphthalenyl]amino]carbonyl)-1,2,4-benzenetricarboxylic acid (A-317491 - the selective P2X3 and P2X2/3 receptor antagonist: 20, 60, 180, 540 μg/knee, i.a., Oliveira et al., 2009). The drugs were obtained from Sigma-Aldrich (MO, USA) and dissolved in 25 μL sterile 0.9% saline solution.

Estrus phase determination of estrous cycle

Estrus phase in female rats was determined by daily microscope examination of vaginal smears taken by gentle lavage, between 9:00 and 10:00 a.m. Estrus phase was identified by the predominance (80 %) of anucleated cornified cells in rats with at least two consecutive regular 4-day cycles (Smith et al., 1975, Marcondes et al., 2002). This phase was chosen because it

represent phase of low ovarian hormonal level, 17β-estradiol and progesterone (Butcher et al., 1974, Spornitz et al., 1999).

Gait disturbance - Rat knee-joint incapacitation test

We used the rat knee-joint incapacitation test, as described previously (Tonussi and Ferreira, 1992). Briefly, 3 hours after carrageenan injection into their right knee joints, rats were put to walk on a steel rotary cylinder (30 cm wide x 50 cm diameter), covered with a fine-mesh non-oxidizable wire screen, which rotates at 3 rpm. Specially, designed metal gaiters were wrapped around both hind paws. After placement of the gaiters, rats were placed to walk in the cylinder and the right paw was then connected via a simple circuit to microcomputer data input/output port. The paw elevation time (PET) is the total time that the rat walks failing to touch the cylinder surface with the injected hindpaw, during a 60 sec period, which is directly proportional to the gait disturbance. Incapacitation was quantified as an increase in the PET, 3 hours after carrageenan injection into the right knee joint. To minimize variations in PET, all rats were introduced to the experimental environment and trained on the apparatus to habituation into the equipment before the testing sessions. To confirm the local effect of some test agents, they were injected into the contralateral rat's knee joint and the test was performed on the ipsilateral knee joint. The rat knee-joint incapacitation test provides automated measurements, which are independent of the subjectivity of the observer (Tonussi and Ferreira, 1992).

Tissue Preparation

Three hours after the carrageenan (300 μg/knee) or sterile 0.9% saline solution injection, the rats were anesthetized with sodium pentobarbital (120 mg/kg i.p.) and fixed with 4 %

paraformaldehyde (PFA, in 0.1 M phosphate buffer (PB), pH 7.4) by perfusion through the ascending aorta. The whole knee joints were rapidly removed and kept in the same fixative for 24 hours at 4ºC. The fixed specimens were decalcified for 8 weeks with 10% ethylenediaminetetraacetic acid (EDTA) in 0.01 M phosphate buffered saline (PBS) at 4ºC with three fresh solution changes per week (Ando et al., 2010). After the complete demineralization, the decalcified specimens were then rinsed thoroughly in PBS, placed in 30% sucrose overnight and embedded in OCT compound (Sakura Finetek, Torrace, California). All samples were rapidly frozen and stored at -80 ºC until being cut on cryostat. Serial sections were then cryosectioned at 20µm using a cryostat which were obtained at the medial midcondylar region in sagittal plane (Hagiwara et al., 2006, Ando et al., 2009).

Immunohistochemistry

Immunohistochemistry labeling was performed using the immunofluorescence method. Nonspecific binding sites were blocked with 10 % NGS (normal goat serum) for 30 minutes. Sections were then rinsed twice for 5 minutes in 0.1M PBS and incubated in primary antibody (1:1000, guinea pig Anti-P2X3 Receptor, AB5896, Chemicon-Millipore, MA, USA) diluted in 1% NGS and 0.05% Triton X-100 in 0.1M PBS and applied to the tissues overnight (4ºC) in a humid atmosphere. Sections were then rinsed twice for 5 minutes in 0.1M PBS and incubated in secondary antibody (1:1000, goat anti-guinea pig IgG-Alexa488, Life Technologies, Grand Island, NY, USA) diluted in 1 % NGS and 0.05 % Triton X-100 in 0.1M PBS for 1 hour at room temperature. Sections were then rinsed twice for 5 minutes in 0.1M PBS and incubated with TO-PRO3 (1:4000, 30 minutes; Invitrogen, Carlsbad, CA, USA) for nuclear staining. After a final washing, the slides were mounted using Vectashield (Vector Laboratories, Burlingame, CA,

USA). Negative controls were prepared without incubation in primary antibody to confirm that there was no non-specific binding of the secondary antibody. After staining, sections were examined with the Confocal Bio-Rad MRC 1024 Microscope and the images were taken with a 20× objective lens of the three specific regions of the knee joint: the articular cartilage covering the femoral condyle, the articular cartilage covering the tibial plateau and the meniscus. Five randomly selected knee sections were chosen for each sample (six samples per group) and were digitally imaged and stored for later analysis The density (mean, arbitrary units) of each section and the number of positive cells (chondrocytes) were quantified by manually counting total numbers in a given area using Image J (National Institutes of Health). Specifically, a standard size (average area of 75,190 μm^2 for the layer of cartilage covering the femoral condyle, 62,410 μm^2 for the layer of cartilage covering the tibial plateau and 93,630 μm^2 for meniscus) was applied to each section. Cells (chondrocytes) were counted if they were positively stained for P2X3 receptor.

Synovial lavage fluid

Under deep anaesthesia (induced by the intraperitoneal injection of 80 mg/kg ketamine and 20 mg/kg xylazine) the rats were then killed by cervical dislocation, the skin overlying the knee was excised, the patellar ligament was dissected and a 26-gauge needle connected to a 100 μL Hamilton syringe was inserted through the joint capsule. The knee joint cavity was washed twice by injecting and immediately aspirating 100 μL of phosphate-buffered saline solution (PBS) containing 4 mM EDTA (Ekundi-Valentim et al., 2010).

ELISA procedure

An adaptation of ELISA (Enzyme-Linked Immunosorbent Assay) (Safieh-Garabedian et al., 1995) was used to quantify the cytokines of the rat's knee joint. Briefly, the synovial lavage fluid was homogenized in solution of phosphate buffered saline (PBS) containing 0.4 M NaCl, 0.05% Tween 20, 0.5% bovine serum albumine (BSA), 0.1 mM phenyl-methylsulfonyl fluoride, 0.1 mM benzotonic chloride, 10 mM EDTA, and 20 KL/mL aprotinine (Sigma, USA). The samples were centrifuged at 10,000 rpm for 15 minutes at 4ºC and the supernatants were stored at -70ºC for posterior use to evaluate the protein levels of TNF-α, IL-1β, IL-6 and CINC-1 in the rat's knee joint. The cytokines were quantified by the following kits: TNF-α: Rat TNF-α/ TNFSF1A DuoSet ELISA Kit (R&D Systems, catalog number DY510); IL-1β: Rat IL-1β/IL-1F2 DuoSet ELISA Kit (R&D Systems, catalog number DY501), IL-6: Rat IL-6 DuoSet ELISA Kit (R&D Systems, catalog number: DY506) and CINC-1: Rat CXCL1/CINC-1 DuoSet ELISA Kit (R&D Systems, catalog number DY515). All procedures followed the instructions of the manufacturer R&D Systems. All samples and standards were run in triplicate and the procedures were repeated twice to guarantee the authenticity of the results. In the present study, the levels of cytokines (TNF-α, IL-1β, IL-6 e CINC-1) were assessed three hours after carrageenan administration.

Measurement of myeloperoxidase activity

Myeloperoxidase is one of the enzymes released from neutrophils and directly associated to tissue injury. Although monocytes/macrophages and fibroblasts also contain myeloperoxidase, neutrophils show the highest intracellular levels of this enzyme, that represents up to 5% of neutrophil proteins (Klebanoff, 1991). Therefore, the measurement of myeloperoxidase's activity was used as a marker of neutrophil migration (Klebanoff, 1991) in the knee joint of rats after

application of the stimulus. Three hours after carrageenan (300μg/knee) or 0.9% saline solution injection in the knee joint, synovial lavage fluid was collected and homogenized in 500μL of buffer 1 (0.1 M NaCl, 0.02 M $NaPO_4$,1.015 M Na EDTA, pH 5.4) followed by centrifugation at 3000 rpm for 15 min. The pellet was resuspended in 500 μL of buffer 1 and subjected to hypotonic lyses by the addition of 500 μL of 0.2% NaCl followed 30 seconds later by addition of 500 μL of 1.6% NaCl in 5% glucose. After a further centrifugation, the pellet was resuspended in 0.05 M $NaPO_4$ buffer (pH 5.4) containing 0.5% hexadecyl-trimethylammonium bromide (HTAB). After that, the samples were snap-frozen in liquid nitrogen and thawed, three times, and centrifuged at 10,000 rpm for 15 min.

The myeloperoxidase kinetic-colorimetric assay was conducted, as previously described (Bradley et al., 1982, Torres-Chavez et al., 2012). Fifty microliters of each sample (supernatant) and 0.08 M $NaPO_4$ were dropped into wells of a 96-well microplate. 25 μl of 3,3´,3,3´-tetramethylbenzidine (TMB) was added in each well and the reaction was initiated by the addition of 100 μL of H_2O_2. The reaction was stopped 5 minutes later by the addition of 50 μL of 4M H_2SO_4. The optical density was read at 450 nm using an Asys UVM340 (3 readings at intervals of 30 seconds). Results were calculated by comparing the optical density of rat's knee joint synovial lavage fluid supernatant with a standard curve of neutrophil (>95% purity). All procedures were repeated twice to guarantee the authenticity of the results. The results were presented as number of neutrophils/knee.

Statistical analysis

To determine if there were significant differences ($p<0.05$) between treatment groups, One-way ANOVA or T-test was performed. If there was a significant between-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. For data shown in Fig. 3B a Two-way repeated measures ANOVA with one between subjects factor (i.e., treatment) and one within-subjects factor (i.e., time) were used to determine whether there were significant ($p<0.05$) differences among the groups. If there was a significant between-subjects main effect of treatment group, post hoc contrasts using the Bonferroni test were performed to determine the basis of the significant difference. Data are expressed in figures as means ± S.E.M.

Results

Carrageenan-induced articular hyperalgesia in males and estrus females

In males and estrus females rats, the intra-articular administration of carrageenan (300 µg/knee) induced hyperalgesia in the rat's knee joint, measured 3 hours after the carrageenan administration (Fig. 1, $P<0.05$, one-way ANOVA post hoc Tukey test). The PET induced by intra-articular administration of 0.9% NaCl (50 µL/knee) was similar to that of untreated animals (Naive) (Fig. 1, $P>0.05$, one-way ANOVA post hoc Tukey test). The hyperalgesic response induced by carrageenan (300 µg/knee) in estrus females rats was not significantly different from that induced by carrageenan (300 µg/knee) in males rats (Fig. 1, $P>0.05$, one-way ANOVA post hoc Tukey test). Therefore, this equi-hyperalsegic dose of carrageenan (300 µg/knee) was used in subsequent experiments.

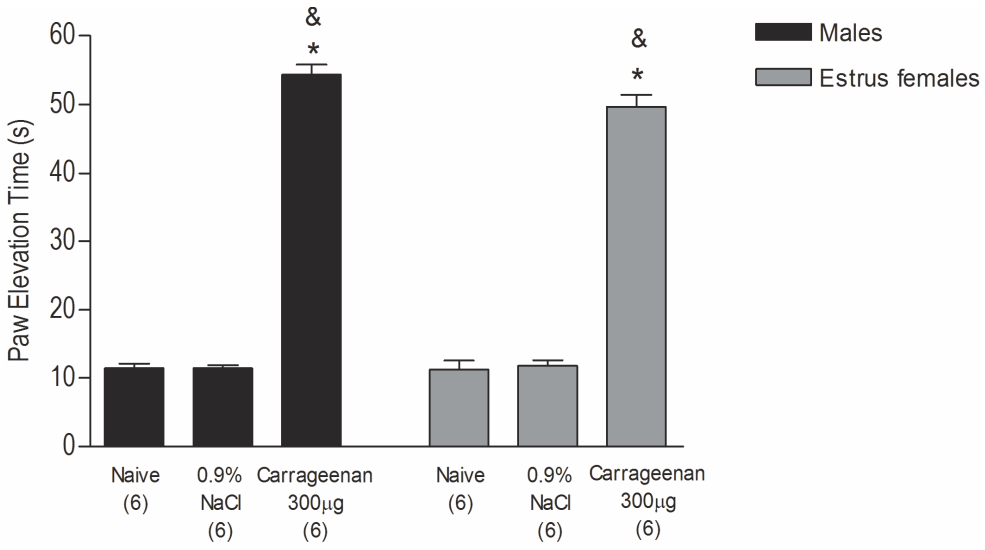

Figure 1 - Articular hyperalgesia induced by carrageenan in males and estrus females rats knee joint. Carrageenan (300 µg/knee, i.a.) induced an articular hyperalgesia in the knee joint of male and estrus female rats. The symbol "*" indicates a response significantly greater than that induced by 0.9% NaCl and Naive groups ($p<0.05$, Tukey test). The symbol "&" indicates equi-hyperalsegic responses induced by carrageenan (300 µg/knee, i.a.) in the knee joint of male and estrus female rats ($p>0.05$, Tukey test). In this and in the subsequent figures the articular hyperalgesia was measured 3 hours after the intra-articular (i.a.) carrageenan administration and the number of rats used are in parentheses.

The blockade of P2X3 and P2X2/3 receptors reduces the carrageenan-induced articular hyperalgesia in males and females in the same manner

To verify whether the activation of P2X3 and P2X2/3 receptors contributes to carrageenan-induced articular hyperalgesia in males and estrus females, and if so, to compare the effect of the P2X3 and P2X2/3 receptor antagonist A-317491 between males and estrus females, A-317491 was co-administrated with carrageenan (300 µg/knee) into the right knee joint of male and estrus female rats, and the hyperalgesic response was evaluated 3 hours after their administration. A-317491 at doses of 60, 180 and 540 µg/knee, but not 20µg/knee, significantly reduced the carrageenan-induced articular hyperalgesia (Fig. 2A and B, $P<0.05$, one-way

ANOVA post hoc Tukey test) in males and estrus females. The highest dose of A-317491 (540 µg/knee) did not affect the carrageenan-induced articular hyperalgesia when applied on the contralateral knee joint (Fig. 2A and B, P>0.05, one-way ANOVA post hoc Tukey test) of males and estrus females, confirming their local peripheral action. When A-317491 plus only 0.9% NaCl was applied in the right knee joint, it had no effect by itself (Fig. 2A and B, P>0.05, one-way ANOVA post hoc Tukey test) in males and estrus females.

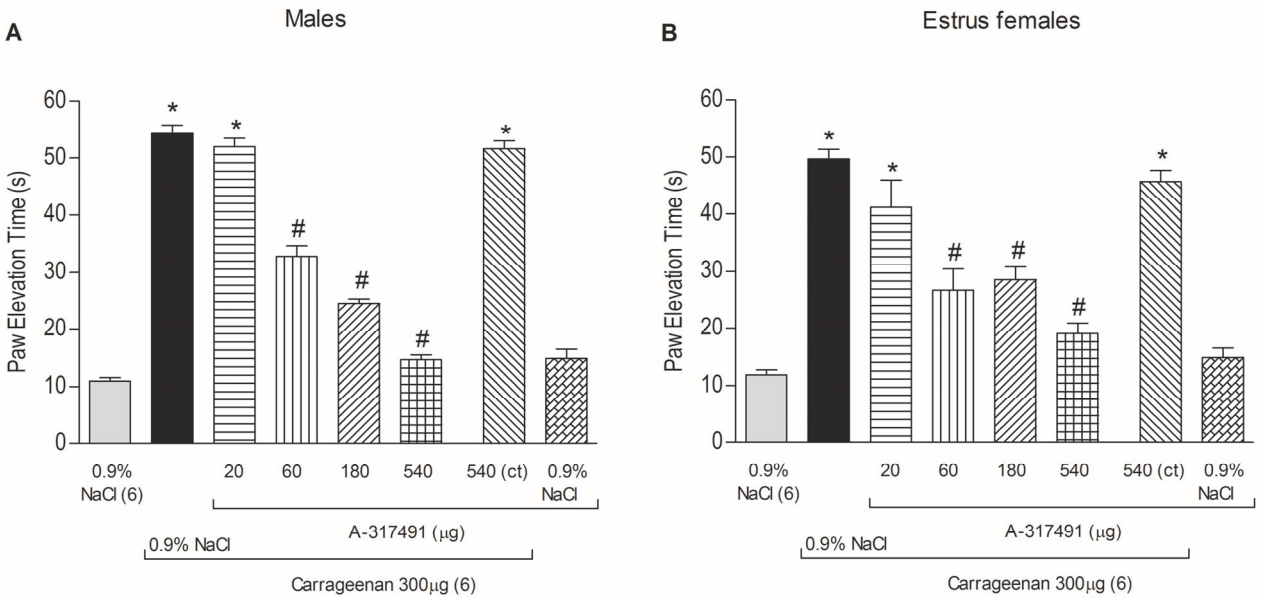

Figure 2 - Effect of the P2X3 and P2X2/3 receptors antagonist A-317491 on carrageenan-induced articular hyperalgesia in the knee joint of males and estrus females. Co-administration of A-317491 (60, 180 and 540 µg/knee) with carrageenan (300 µg/knee) significantly reduced carrageenan-induced articular hyperalgesia in males (**A**) and estrus females (**B**), as indicated by the symbol "#" ($p<0.05$, Tukey test). The highest dose of A-317491 (540 µg/knee) injected in the contralateral knee joint (ct) did not affect the carrageenan-induced articular hyperalgesia ($p>0.05$, Tukey test, **A** and **B**). A-317491 injected only with 0.9% NaCl had no effect by itself ($p>0.05$, Tukey test, **A** and **B**). The symbol "*" indicates a response significantly greater than that induced by 0.9% NaCl ($p<0.05$, Tukey test, **A** and **B**).

Because no differences were found in the effect of the different doses of the P2X3 and P2X2/3 receptor antagonist A-317491 on carrageenan-induced articular hyperalgesia between males and estrus females, the subsequent experiments were performed only in male rats.

Effect of blockade of articular P2X3 and P2X2/3 receptors on the development of carrageenan-induced articular hyperalgesia

To characterize the period of time at which the activation of P2X3 and P2X2/3 receptors contributes to the development of carrageenan-induced hyperalgesia in the knee joint, the A-317491 was co-administrated (0 hour) with carrageenan or administrated ½, 1, 2 or 3 hours after the carrageenan administration. Co-administration of A-317941 (540 μg/knee) with carrageenan (300 μg/knee) (Fig. 3A, $P<0.05$, one-way ANOVA post hoc Tukey test), but not its administration ½, 1, 2 or 3 hours after the carrageenan administration (Fig. 3A, $P>0.05$, one-way ANOVA post hoc Tukey test) significantly reduced the carrageenan-induced articular hyperalgesia.

In another set of experiments, A-317491 (540 μg/knee) was co-administrated with carrageenan (300 μg/knee) and the measurements were taken ½, 1, 2, 3, 4, 5, 6 and 24 hours later. The carrageenan-induced articular hyperalgesia reached its maximum 3 hours after its administration (Fig. 3B). A-317491 blocked the carrageenan-induced articular hyperalgesia 1, 2, 3, 4, 5 and 6 hours after its co-administration (Fig. 3B, $P<0.05$, two-way ANOVA post hoc Bonferroni test).

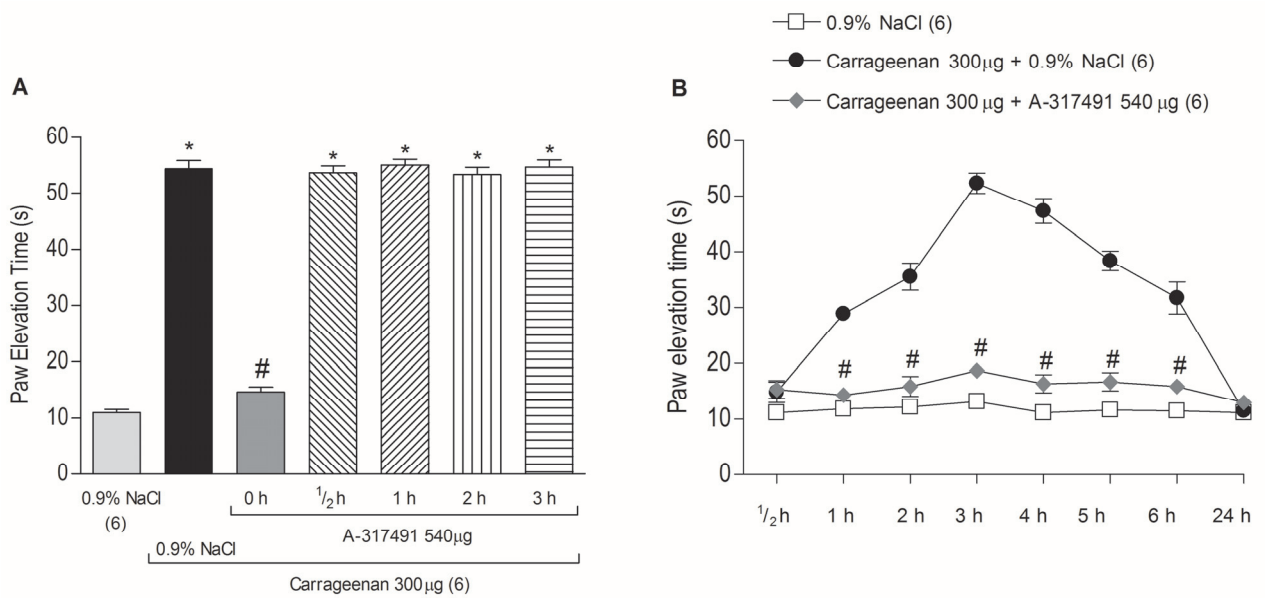

Figure 3 - Temporal analysis of the effect of the P2X3 and P2X2/3 receptors antagonist A-317491 on carrageenan-induced articular hyperalgesia.

A - Co-administration (0 h) of A-317491 (540 µg/knee) with carrageenan (300 µg/knee), but not its administration ½, 1, 2 or 3 hours after the carrageenan administration ($p>0.05$, Tukey test) significantly reduced carrageenan-induced articular hyperalgesia, as indicated by the symbol "#" ($p<0.05$, Tukey test). The symbol "*" indicates a response significantly greater than that induced by 0.9% NaCl ($p<0.05$, Tukey test). **B -** The temporal analysis of the effect of the co-administration of A-317491 (540 µg/knee) with carrageenan showed that it blocked the hyperalgesic response 1, 2, 3, 4, 5 and 6 hours after its co-administration, as indicated by the symbol "#" ($p<0.05$, Tukey test).

Carrageenan increased the expression of the P2X3 receptor on the chondrocytes of the articular cartilage of the knee joint

To test the hypothesis that the local inflammation induced by carrageenan in the rat's knee joint increases the expression of the P2X3 receptors on the chondrocytes of the articular cartilage covering the femoral condyle, the tibial plateau and meniscus cartilage of the knee joint, the carrageenan (300 µg/knee) or 0.9% NaCl (50µL) was administered into the rat's knee joint and

the expression of P2X3 receptor was quantified 3 hours after the administration by immunofluorescence.

The intra-articular administration of carrageenan (300 μg/knee) increased the expression of P2X3 receptor on the chondrocytes of the articular cartilage covering the femoral condyle (Fig. 4, $P<0.05$, one-way ANOVA post hoc Tukey test), the tibial plateau (Fig. 5, $P<0.05$, one-way ANOVA post hoc Tukey test) and the chondrocytes of the cartilage that forms the meniscus (Fig. 6, $P<0.05$, one-way ANOVA post hoc Tukey test) when compared with the 0.9% NaCl treated and with untreated animals (Naive). The intra-articular injection of 0.9% NaCl alone (50 μL/knee) did not affect the expression of P2X3 receptor in the chondrocytes of the three regions analyzed when compared with the Naive group ($P>0.05$, one-way ANOVA post hoc Tukey test).

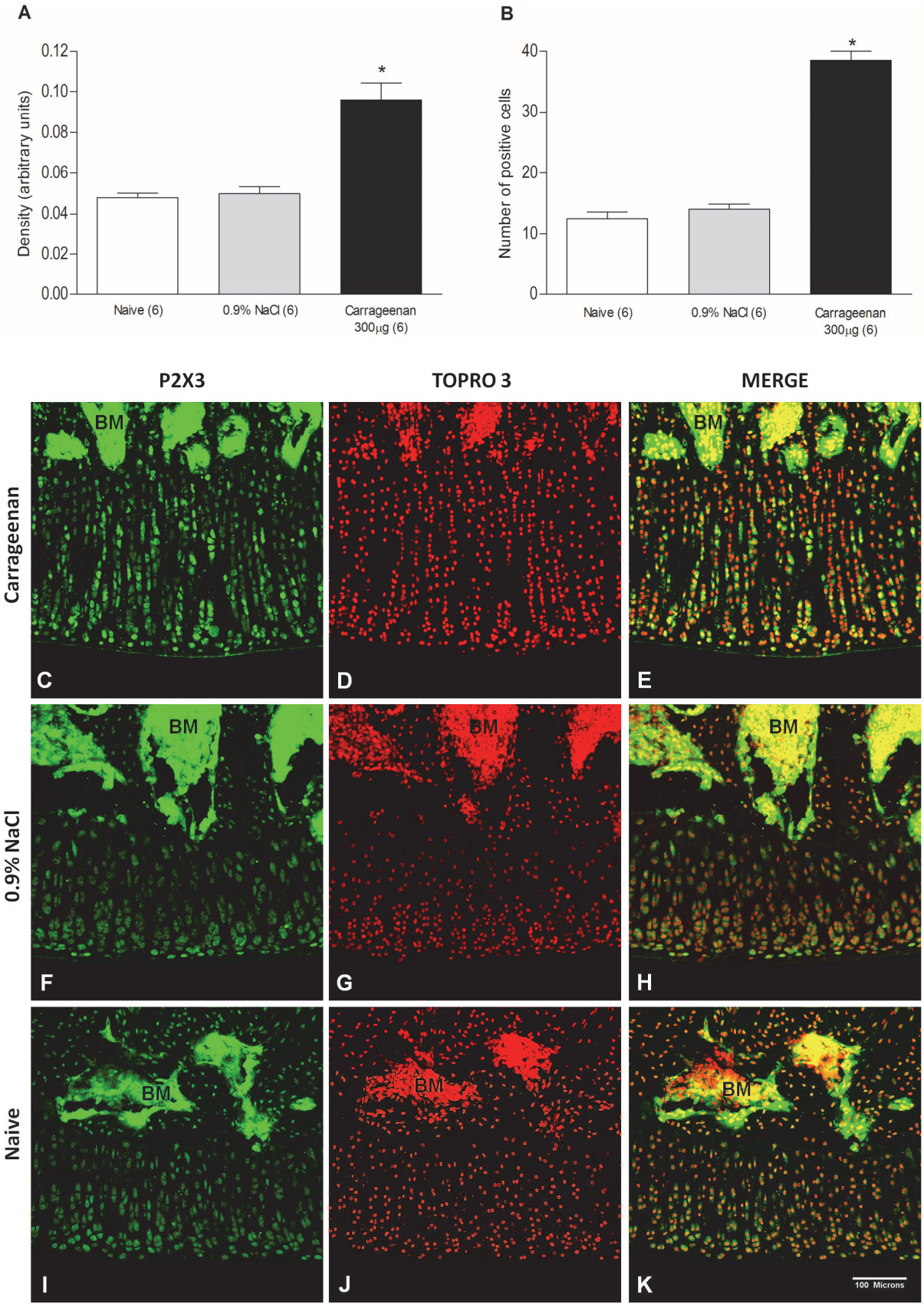
A
Density (arbitrary units)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
*
Naive (6)
0.9% NaCl (6)
Carrageenan 300μg (6)
B
Number of positive cells
40
30
20
10
0
*
Naive (6)
0.9% NaCl (6)
Carrageenan 300μg (6)
P2X3
TOPRO 3
MERGE
Carrageenan
0.9% NaCl
Naive
BM
C
D
E
F
G
H
I
J
K
100 Microns

Figure 4 - Effect of intra-articular injection of carrageenan on P2X3 receptors expression in the chondrocytes of the articular cartilage of the femoral condyle of the rats knee joint. The carrageenan administration (300 μg/knee) significantly increased the expression of P2X3 receptor in the chondrocytes of the articular cartilage covering the femoral condyle (**A**, **B** and green: **C**). The symbol "*" indicates an expression significantly greater when compared with 0.9% NaCl (**A**, **B** and green: **F**) and naive groups (**A**, **B** and green: **I**) rats ($p<0.05$, Tukey test). Red: TOPRO 3 (nuclear marker). BM = Bone Marrow. Scale bar of 100 μm.

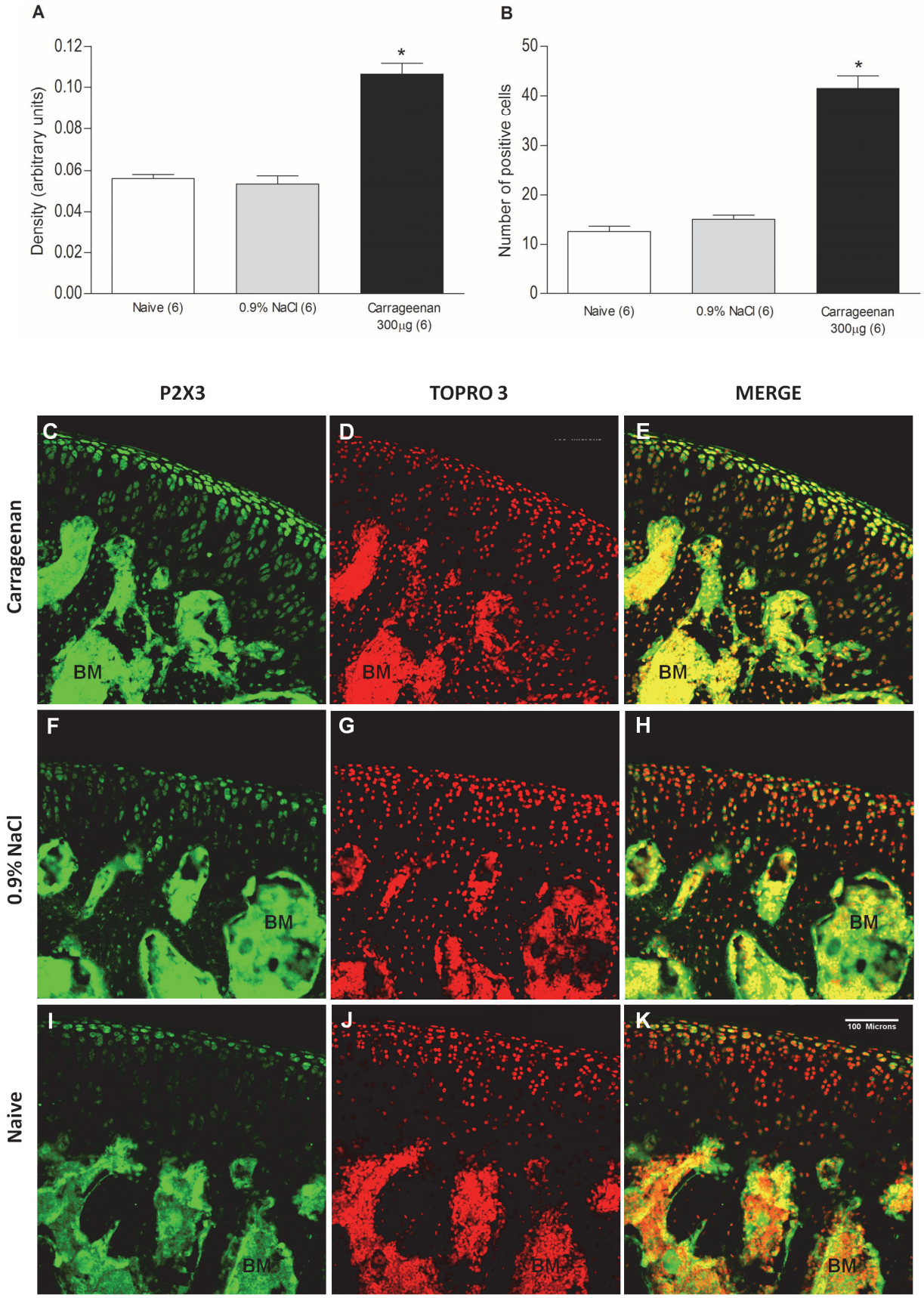
A
Density (arbitrary units)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
*
Naive (6)
0.9% NaCl (6)
Carrageenan 300µg (6)
B
Number of positive cells
50
40
30
20
10
0
*
Naive (6)
0.9% NaCl (6)
Carrageenan 300µg (6)
P2X3
TOPRO 3
MERGE
Carrageenan
0.9% NaCl
Naive
C
D
E
F
G
H
I
J
K
BM
100 Microns

Figure 5 - Effect of intra-articular injection of carrageenan on P2X3 receptors expression in the chondrocytes of the articular cartilage of the tibial plateau of the rat's knee joint. The carrageenan administration (300 µg/knee) significantly increased the expression of P2X3 receptor in the chondrocytes of the articular cartilage covering the tibial plateau (**A**, **B** and green: **C**). The symbol "*****" indicates an expression significantly greater when compared with 0.9% NaCl (**A**, **B** and green: **F**) and naive groups (**A**, **B** and green: **I**) rats ($p<0.05$, Tukey test). Red: TOPRO 3 (nuclear marker). BM = Bone Marrow. Scale bar of 100 µm.

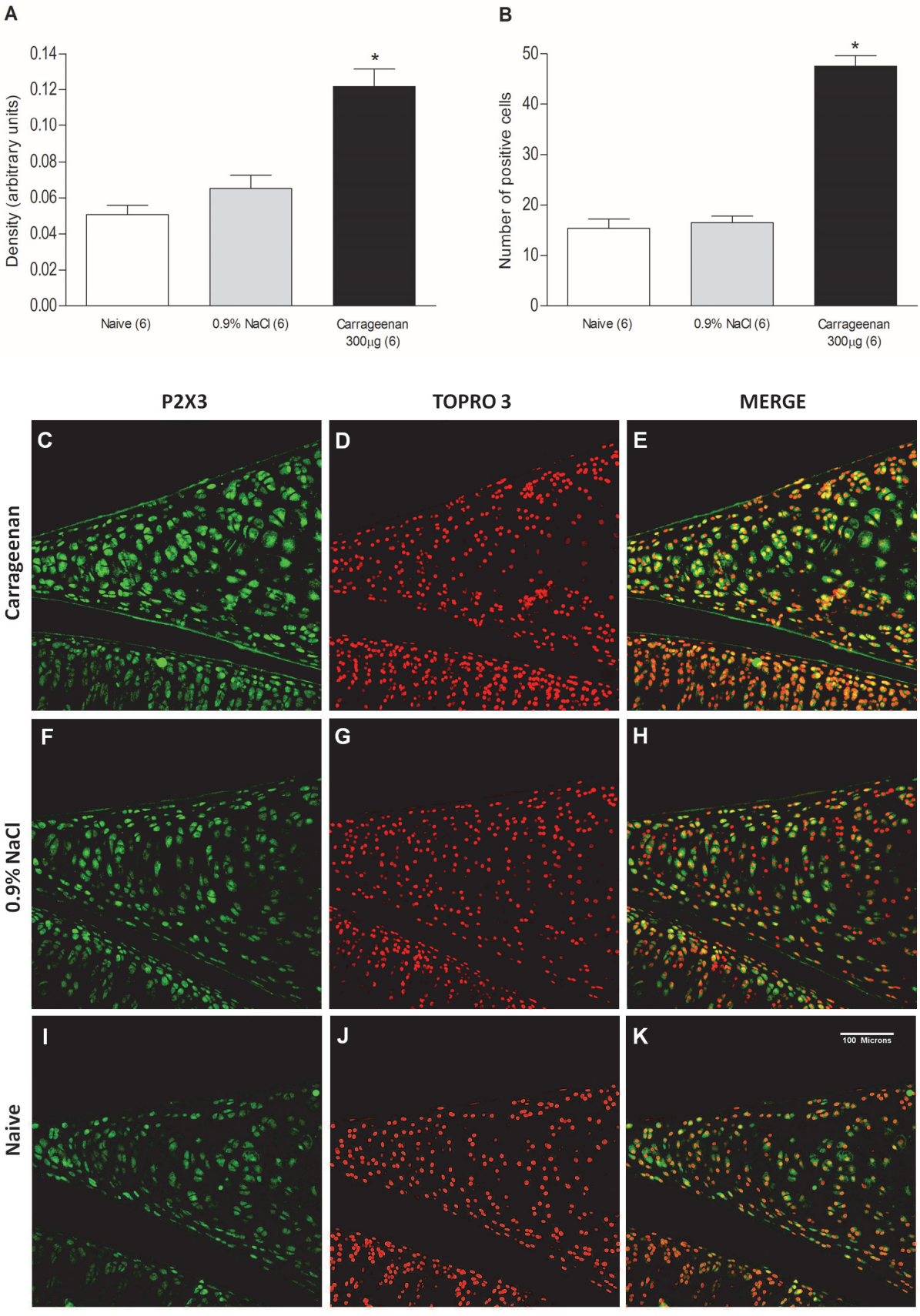
A
Density (arbitrary units)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
*
Naive (6)
0.9% NaCl (6)
Carrageenan 300µg (6)
B
Number of positive cells
50
40
30
20
10
0
*
Naive (6)
0.9% NaCl (6)
Carrageenan 300µg (6)
P2X3
TOPRO 3
MERGE
Carrageenan
0.9% NaCl
Naive
C
D
E
F
G
H
I
J
K
100 Microns

Figure 6 - Effect of intra-articular injection of carrageenan on P2X3 receptors expression in the chondrocytes of the cartilage that forms the meniscus of the rat's knee joint. The carrageenan administration (300 μg/knee) significantly increased the expression of P2X3 receptor in the chondrocytes of the cartilage that forms the meniscus (**A**, **B** and green: **C**). The symbol "*" indicates an expression significantly greater when compared with 0.9% NaCl (**A**, **B** and green: **F**) and naive groups (**A**, **B** and green: **I**) rats ($p<0.05$, Tukey test). Red: TOPRO 3 (nuclear marker). Scale bar of 100 μm.

The blockade of P2X3 and P2X2/3 receptors reduces the carrageenan-induced local increase cytokines concentration

To test the hypothesis that P2X3 and P2X2/3 receptors activation contributes to the carrageenan-induced articular hyperalgesia in the rats knee joint through an indirect sensitization of primary afferent nociceptors dependent on the previous release of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and CINC-1 at the inflamed joint, the selective P2X3 and P2X2/3 receptor antagonist A-317491 (540 μg/knee), was co-administrated with carrageenan (300 μg/knee) in the rats knee joint and the local concentration of TNF-α, IL-1β, IL-6 and CINC-1 was quantified 3 hours later by ELISA procedure.

The carrageenan administration (300 μg/knee) significantly increased the local concentration of TNF-α (Fig. 7A), IL-1β (Fig. 7B), IL-6 (Fig. 7C) and CINC-1 (Fig. 7D) when compared with 0.9% NaCl treated and with untreated rats (Naive) ($P<0.05$, one-way ANOVA post hoc Tukey test). The co-administration of A-317491 (540 μg/knee) with carrageenan significantly reduced the local concentration of TNF-α (Fig. 7A), IL-6 (Fig. 7C) and CINC-1 (Fig. 7D) ($P<0.05$, one-way ANOVA post hoc Tukey test), but not the local concentration of IL-1β (Fig. 7B) ($P>0.05$, one-way ANOVA post hoc Tukey test) induced by carrageenan. The intra-articular injection of 0.9% NaCl alone did not affect the endogenous concentration of TNF-α, IL-

1β, IL-6 and CINC-1 when compared with naive rats (P>0.05, one-way ANOVA post hoc Tukey test).

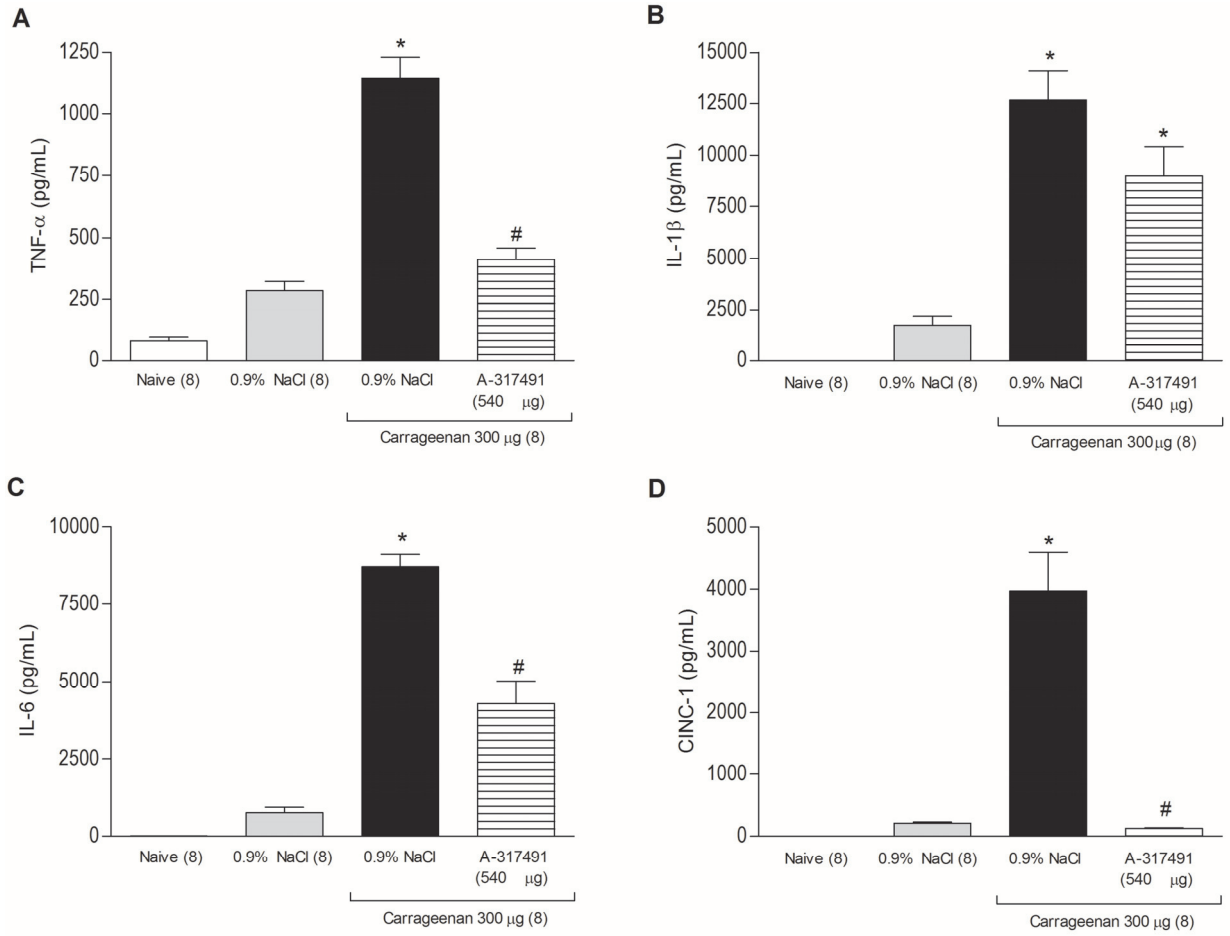

Figure 7 - Effect of the P2X3 and P2X2/3 receptors antagonist A-317491 on carrageenan-induced local increase of pro-inflammatory cytokines concentration. The carrageenan administration (300 µg/knee) significantly increased the local concentration of TNF-α (**A**), IL-1β (**B**), IL-6 (**C**) and CINC-1 (**D**) 3 hours after its administration. The co-administration of A-317491 (540 µg/knee) with carrageenan significantly reduced the local concentration of TNF-α (**A**), IL-6 (**C**) and CINC-1 (**D**), as indicated by the symbol "#" ($p<0.05$, Tukey test), but not the local concentration of IL-1β (**B**) ($p>0.05$, Tukey test). The intra-articular injection of 0.9% NaCl alone did not significantly affect the local endogenous concentration of TNF-α, IL-1β, IL-6 and CINC-1 when compared with naive rats ($p>0.05$, Tukey test). The symbol "*" indicates a response significantly greater than that induced by 0.9% NaCl and naive rats ($p<0.05$, Tukey test).

The blockade of P2X3 and P2X2/3 receptors reduced the carrageenan-induced local neutrophil migration

To test the hypothesis that P2X3 and P2X2/3 receptors activation contributes to the carrageenan-induced articular hyperalgesia in the rats knee joint through neutrophil migration, the A-317491 (540 µg/knee) was co-administrated with carrageenan (300 µg/knee) in the knee joint and the myeloperoxidase's activity was quantified 3 hours after its administration.

The carrageenan administration (300 µg/knee) significantly increased the myeloperoxidase's activity when compared with 0.9% NaCl and untreated rats (naive) (Fig. 8, $P<0.05$, one-way ANOVA post hoc Tukey test). The co-administration of A-317491 (540 µg/knee) with carrageenan significantly reduced the myeloperoxidase's activity induced by carrageenan (Fig. 8, $P<0.05$, one-way ANOVA post hoc Tukey test). The intra-articular injection of 0.9% NaCl alone did not affect the myeloperoxidase's activity when compared with naive rats (Fig. 8, $P>0.05$, one-way ANOVA post hoc Tukey test).

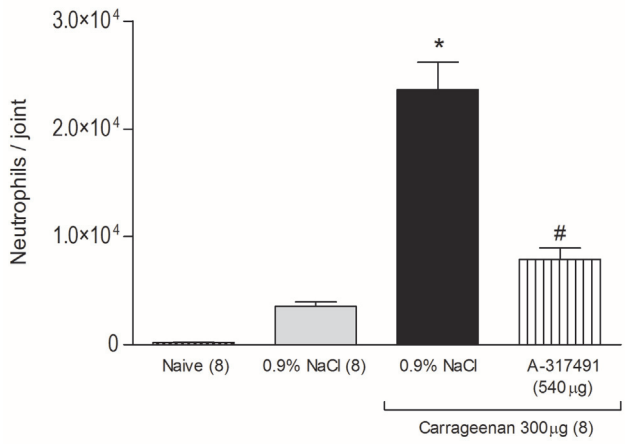

Figure 8 - Effect of the P2X3 and P2X2/3 receptors antagonist A-317491 on carrageenan-induced neutrophil migration. The carrageenan administration (300 µg/knee) significantly increased the myeloperoxidase's activity into the rat's knee joint 3 hours after its administration. The co-administration of A-317491 (540 µg/knee) with carrageenan significantly reduced the carrageenan-induced increase of myeloperoxidase's activity, as indicated by the symbol "#"

($p<0.05$, Tukey test). The symbol "*" indicates a response significantly greater than that induced by 0.9% NaCl and naive groups ($p<0.05$, Tukey test).

Discussion

In this study we showed, for the first time, that the activation of P2X3 and P2X2/3 receptors by endogenous ATP contributes to carrageenan-induced articular hyperalgesia in the knee joint of males and estrus females in a similar manner. We also showed that carrageenan-induced articular inflammation increases the expression of P2X3 receptors in chondrocytes of articular cartilage of the knee joint. Finally, we showed that the activation of P2X3 and P2X2/3 receptors contributes to carrageenan-induced articular hyperalgesia through an indirect sensitization of the primary afferent nociceptors dependent on the previous release of pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and chemokine-induced chemoattractant-1 (CINC-1, analog to IL-8 in rats) and on the migration of neutrophils to the inflamed knee joint.

The intra-articular carrageenan administration (300 μg/knee) induced an articular hyperalgesia in the knee joint of males and estrus females rats (low levels of ovarian hormones) of similar magnitude. Therefore, the blockade of P2X3 and P2X2/3 receptors in the rat's knee joint attenuated the carrageenan-induced articular hyperalgesia in males and estrus females in a similar manner. Although earlier reports have shown sex differences in the action of opioids agonists (Cai et al., 2001, Clemente et al., 2004, Clemente-Napimoga et al., 2009) and of β-adrenoceptor antagonist (Favaro-Moreira et al., 2012) on TMJ nociception between males and females, selective kappa opioid agonists are equally powerful to attenuate the edema of experimental arthritis in both male and female animals (Binder et al., 2000). Taken together,

these findings suggest that sex differences in analgesia depend, at least in part, on the particular receptor type under study.

We showed that the P2X3 and P2X2/3 receptors antagonist A-317491 (Jarvis et al., 2002) blocked the carrageenan-induced articular hyperalgesia in the rat knee joint from 1 to 6 hours after its administration and did not affect the carrageenan-induced articular hyperalgesia when applied in the contralateral paw. Although spinal P2X3 and P2X2/3 receptors also contribute to nociception (McGaraughty et al., 2003), the lack of effect of the contralateral administration of A-317491 confirmed that only P2X3 and P2X2/3 receptors in the knee joint of male and estrus female rats were targeted.

The presented results reinforce the findings from our research group that during the inflammatory process, ATP is released from injured cells such as by macrophages, platelets, neutrophils and dying cells (Filippini et al., 1990, Dubyak and el-Moatassim, 1993, Ferrari et al., 1997, Beigi et al., 1999, Sikora et al., 1999, Mizumoto et al., 2003, Campwala and Fountain, 2013) to the extracellular milieu and contributes to the development of inflammatory hyperalgesia induced by carrageenan in the subcutaneous tissue and in the TMJ region of rats via P2X3 and P2X2/3 receptor activation (Oliveira et al., 2005, Oliveira et al., 2009, Teixeira et al., 2010). Further, they are also consistent with a previous report that the activation of P2X3 receptor is involved in the mechanical hypersensitivity induced by Complete Freund's adjuvant (CFA) of the inflamed knee joint (Seino et al., 2006) and with the observation that extracellular ATP is often found in the synovial fluid of patients with arthropathies (Ryan et al., 1991, Kumahashi et al., 2011).

Similarly to what occurs in the subcutaneous tissue of rat paw (Oliveira et al., 2009), in the articular tissue of the knee joint, P2X3 and P2X2/3 receptors seem to be essential to the

development, but not to the maintenance of the hyperalgesic response. This is because A-317491 blocked carrageenan-induced articular hyperalgesia only when it was co-administered with carrageenan, but not when it was administered ½, 1, 2 or 3 hours after the carrageenan administration.

It is broadly accepted that carrageenan induces hyperalgesia by two distinct pathways that ultimately result in the local release of prostaglandins and sympathomimetic amines (Nakamura and Ferreira, 1987, Cunha et al., 1991, Cunha et al., 1992, Cunha et al., 2005). These inflammatory mediators directly sensitize the primary afferent nociceptor (Taiwo et al., 1989, Gold et al., 1996, Khasar et al., 1999, Rush and Waxman, 2004). Therefore, the blockade of carrageenan-induced articular hyperalgesia by the co-administration of P2X3 and P2X2/3 receptors antagonist with carrageenan, suggests that the articular activation of P2X3 and P2X2/3 receptors may be crucial to the sensitization of primary afferent nociceptors mediated by PGE_2 and sympathomimetic amines.

Because the P2X3 receptor is also expressed in chondrocytes (Varani et al., 2008) and in degenerative diseases such as OA these cells are associated to cartilage damage, increased production of matrix-degrading enzymes and inflammatory mediators such as prostaglandins (PGE) (de Mattei et al., 2002, Varani et al., 2008, Lee et al., 2013), we tested the hypothesis that the local inflammation induced by carrageenan in the rat knee joint increases the expression of the P2X3 receptor in the chondrocytes of the articular cartilage. The current results demonstrated that the carrageenan-induced knee joint inflammation increased the expression of the P2X3 receptor in the chondrocytes of the articular cartilage covering the femoral condyle, tibial plateau and meniscus cartilage of the rat's knee joint.

The chondrocytes, the unique cell type residing in cartilage (Picher et al., 2003 Maldonado and Nam, 2013), can release PGE_2 (Chowdhury et al., 2008), a major contributor of inflammatory pain in arthritis conditions, that acts through a variety of prostanoids receptors expressed in peripheral sensory neurons and spinal cord (Dray and Read, 2007). Previous study *in vitro* showed that ATP and α,β-meATP (P2X3 agonist, Gever et al., 2006) increase the PGE_2 production by chondrocytes in the absence and in the presence of the pro-inflammatory cytokine IL-1β, an effect that was blocked by the selective P2X3 and P2X2/3 receptors antagonist A317491, indicating a role for P2X3 receptors in PGE_2 release by chondrocytes (Varani et al., 2008). Moreover, previous studies *in vitro* have been shown that IL-1β stimulates the production of pro-inflammatory cytokines by chondrocytes (Guerne et al., 1990, Lotz et al., 1992, Aida et al., 2006). Taken together, these findings suggest that the increase on P2X3 receptor expression in chondrocytes during articular inflammation and its activation by ATP released from injured or dead cells might induce the PGE_2 released by chondrocytes, which in turn, activates the prostanoids receptors expressed in primary afferent nociceptors. These data suggest that activation of P2X3 and P2X2/3 receptors expressed in chondrocytes contributes to carrageenan-induced articular hyperalgesia in addition to those expressed in the peripheral terminals of primary afferent nociceptors that innervate the knee joint, as previously demonstrated (Dowd et al., 1998).

Although the mechanisms involved in the development of articular hyperalgesia resulting from OA are not well elucidated, several studies have suggested the involvement of pro-inflammatory cytokines in articular inflammatory processes (Smith et al., 1997, Fiorito et al., 2005, Pearle et al., 2007). The increased concentration of pro-inflammatory cytokines such as IL-1β, TNF-α, IL-6 and IL-8, has been demonstrated in the synovial fluid of joints with experimental

OA in rats (Gong et al., 2011, Rocha et al., 2011) and in the knee synovial fluid of patients with OA (Orita et al., 2011). A broad array of cytokines, including IL-1, IL-6, IL-18, TNF-α and chemokines, are produced by macrophages and fibroblasts of the synovium (Firestein, 2003), as well as by chondrocytes (Guerne et al., 1990, Lotz et al., 1992, Aida et al., 2006) that can activate either themselves or their neighboring cells.

It is well known that carrageenan-induced hyperalgesia is mediated by the previous release of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6 and IL-8 (Cunha et al., 1992, Lorenzetti et al., 2002). Therefore, we tested the hypothesis that the P2X3 and P2X2/3 receptors activation contributes to carrageenan-induced articular hyperalgesia by an indirect sensitization of primary afferent nociceptors dependent on the previous release of pro-inflammatory cytokines: TNF-α IL-1β, IL-6 and CINC-1.

The results obtained showed that the peripheral P2X3 and P2X2/3 receptors activation by endogenous ATP is important to increase the concentration of TNF-α, IL-6 and CINC-1, but not of IL-1β. Previous data from our laboratory demonstrated that the administration of P2X3 and P2X2/3 receptors antagonist did not alter the carrageenan-induced increase of IL-1β concentration in the subcutaneous tissue of rat paw (Oliveira et al., 2009). Also, in the muscle tissue, the carrageenan increases the concentration of IL-1β, but not of TNF-α (Loram et al., 2007). Therefore, the release of IL-1β does not always depends on the prior release of TNF-α, as previously suggested (Cunha et al., 1992). Another possibility is that the residual concentration of TNF-α in the knee joint observed after the administration of A-317491, could be enough to keep the concentration of IL-1β elevated.

The importance of TNF-α and IL-6 to the development of arthropathies was demonstrated by the ability of anti-TNF-α and anti-IL-6 antibodies to prevent the progression of bone and

cartilage damage, relieving pain symptoms in patients (Lipsky et al., 2000, Grunke and Schulze-Koops, 2006, Garnero et al., 2010, Tanaka et al., 2013) and by their ability in the prevention of collagen-induced arthritis in mice (Williams et al., 1992, Takagi et al., 1998). Thus, the findings of current study indicate that the essential role of peripheral P2X3 and P2X2/3 receptors activation in the development of carrageenan-induced articular hyperalgesia is mediated, at least in part, by the release of TNF-a and IL-6.

The pro-inflammatory chemokine CINC-1 is responsible for stimulating the release of sympathomimetic amines (Cunha et al., 1991, Lorenzetti et al., 2002), which directly sensitize primary afferent nociceptors (Nakamura and Ferreira, 1987) and contributes to the influx and activation of polymorphonuclear cells into the joint (Moon et al., 2010). This leads to the release of enzymes that decrease the expression of collagen by fibroblasts, which may result in the destruction of cartilage and bone, and consequently pain (Badolato and Oppenheim, 1996). The current results also demonstrated that the peripheral P2X3 and P2X2/3 receptors activation is important to carrageenan-induced increase of CINC-1 concentration in the knee joint. It explains, at least in part, the anti-hyperalgesic effect of the co-administration of P2X3 and P2X2/3 receptors antagonist in the knee joint of rats, suggesting that blockade of carrageenan-induced CINC-1 release by the P2X3 and P2X2/3 receptors antagonist may reduce the release of sympathomimetic amines, since the synovium is innervated by sympathetic nerve fibers in OA joints (Weidler et al., 2005).

In patients with OA, it was also demonstrated an intense neutrophil migration (Jones et al., 1991). Because neutrophils are involved in the genesis of inflammatory hyperalgesia (Cunha et al., 2008), and their infiltration into synovial tissues can cause proteolytic enzymes release which contributes significantly to the tissue damage (Edwards and Hallett, 1997), we also

investigated the involvement of articular P2X3 and P2X2/3 receptors activation on neutrophil migration induced by intra-articular administration of carrageenan, which induces neutrophil recruitment into the knee joint (Ekundi-Valentim et al., 2010).

Our data demonstrated that the blockade of the P2X3 and P2X2/3 receptors reduced the carrageenan-induced neutrophil migration to the rat's knee joint, showing that the peripheral P2X3 and P2X2/3 receptors activation by endogenous ATP is important to induce neutrophil migration. This data indicate an important difference between the pathophysiology of inflammatory hyperalgesia in the articular and subcutaneous tissues, because although P2X3 receptor activation by endogenous ATP, is important for the release of CINC-1 in the subcutaneous tissue, it has a minor importance for leukocyte migration in this tissue (Oliveira et al., 2009).

In summary, we conclude that the activation of P2X3 and P2X2/3 receptors by endogenous ATP plays a crucial role in the development of carrageenan-induced articular hyperalgesia in the knee joint of males and estrus females in a similar manner. Furthermore, during knee joint inflammation the expression of P2X3 receptors in chondrocytes is increased, suggesting that their activation may contribute to the increased release of inflammatory mediators, which in turn, activate the primary afferent nociceptors. This suggestion is supported by our findings that the essential role played by P2X3 and P2X2/3 receptors in the development of carrageenan-induced articular hyperalgesia is mediated by an indirect sensitization of the primary afferent nociceptors dependent on the previous release of pro-inflammatory cytokines and on neutrophil migration in the knee joint. Taken together, these findings suggest that selective antagonists for the P2X3 and P2X2/3 receptors could be potential targets for drug development for inflammatory joint diseases treatment.

Acknowledgements

We thank the Central Microscopy Facility of University of Iowa, Iowa City, IA, USA. This work was supported by a Grant from Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP, Brazil (2009/16854-3 and 2010/05381-4).

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III. CAPÍTULO 02

Intra-articular blockade of P2X7 purinergic receptor reduces the articular hyperalgesia and inflammation in the knee joint experimental synovitis especially in female rats

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Abstract

Knee osteoarthritis (OA) is a common degenerative joint disease which causes physical disability, synovitis, joint swelling and inflammatory pain and affects a greater proportion of women than men. Although the activation of P2X7 receptor contributes to the development of inflammatory hyperalgesia and inflammation, it is not known whether P2X7 receptor plays a role in carrageenan-induced inflammatory hyperalgesia in the knee joint. In this study, we investigated whether peripheral P2X7 receptor activation by endogenous ATP contributes to carrageenan-induced hyperalgesia in the knee joint of rats, and, if so, whether this contribution depends on the previous release of the pro-inflammatory cytokines and/or the migration of neutrophils to the inflamed knee joint. Because females with lower levels of ovarian hormones are more responsive to some analgesic drugs, we also investigated whether the effect induced by the blockade of P2X7 receptors in the knee joint differs between male and estrus female rats (low levels of ovarian hormones). Blockade of P2X7 in the rat's knee joint significantly reduced the articular hyperalgesia, the increase of concentration of pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and CINC-1 and the neutrophil migration induced by the carrageenan injection into the knee joint of males and estrus females. However, a lower dose of the selective P2X7 receptor antagonist A-740003 was sufficient to significantly reduce the hyperalgesic responses, the pro-inflammatory cytokines concentration and the neutrophil migration in the knee joint of estrus females, but not of males. We conclude that activation of P2X7 receptors by endogenous ATP is essential to the development of the articular hyperalgesia mediated by the previous release of pro-inflammatory cytokines and neutrophil migration in both males and females rat knee joint. However, estrus females are more responsive than males to the anti-hyperalgesic and anti-inflammatory effects induced by the blockade of P2X7 receptors in the rat knee joint.

Keywords: articular hyperalgesia, P2X7 receptors, male and female rats, knee joint, pro-inflammatory cytokines, neutrophils migration.

Introduction

Knee osteoarthritis (OA) is one of the most common rheumatic disorders. It affects a greater proportion of women than men (Nevitt and Felson, 1996, Srikanth et al., 2005, Felson, 2006) and it is characterized by synovial inflammation and cartilage breakdown due to an imbalance between extracellular matrix destruction and repair, which is directly linked to clinical symptoms such as physical disability, synovitis, joint swelling and inflammatory pain (Hunter et al., 2009, Sellam and Berenbaum, 2010, Orita et al., 2011).

The P2X purinergic receptors are ligand-gated ionotropic channels, which open in response to the binding of extracellular adenosine 5'-triphosphate (ATP). Among the seven P2X purinergic receptor subtypes (P2X1-P2X7) (Buell et al., 1996), the P2X7 subtype has been implicated in processes of pain and inflammation (Dell'Antonio et al., 2002a, Dell'Antonio et al., 2002b, Fulgenzi et al., 2005, Donnelly-Roberts and Jarvis, 2007, Lister et al., 2007, McGaraughty et al., 2007, Fulgenzi et al., 2008, Honore et al., 2009, Teixeira et al., 2010a and b).

In the peripheral tissue, the P2X7 receptor is selectively expressed in mast cells, lymphocytes, fibroblasts, erythrocytes, monocytes, peripheral macrophages and type B synoviocytes (FLS cells) (Surprenant et al., 1996, Collo et al., 1997, Mancino et al., 2001, Caporali et al., 2008), where it plays an important role in the development of the inflammatory processes by modulating the production of inflammatory mediators, such as IL-1β, IL-6, IL-8, TNFα, monocyte chemoattractant protein-1 (MCP-1) and interferon gamma-induced protein (IP-

10) (Chessell et al., 2005, Fulgenzi et al., 2005, Ferrari et al., 2006, Honore et al., 2006, Lister et al., 2007, Mingam et al., 2008). Although the occurrence of OA is very common in the knee joint (Hunter and Eckstein, 2009), it is not known whether P2X7 receptor play a role in carrageenan-induced inflammatory hyperalgesia in this joint. In the temporomandibular joint (TMJ) they do not, whereas functional P2X7 receptors are expressed in the TMJ region (Teixeira et al., 2010a).

In this study, we investigated whether the P2X7 receptor activation by endogenous ATP contributes to carrageenan-induced articular hyperalgesia in rats knee joint, and, if so, whether this contribution depends on the previous release of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and chemokine-induced chemoattractant-1 (CINC-1, analog to IL-8 in rats) and/or migration of neutrophils to the inflamed joint. Because the involvement of some receptors in pain and analgesia processes is sex-dependent, for example females with lower levels of ovarian hormones are more responsive to some analgesic drugs than males rats (Clemente et al., 2004, Clemente-Napimoga et al., 2009, Favaro-Moreira et al., 2012), we also investigated whether the effect induced by the blockade of P2X7 receptors in the knee joint differs between male and estrus female rats (low levels of ovarian hormones).

Materials and Methods

Animals

Male and female Wistar rats (200-250g) obtained from the Multidisciplinary Center for Biological Research (CEMIB - UNICAMP, SP, Brazil) were used in this study. The animals were housed in plastic cages with soft bedding (five/cage) on a 12:12 light cycle (lights on at 06:00 A.M.) with food and water available *ad libitum*. They were maintained in a temperature-

controlled room (±23°C) and handled for at least one week prior to the experiments. Each animal was used once and the number of animals per group was kept to a minimum. Experimental protocols were approved by the Committee on Animal Research of the University of Campinas (protocol number: 2049-1) and were conformed to IASP guidelines for the study of the pain in animals (Zimmermann, 1983).

General Procedures

Testing sessions took place during light phase (between 09:00 AM and 5:00 PM) in a quiet room maintained at 23ºC (Rosland, 1991). During the tests the animals had no access to water or food. The animals were habituated for 1 hour prior to the experiment to minimize stress.

Drugs administration

Drugs or their vehicle were intra-articularly administrated in the right rat's knee joint by a 26-gauge needle that was connected to a catheter of polyethylene and also to a Hamilton syringe. The volume of injection was 50 µL.

Carrageenan-induced knee joint inflammation (synovitis)

Under brief inhalation of isoflurane anesthesia, rats were subjected to intra-articular (i.a.) injection of λ-carrageenan dissolved in 25 µL sterile 0.9% saline solution into their right knee joints (Tonussi and Ferreira, 1992, Ekundi-Valentim et al., 2010). The injection site was shaved and treated with an antiseptic solution of iodine alcohol. The other drugs were also injected into the knee joint in the same manner that the carrageenan and the control animals received vehicle or sterile 0.9% saline solution.

Drugs and doses

The following drugs were used: λ-carrageenan (Cg; 300 μg/knee, i.a., Tonussi and Ferreira, 1992, Tonussi and Ferreira, 1999) obtained from Sigma-Aldrich (MO, USA) and [*N*-(1-{[(Cyanoimino)(5-quinolinylamino)methyl]amino}-2,2-dimethylpropyl)-2-(3,4-dimethoxyphenyl) acetamide] (A-740003 - the selective P2X7 receptor antagonist: 142, 284, 568 μg/knee, i.a., Honore et al., 2006), dissolved in 25 μL of dimethyl sulfoxide (DMSO, 50%) and propylene glycol (50%), obtained from Tocris Bioscience (Ellisville, MO).

Estrus phase determination

Estrus phase in female rats was determined by daily microscope examination of vaginal smears taken by gentle lavage, between 9:00 and 10:00 a.m. Estrus phase was identified by the predominance (80 %) of anucleated cornified cells in rats with at least two consecutive regular 4-day cycles (Smith et al., 1975, Marcondes et al., 2002). This phase was chosen because it represent phase of low levels of ovarian hormones, 17β-estradiol and progesterone (Butcher et al., 1974, Spornitz et al., 1999).

Gait disturbance - Rat knee joint incapacitation test

We used the rat knee-joint incapacitation test, as described previously (Tonussi and Ferreira, 1992). Briefly, 3 hours after carrageenan injection into their right knee joints, rats were put to walk on a steel rotary cylinder (30 cm wide x 50 cm diameter), covered with a fine-mesh non-oxidizable wire screen, which rotates at 3 rpm. Specially, designed metal gaiters were wrapped around both hind paws. After placement of the gaiters, rats were placed to walk in the

cylinder and the right paw was then connected via a simple circuit to microcomputer data input/output port. The paw elevation time (PET) is the total time that the rat walks failing to touch the cylinder surface with the injected hindpaw, during a 60 sec period, which is directly proportional to the gait disturbance. Incapacitation was quantified as an increase in the PET, 3 hours after carrageenan injection into the right knee joint. To minimize variations in PET, all rats were introduced to the experimental environment and trained on the apparatus to habituation into the equipment before the testing sessions. To confirm the local effect of some test agents, they were injected into the contralateral rat's knee joint and the test was performed on the ipsilateral knee joint. The rat knee-joint incapacitation test provides automated measurements, which are independent of the subjectivity of the observer (Tonussi and Ferreira, 1992).

Synovial lavage fluid

Under deep anaesthesia (induced by the intraperitoneal injection of 80 mg/kg ketamine and 20 mg/kg xylazine) the rats were then killed by cervical dislocation, the skin overlying the knee was excised, the patellar ligament was dissected and a 26-gauge needle connected to a 100 μL Hamilton syringe was inserted through the joint capsule. The knee joint cavity was washed twice by injecting and immediately aspirating 100 μL of phosphate-buffered saline solution (PBS) containing 4 mM EDTA (Ekundi-Valentim et al., 2010).

ELISA procedure

An adaptation of ELISA (Enzyme-Linked Immunosorbent Assay) (Safieh-Garabedian et al., 1995) was used to quantify the cytokines of the rat's knee joint. Briefly, the synovial lavage fluid was homogenized in solution of phosphate-buffered saline (PBS) containing 0.4 M NaCl,

0.05 % Tween 20, 0.5 % bovine serum albumine (BSA), 0.1 mM phenyl-methylsulfonyl fluoride, 0.1 mM benzotonic chloride, 10 mM EDTA, and 20 KL/mL aprotinine (Sigma, USA). The samples were centrifuged at 10000 rpm for 15 minutes at 4ºC and the supernatants were stored at -70ºC for posterior use to evaluate the protein levels of TNF-α, IL-1β, IL-6 and CINC-1 in the rat's knee joint. The cytokines were quantified by the following kits: TNF-α: Rat TNF-α/ TNFSF1A DuoSet ELISA Kit (R&D Systems, catalog number DY510); IL-1β: Rat IL-1β/IL-1F2 DuoSet ELISA Kit (R&D Systems, catalog number DY501), IL-6: Rat IL-6 DuoSet ELISA Kit (R&D Systems, catalog number: DY506) and CINC-1: Rat CXCL1/CINC-1 DuoSet ELISA Kit (R&D Systems, catalog number DY515). All procedures followed the instructions of the manufacturer R&D Systems. All samples and standards were run in triplicate and the procedures were repeated twice to guarantee the authenticity of the results. In the present study, the levels of cytokines (TNF-α, IL-1β, IL-6 e CINC-1) were assessed three hours after carrageenan administration.

Measurement of myeloperoxidase activity

Myeloperoxidase is one of the enzymes released from neutrophils and directly associated to tissue injury. Although monocytes/macrophages and fibroblasts also contain myeloperoxidase, neutrophils show the highest intracellular levels of this enzyme, that represents up to 5 % of neutrophil proteins (Klebanoff, 1991). Therefore, the measurement of myeloperoxidase's activity was used as a marker of neutrophil migration (Klebanoff, 1991) in the rat's knee joint after application of the stimulus. Three hours after injection of the inflammatory agent in the knee joint, synovial lavage fluid was collected and homogenized in 500μL of buffer 1 (0.1 M NaCl, 0.02 M $NaPO_4$,1.015 M Na EDTA, pH 5.4) followed by centrifugation at 3000 rpm for 15 min.

The pellet was resuspended in 500 µL of buffer 1 and subjected to hypotonic lyses by the addition of 500 µL of 0.2 % NaCl followed 30 seconds later by addition of 500 µL of 1.6% NaCl in 5% glucose. After a further centrifugation, the pellet was resuspended in 0.05 M $NaPO_4$ buffer (pH 5.4) containing 0.5% hexadecyl-trimethylammonium bromide (HTAB). After that, the samples were snap-frozen in liquid nitrogen and thawed, three times, and centrifuged at 10,000 rpm for 15 min.

The myeloperoxidase kinetic-colorimetric assay was conducted, as previously described (Bradley et al., 1982, Torres-Chavez et al., 2012). Fifty microliters of each sample (supernatant) and 0.08 M $NaPO_4$ were dropped into wells of a 96-well microplate. 25 µL of 3,3′,3,3′-tetramethylbenzidine (TMB) was added in each well and the reaction was initiated by the addition of 100 µL of H_2O_2. The reaction was stopped 5 min later by the addition of 50 µL of 4M H_2SO_4. The optical density was read at 450 nm using an Asys UVM340 (3 readings at intervals of 30 seconds). Results were calculated by comparing the optical density of rat's knee joint synovial lavage fluid supernatant with a standard curve of neutrophil (>95% purity). All procedures were repeated twice to guarantee the authenticity of the results. The results were presented as number of neutrophils/knee.

Statistical analysis

To determine if there were significant differences ($p<0.05$) between treatment groups, One-way ANOVA or T-test was performed. If there was a significant between-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. For data shown in Fig. 2B a Two-way repeated measures ANOVA with one between subjects factor (i.e., treatment) and one

within-subjects factor (i.e., time) were used to determine whether there were significant ($p<0.05$) differences among the groups. If there was a significant between-subjects main effect of treatment group, post hoc contrasts using the Bonferroni test were performed to determine the basis of the significant difference. Data are expressed in figures as means ± S.E.M.

Results

Effect of blockade of P2X7 receptors on carrageenan-induced articular hyperalgesia

The hyperalgesic and inflammatory responses of males and estrus females administered carrageenan (300 μg/knee) were not significantly different from each other (Fig. 1, 3 and 4, $P>0.05$, t test). Therefore, this equi-hyperalgesic and equi-inflammatory dose of carrageenan was used to compare the effect of P2X7 receptor blockade into the knee joint among males and estrus females.

Co-administration of the selective P2X7 receptor antagonist A-740003 with carrageenan into the rat's knee joint significantly reduced carrageenan-induced articular hyperalgesia (Fig. 1, $P<0.05$, one-way ANOVA post hoc Tukey test) measured 3 hours after the carrageenan administration in both males and females and in a dose related fashion. However, the lowest dose of A-740003 (142 μg/knee) significantly reduced the carrageenan-induced articular hyperalgesia in females but not in males, suggesting that carrageenan-induced hyperalgesia is significantly more responsive to P2X7 receptor antagonist A-740003 in females than in males.

The highest dose of A-740003 (568 μg/knee) did not affect the carrageenan-induced articular hyperalgesia when administered on the contralateral knee joint in both males and estrus females (Fig. 1A and B, $P>0.05$, one-way ANOVA post hoc Tukey test), confirming its local

action. Co-administration of the highest dose of A-740003 (568 µg/knee) with the vehicle of carrageenan (0.9% NaCl) had no effect by itself (Fig. 1A and B, P>0.05, one-way ANOVA post hoc Tukey test) in both males and estrus females. Furthermore, the co-administration of 0.9% NaCl with the vehicle of A-740003 [dimethyl sulfoxide (50 %, DMSO) plus propylene glycol (50 %)] had no effect by itself either (Fig. 1A and B, P>0.05, one-way ANOVA post hoc Tukey test).

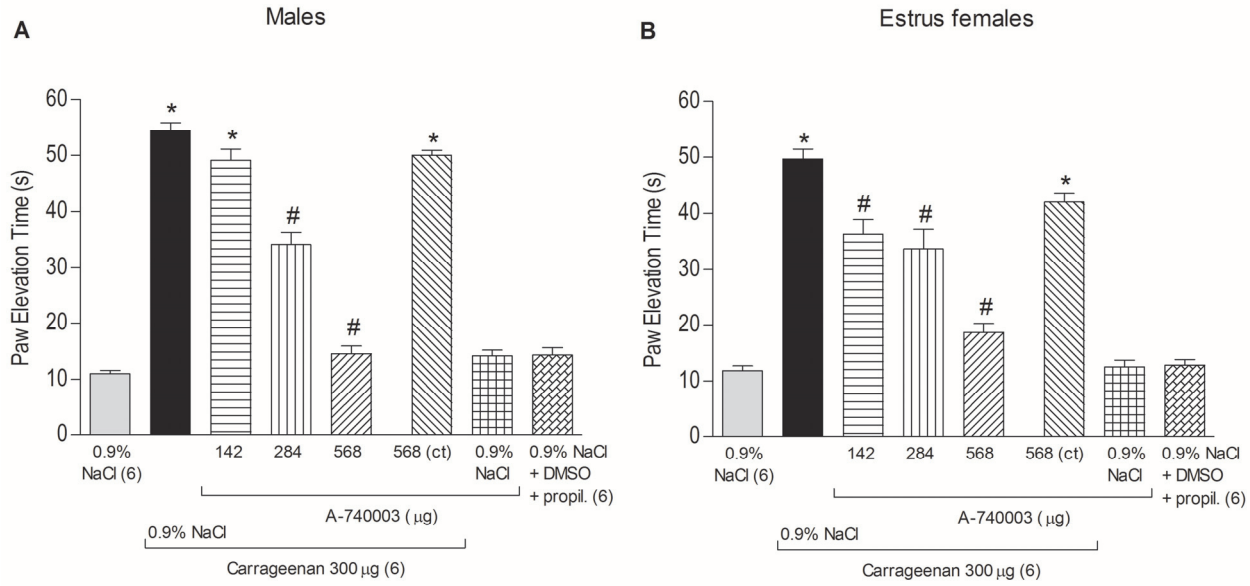

Figure 1 - Effect of the P2X7 receptor antagonist on carrageenan-induced articular hyperalgesia in males and estrus females rat's knee joint. Co-administration of A-740003 in males (**A,** 284 and 568 µg/knee) and in estrus females (**B**, 142, 284 and 568 µg/knee) with carrageenan (300 µg/knee) significantly reduced carrageenan-induced articular hyperalgesia, as indicated by the symbol "#" ($p<0.05$, Tukey test). The highest dose of A-740003 (568 µg/knee) injected in the contralateral knee joint (ct) did not affect the carrageenan-induced articular hyperalgesia ($p>0.05$, Tukey test, **A** and **B**). A-740003 injected only plus 0.9% NaCl did not induce articular hyperalgesia by itself ($p>0.05$, Tukey test, **A** and **B**). The administration of DMSO (50%) plus propylene glycol (50%) did not induce articular hyperalgesia by itself ($p>0.05$, Tukey test, **A** and **B**) in males and estrus females knee joint. The symbol "*" indicates a response significantly greater than that induced by 0.9% NaCl group ($p<0.05$, Tukey test, **A** and **B**).

Effect of blockade of articular P2X7 receptors on the development of carrageenan-induced articular hyperalgesia

Co-administration of A-740003 (568 µg/knee) with carrageenan (300 µg/knee), but not its administration ½, 1, 2 and 3 hours after the carrageenan administration, significantly reduced carrageenan-induced hyperalgesia measured 3 hours after the carrageenan administration (Fig. 2A, $P<0.05$, one-way ANOVA post hoc Tukey test). The carrageenan-induced articular hyperalgesia reached its maximum 3 hours after its administration (Fig. 2B). Co-administration of A-740003 (568 µg/knee) with carrageenan (300 µg/paw) blocked the hyperalgesic responses 1, 2, 3, 4, 5 and 6 hours after the carrageenan administration (Fig. 2B, $P<0.05$, two-way ANOVA post hoc Bonferroni test).

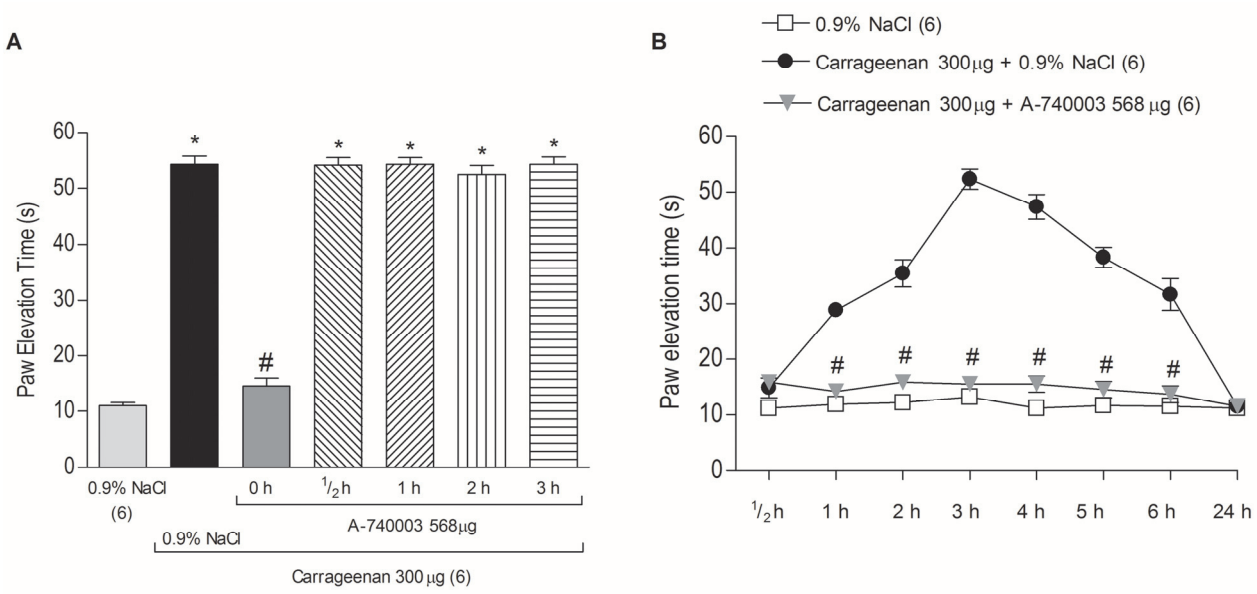

Figure 2 - Effect of P2X7 receptor antagonist co-administered or administrated ½, 1, 2, and 3 hours after the carrageenan administration on carrageenan-induced articular hyperalgesia and the temporal analysis. (A) Co-administration (0 h) of A-740003 (568 µg/knee) with the carrageenan (Cg, 300 µg/knee), but not its administration ½, 1, 2 or 3 hours after carrageenan administration ($p>0.05$, Tukey test) significantly reduced carrageenan-induced articular hyperalgesia in the males rat's knee joint, as indicated by the symbol "#" ($p<0.05$,

Tukey test). The symbol "*" indicates a response significantly greater than that induced by 0.9% NaCl group ($p<0.05$, Tukey test). **(B)** The temporal analysis of the effect of the co-administration of A-740003 (568 μg/knee) with carrageenan showed that it blocked the hyperalgesic response 1, 2, 3, 4, 5 and 6 hours after its co-administration, as indicated by the symbol "#" ($p<0.05$, Tukey test).

Effect of blockade of articular P2X7 receptors on carrageenan-induced local increase in cytokines concentration

To verify whether the activation of P2X7 receptor by endogenous ATP contributes to the release of pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and CINC-1 induced by carrageenan at the knee joint, A-740003 (142 and 568 μg/knee for males and 142 μg/knee for estrus females) was co-administered with carrageenan (300 μg/knee) into the knee joint and the local concentrations of TNF-α, IL-1β, IL-6 and CINC-1 were quantified 3 hours after the carrageenan administration.

The carrageenan administration (300 μg/knee) significantly increased the local concentration of TNF-α (Fig. 3A and B), IL-1β (Fig. 3C and D), IL-6 (Fig. 3E and F) and CINC-1 (Fig. 3G and H) 3 hours after its administration into the knee joint of males and estrus females rats ($P<0.05$, one-way ANOVA post hoc Tukey test) when compared with the 0.9% NaCl administration and with untreated animals (Naive). Co-administration of A-740003 (568 μg/knee, but not 142 μg/knee) with carrageenan significantly reduced the local concentration of TNF-α (Fig. 3A), IL-1β (Fig. 3C), IL-6 (Fig. 3E) and CINC-1 (Fig. 3G) ($P<0.05$, one-way ANOVA post hoc Tukey test) induced by carrageenan into the knee joint of male rats. However, in estrus females the co-administration of the lowest dose of A-740003 (142 μg/knee) with carrageenan was sufficient to significantly reduce the local concentration of TNF-α (Fig. 3B), IL-6 (Fig. 3F) and CINC-1 (Fig. 3H) ($P<0.05$, one-way ANOVA post hoc Tukey test), but not of IL-1β (Fig.

3D) ($P>0.05$, one-way ANOVA post hoc Tukey test) induced by carrageenan into the knee joint. The intra-articular injection either the vehicle of carrageenan (0.9% NaCl) or the vehicle of A-740003 [DMSO (50%) plus propylene glycol (50%)] did not affect the concentrations of TNF-α, IL-1β, IL-6 and CINC-1 when compared with those of the knee joint of untreated males and estrus females (Fig. 3, $P>0.05$, one-way ANOVA post hoc Tukey test).

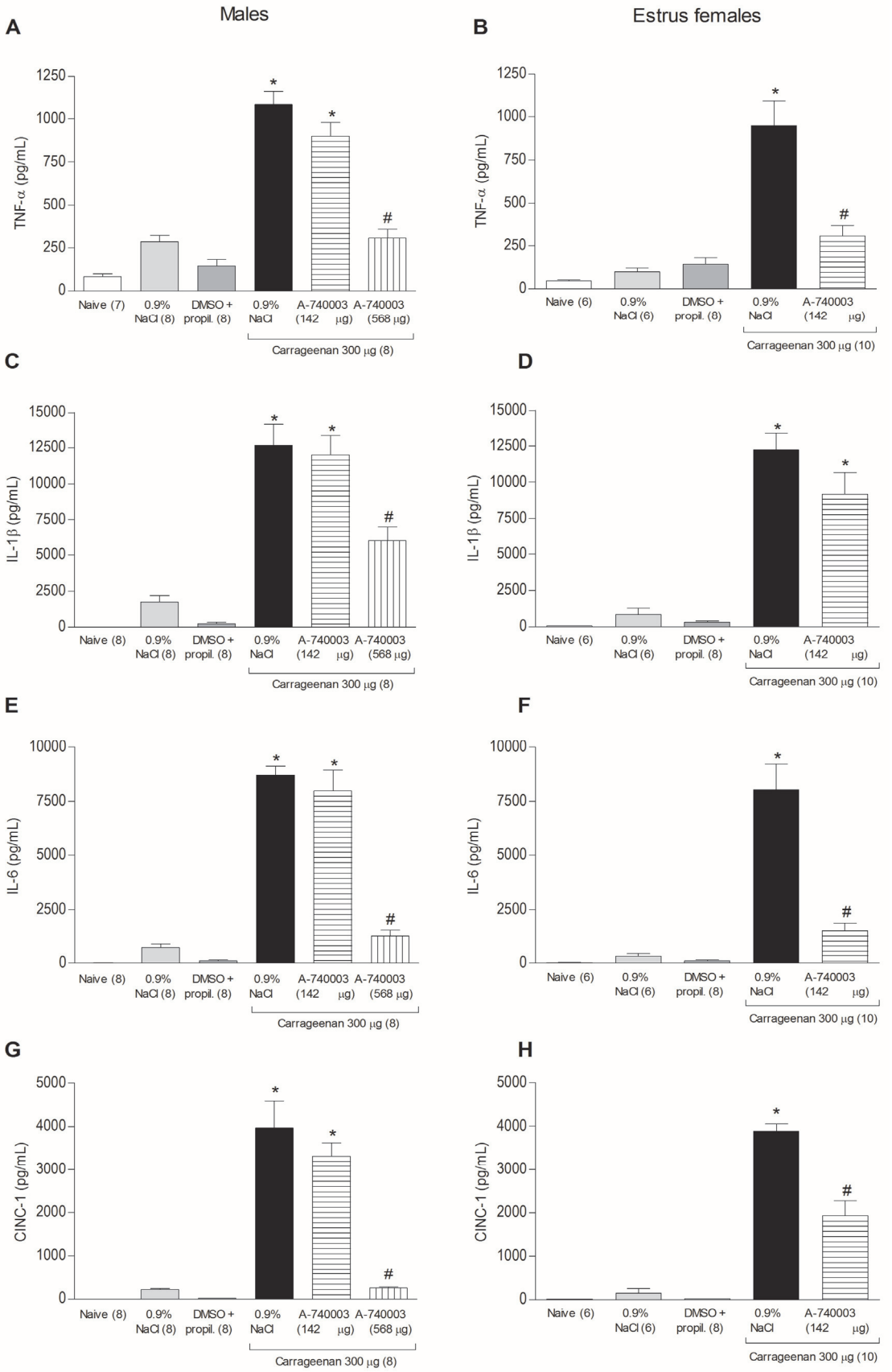
Males
Estrus females
A
B
C
D
E
F
G
H
TNF-α (pg/mL)
IL-1β (pg/mL)
IL-6 (pg/mL)
CINC-1 (pg/mL)
Naive (7)
Naive (8)
Naive (6)
0.9% NaCl (8)
0.9% NaCl (6)
DMSO + propil. (8)
DMSO + propil. (6)
0.9% NaCl
A-740003 (142 μg)
A-740003 (568 μg)
Carrageenan 300 μg (8)
Carrageenan 300 μg (10)

Figure 3 - Effect of P2X7 receptor antagonist on carrageenan-induced local increase of pro-inflammatory cytokines concentration in males and estrus females rat's knee joint. The carrageenan administration (300 μg/knee) significantly increased the local concentration of TNF-α (**A** and **B**), IL-1β (**C** and **D**), IL-6 (**E** and **F**) and CINC-1 (**G** and **H**) in the knee joint of males and estrus females rats 3 hours after its administration. In males rats, the co-administration of A-740003 (568 μg/knee, but not 142 μg/knee) with carrageenan significantly reduced the local concentration of TNF-α (**A**), IL-1β (**C**), IL-6 (**E**) and CINC-1 (**G**) in the knee joint, as indicated by the symbol "#" ($p<0.05$, Tukey test). In estrus females rats, the co-administration of A-740003 (142 μg/knee) with carrageenan significantly reduced the local concentration TNF-α (**B**), IL-6 (**F**) and CINC-1 (**G**) in the knee joint, as indicated by the symbol "#" ($p<0.05$, Tukey test), but not the local concentration of IL-1β (**D**) ($p>0.05$, Tukey test). The intra-articular injection in both males and estrus females knee joint of either 0.9% NaCl alone or DMSO (50%) plus propylene glycol (50%) did not significantly affect the local endogenous concentration of TNF-α, IL-1β, IL-6 and CINC-1 when compared with naive group ($p>0.05$, Tukey test). The symbol "*" indicates a response significantly greater than that induced by naïve and vehicle groups ($p<0.05$, Tukey test).

Effect of blockade of articular P2X7 receptors on carrageenan-induced local neutrophil migration

To verify whether the activation of P2X7 receptor by endogenous ATP contributes to the neutrophil migration induced by carrageenan at the knee joint, A-740003 (142 and 568 μg/knee for males and 142 μg/knee for estrus females) was co-administrated with carrageenan (300 μg/knee) in the knee joint of males and estrus females and the myeloperoxidase's activity was quantified 3 h after the carrageenan administration.

The carrageenan administration (300 μg/knee) significantly increased the myeloperoxidase's activity in males (Fig. 4A) and in estrus females (Fig. 4B) when compared with the 0.9% NaCl administration and with untreated animals (Naive) ($P<0.05$, one-way ANOVA post hoc Tukey test). Co-administration of A-740003 (568 μg/knee, but not 142

μg/knee) with carrageenan significantly reduced the myeloperoxidase's activity induced by carrageenan in the knee joint of male rats (Fig. 4A, P<0.05, one-way ANOVA post hoc Tukey test). However, in estrus females, the co-administration of the lowest dose of A-740003 (142 μg/knee) with carrageenan was also sufficient to significantly reduce the myeloperoxidase's activity induced by carrageenan in the knee joint of estrus female rats (P<0.05, one-way ANOVA post hoc Tukey test). The intra-articular injection of either the vehicle of carrageenan (0.9% NaCl) or the vehicle of A-740003 [DMSO (50 %) plus propylene glycol (50 %)] did not affect the basal myeloperoxidase's activity when compared with untreated males and estrus females (naive groups) (P>0.05, one-way ANOVA post hoc Tukey test).

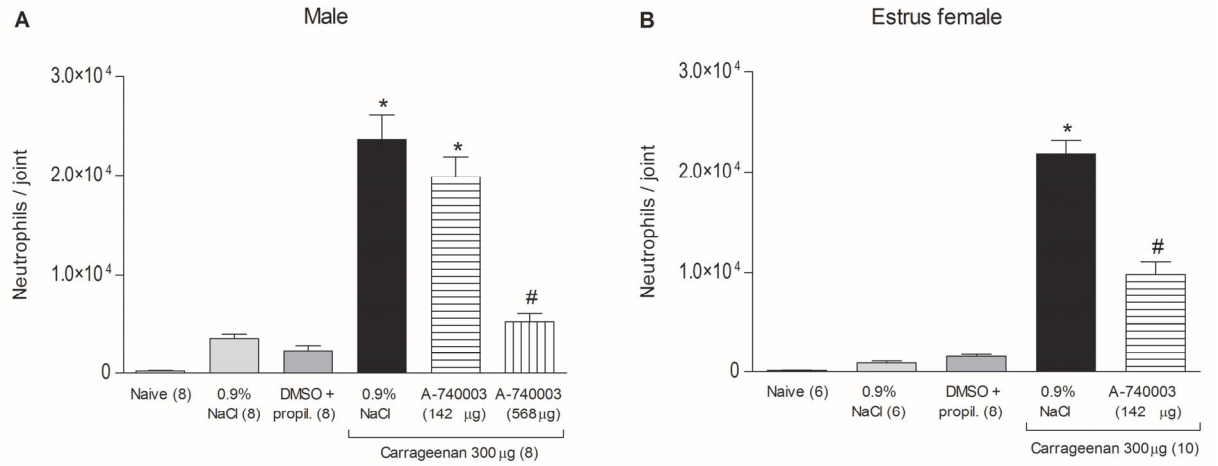

Figure 4 - Effect of P2X7 receptor antagonist on carrageenan-induced neutrophil migration in males and estrus females rat's knee joint. The carrageenan administration (Cg, 300μg/knee) significantly increased the myeloperoxidase's activity in the knee joint of males (**A**) and estrus females rats (**B**). The co-administration of A-740003 in males (**A**, 568 μg/knee, but not 142 μg/knee) and in estrus females (**B**, 142 μg/knee) with carrageenan significantly reduced the carrageenan-induced increase of myeloperoxidase's activity, as indicated by the symbol "#" (p<0.05, Tukey test). The intra-articular injection in both males and estrus females knee joint of either 0.9% NaCl alone or DMSO (50 %) plus propylene glycol (50 %) did not affect the myeloperoxidase's activity when compared with naive group (p>0.05, Tukey test). The symbol

"*" indicates a response significantly greater than that induced by vehicle and naive groups ($p<0.05$, Tukey test).

Discussion

In this study, we showed that blockade of P2X7 receptors in the rat knee joint significantly reduces the articular hyperalgesia mediated by the previous release of pro-inflammatory cytokines and neutrophil migration in the rat knee joint of both males and estrus females. However, estrus females are more responsive than males to the anti-hyperalgesic and anti-inflammatory effects induced by the blockade of P2X7 receptor in the rat knee joint.

The activation of P2X7 receptor by endogenous ATP during inflammation, not only contributes but it is essential to the development, but not to the maintenance of the articular hyperalgesia in the knee joint of males and estrus females. This is because the selective P2X7 receptor antagonist A-740003 (Honore et al., 2006) blocked carrageenan-induced articular hyperalgesia when it was co-administered with carrageenan, but not when it was administered ½, 1, 2 or 3 hours after the carrageenan administration.

A-740003 blocked the carrageenan-induced articular hyperalgesia in the rat knee joint from 1 to 6 hours after its administration and did not affect the carrageenan-induced articular hyperalgesia when applied in the contralateral paw. Although the activation of spinal P2X7 receptor contributes to the mechanical hyperalgesia (Clark et al., 2010), the finding that the administration of A-740003 in the contralateral knee joint did not affect carrageenan-induced articular hyperalgesia, confirms that only articular P2X7 receptors of the knee joint were targeted. These findings are consistent with the involvement of peripheral P2X7 receptors in collagen-

induced arthritis (Labasi et al., 2002, Broom et al., 2008) and in carrageenan-induced mechanical hyperalgesia in the subcutaneous tissue (Teixeira et al., 2010b). However, these receptors are not involved in processes of pain and inflammation in all joints. Although functional P2X7 receptors are expressed in the temporomandibular joint, they do not play a role in the carrageenan-induced inflammatory hyperalgesia in this joint (Teixeira et al., 2010a).

The mechanisms involved in the development of articular pain resulting from OA are not well elucidated. However, in patients with OA (Orita et al., 2011) and in rats with experimental OA (Gong et al., 2011, Rocha et al., 2011), the concentration of pro-inflammatory cytokines such as IL-1β, TNF-α, IL-6 and CINC-1 (or IL-8 in human) is increased in the synovial fluid. These pro-inflammatory cytokines contribute to the destruction of cartilage and bone, and consequently to pain development in the joints (Goldring et al., 1994, Attur et al., 1998, Mengshol et al., 2000).

In addition, it has been demonstrated in the carrageenan model, that TNF-α triggers the release of IL-1β, IL-6 and CINC-1, that ultimately induce the synthesis of the final inflammatory mediators prostaglandins and sympathomimetic amines, respectively (Cunha et al., 1991, Cunha et al., 1992, Ferreira et al., 1993) which sensitize the primary afferent nociceptor (Taiwo et al., 1989, Gold et al., 1996, Khasar et al., 1999, Rush and Waxman, 2004). Therefore, our findings that the administration of the P2X7 receptor antagonist A-740003 significantly reduced the increase of concentration of pro-inflammatory cytokines induced by carrageenan in the knee joint of males and females, suggest that the essential role that the peripheral P2X7 receptor activation plays in the development of carrageenan-induced articular hyperalgesia in males and females, is mediated, in part, by the previous release of pro-inflammatory cytokines.

This interpretation is in accordance with the finding that the anti-hyperalgesic effect of P2X7 receptor antagonists in the CFA-induced hyperalgesia (Complete Freund's Adjuvant) is

mediated by modulation of IL-1β activity (Honore et al., 2009). In the articular tissue, pro-inflammatory cytokines such as IL-1β, TNF-α, IL-6 and CINC-1 (or IL-8 in human) are produced by synovial macrophages (Firestein, 2003) and by type B synoviocytes (Guerne et al., 1989, Pap et al., 2000, Hayashida et al., 2001), which express the P2X7 receptor (Caporali et al., 2008). Therefore, the high concentration of extracellular ATP released due to the extensive cell injury in the inflamed joints, may activate the P2X7 receptor expressed on these cells, resulting in the release of pro-inflammatory cytokines that contribute to synovitis, to cartilage and bone destruction and to inflammatory pain development in the arthropathies. There is an important difference in the inflammatory response mediated by the activation of P2X7 receptor located in the knee joint and in the subcutaneous tissue. The P2X7 receptor activation by endogenous ATP released during inflammation increases the concentration of IL-1β in the knee joint, but not in the subcutaneous tissue of male rats (Teixeira et al., 2010b).

In patients with OA, in addition to the increased levels of pro-inflammatory cytokines in the synovial fluid, there is also an intense neutrophil migration into the knee joint (Jones et al., 1991). The pro-inflammatory chemokine CINC-1 contributes to the influx and to the activation of polymorphonuclear cells into the joint (Moon et al., 2010), leading to the release of enzymes that decrease the expression of collagen by fibroblasts, which may result in destruction of cartilage and bone and, consequently, pain development (Badolato and Oppenheim, 1996).

The intra-articular administration of the P2X7 receptor antagonist A-740003 significantly reduced the carrageenan-induced neutrophil migration in the knee joint of males and estrus females. This finding suggests that the peripheral P2X7 receptor activation by endogenous ATP is important in the recruitment of neutrophil to the injured joint and is in agreement with those showing that the blockade of P2X7 receptor reduces neutrophil migration in different tissues

(Kim et al., 2010, Martins et al., 2012). Because neutrophils are involved in the genesis of inflammatory hyperalgesia (Cunha et al., 2008), the anti-hyperalgesic effect of the P2X7 receptor antagonist A-740003 on the knee joint, may be mediated, in part, by a decreased concentration of the chemokine CINC-1 into the joint tissue, and by the subsequent decrease of neutrophil migration.

In this study, we showed for the first time, that while males and females respond to the anti-hyperalgesic and anti-inflammatory effects of the P2X7 receptor antagonist A-740003 in the inflamed knee joint, estrus females are more responsive to these effects than males. The evidence is that, the lowest dose of the P2X7 receptor antagonist A-740003 (142 μg) was sufficient to reduce the articular hyperalgesia, the local concentration of the pro-inflammatory cytokines TNF-α, IL-6 and CINC-1, and the neutrophil migration induced by carrageenan in estrus females, but not in males, which responded only to the two highest doses of A-740003.

These findings are of clinical relevance, because OA is more prevalent in women than men (Nevitt and Felson, 1996, Srikanth et al., 2005, Felson, 2006). They are also in accordance with previous reports that the administration of a kappa opioid agonist (Clemente et al., 2004) or of a β-adrenoceptor antagonist (Favaro-Moreira et al., 2012) induces greater TMJ analgesia in females than in males, and especially in females with lower levels of ovarian hormones, such as estrus or diestrus females. On the other hand, we have demonstrated that males and females are similarly responsive to the anti-hyperalgesic effect of P2X3 and P2X2/3 receptor antagonists in the inflamed knee joint (Teixeira et al., 2014 *in press*). Taken together, these findings reinforce the previous suggestion that sex differences in analgesia depend, at least in part, on the particular receptor type under study (Binder et al., 2000, Clemente et al., 2004, Favaro-Moreira et al., 2012).

The peripheral mechanism involved in the greater responsiveness of estrus females to the anti-hyperalgesic effect induced by the P2X7 receptor antagonist A-740003 may be mediated, at least in part, by the greater ability of this antagonist in reducing the release of the pro-inflammatory cytokines TNF-α, IL-6 and CINC-1 and the neutrophil migration induced by carrageenan in the knee joint of estrus females than males. In estrus females, a higher dose of the P2X7 receptor antagonist A-740003 may be necessary to significantly reduce the release of IL-1β induced by carrageenan.

The physiological mechanisms involved in the sex-related differences in the anti-hyperalgesic and anti-inflammatory responses mediated by the blockade of P2X7 receptors in the knee joint are not presently known. However, our findings that estrus females are more responsive to the anti-hyperalgesic and anti-inflammatory effect of the P2X7 receptor antagonist A-740003 than males, suggest that testosterone might attenuate the anti-hyperalgesic effect induced by this antagonist, as previously showed in the antinociceptive effects induced by opiods agonist (Clemente et al., 2004) and by β-blockers (Favaro-Moreira et al., 2012) on the TMJ of male rats.

In summary, we have shown that activation of P2X7 receptors by endogenous ATP is essential to the development of the articular hyperalgesia dependent on the previous release of pro-inflammatory cytokines and neutrophil migration in both males and females rat's knee joint. However, estrus females are more responsive than males to the anti-hyperalgesic and anti-inflammatory effects induced by the blockade of P2X7 receptors in the rat knee joint. The greater responsiveness of estrus females to the anti-hyperalgesic effect induced by the blockade of P2X7 receptors in the rat knee joint, may result from their greater responsiveness to the anti-inflammatory effect induced by the articular blockade of this purinergic receptor. Taken together,

these findings indicate that the P2X7 receptors are a novel anti-inflammatory and anti-hyperalgesic therapeutic target in the treatment of the symptoms of OA in the knee joint, especially in women, who are more affected than men by this disease.

Acknowledgements

This work was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP, Brazil (2009/16854-3 and 2010/05381-4).

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IV. CAPÍTULO 03

P2X3 and P2X2/3 receptors activation induces articular hyperalgesia by an indirect sensitization of the primary afferent nociceptor in the rat's knee joint

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Abstract

P2X3 and P2X2/3 receptors activation plays an important role in articular hyperalgesia mediated by inflammatory agent in the knee joint. In this study, we verify the mechanism by which the activation of the P2X3 and P2X2/3 receptors exclusively by their agonist α,β-meATP contributes to the articular hyperalgesia. The data of this study demonstrated that the administration of α,β-meATP in rat's knee joint induces a dose-dependent articular hyperalgesia that was blocked by the selective P2X3 and P2X2/3 receptors antagonist A-317491. Co-administration of selective antagonists for bradykinin B_1- (DALBK) or B_2-receptors (Bradyzide), β_1- (Atenolol) or β_2-adrenoceptors (ICI 118,551) and the local pre-treatment with cyclooxygenase inhibitor (Indomethacin) or with the nonspecific selectin inhibitor (Fucoidan) significantly reduced α,β-meATP-induced articular hyperalgesia in the rat's knee joint. Local administration of α,β-meATP also induced the release of pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and CINC-1, that was significantly decrease by A-317491. Moreover, the systemic treatment with fucoidan blocked α,β-meATP-induced neutrophil migration and articular hyperalgesia. Taken together, these findings suggest that peripheral P2X3 and P2X2/3 receptors activation induces articular hyperalgesia by an indirect mechanism that involves release of bradykinin, prostaglandins, sympathomimetic amines, pro-inflammatory cytokines and neutrophil migration.

Keywords: α,β-meATP, P2X3 and P2X2/3 receptors, articular hyperalgesia, knee joint, inflammatory mediators, neutrophil migration.

Introduction

Osteoarthritis (OA) is a degenerative joint disease which causes physical disability, inflammatory pain and synovitis and affects the whole joint including cartilage, synovial membrane, bone, muscle and tendons (Woolf and Pfleger, 2003). Although the mechanisms underlying the OA is not yet clear, evidences suggest that adenosine triphosphate (ATP) is an important inflammatory mediator involved in the development of hyperalgesia during OA (Dowd et al., 1998, Seino et al., 2006, Kumahashi et al., 2011, Teixeira et al., 2014b *in press*). Indeed, extracellular ATP is frequently found in synovial fluid of patients with arthropathies (Ryan et al., 1991, Park et al., 1996, Kumahashi et al., 2011).

P2X purinergic receptors (P2X1-P2X7) are membrane ligand-gated ion channels which open in response to ATP (Dubyak and el-Moatassim, 1993, Valera et al., 1994). In particular, the P2X3 receptor is involved in pain mechanisms including inflammatory articular hyperalgesia. For example, it has been demonstrated that the activation of P2X3 receptor, as a consequence of ATP release in inflamed articular tissue, is important to development of articular hyperalgesia in temporomandibular joint (TMJ) (Shinoda et al., 2005, Teixeira et al., 2010) and knee joint (Seino et al., 2006) of rats.

In addition to ATP, others inflammatory mediators, such as bradykinin, prostaglandins, sympathomimetic amines and the pro-inflammatory cytokines released after tissue injury are essential to induce and to maintain the inflammatory hyperalgesia (Cunha et al., 1992, Ferreira et al., 1993a, Verri et al., 2006, Loram et al., 2007, Luiz et al., 2010, Petho and Reeh, 2012, Villarreal et al., 2013). However, inflammatory mediators are released in a subsequent way, in which the release of ATP and P2X3 receptor activation induces the release of TNF-α (de Oliveira

Fusaro et al., 2010), that ultimately induces prostaglandins and sympathomimetic amines release, which directly sensitize primary afferent nociceptors (Gold et al., 1996, Khasar et al., 1999, Rush and Waxman, 2004).

Although P2X3 receptor is expressed on terminals of primary afferent neurons, particularly in the non-peptidergic subpopulation (Bradbury et al., 1998, Burnstock and Knight, 2004, Gever et al., 2006), it has been demonstrated that this purinergic receptor is also expressed in chondrocytes (Varani et al., 2008). Furthermore, we have recently showed that endogenous ATP, via P2X3 and P2X2/3 receptors is essential to development of carrageenan-induced articular hyperalgesia in the rat's knee joint, and that the P2X3 receptor expression in chondrocytes of cartilage is increased during carrageenan-induced articular inflammation (Teixeira et al, 2014b *in press*). Once chondrocytes can release inflammatory mediators (Guerne et al., 1990, Lotz et al., 1992, Aida et al., 2006), it is plausible to hypothesize that P2X3 receptor activation in knee joint induces articular hyperalgesia not only by directly sensitizing primary afferent nociceptor, but also by an indirect mechanism that involves the release of inflammatory mediators.

Therefore, the aim of this study was to verify whether the administration of P2X3 and P2X2/3 receptor agonist α,β-meATP in the rat's knee joint induces articular hyperalgesia which depends on release of bradykinin, prostaglandins, sympathomimetic amines, pro-inflammatory cytokines and on neutrophil migration.

Materials and Methods

Animals

Male Wistar rats (200-250g) obtained from the Multidisciplinary Center for Biological Research (CEMIB) - University of Campinas, were used in this study. The animals were housed in plastic cages with soft bedding (five/cage) on a 12:12 light cycle (lights on at 06:00 A.M.) with food and water available *ad libitum*. They were maintained in a temperature-controlled room (±23°C) and handled for at least one week prior to the experiments. Each animal was used once and the number of animals per group was kept to a minimum. Experimental protocols were approved by the Committee on Animal Research of the University of Campinas (protocol number: 2049-1) and conformed to IASP guidelines for the study of the pain in animals (Zimmermann, 1983).

General Procedures

Testing sessions took place during light phase (between 09:00 AM and 5:00 PM) in a quiet room maintained at 23ºC (Rosland, 1991). During the tests the animals had no access to water or food. The animals were habituated for 1 hour prior to the experiment to minimize stress.

Drugs administration

Drugs or their vehicle were intra-articularly administrated in the right rat's knee joint by a 26-gauge needle that was connected to a catheter of polyethylene and also to a Hamilton syringe (50 µL). The volume of injection was 50 µL.

α,β-meATP-induced knee joint inflammation

Under brief inhalation of isoflurane anesthesia, rats were subjected to intra-articular (i.a.) injection of α,β-meATP dissolved in 25 μL sterile 0.9% saline solution into their right knee joints, in the same manner as demonstrated in carrageenan-induced knee joint inflammation (Tonussi and Ferreira, 1992). The injection site was shaved and treated with an antiseptic solution of iodine alcohol. The other drugs were also injected into the knee joint in that the α,β-meATP and the control animals received vehicle.

Drugs and doses

The following drugs were used: the P2X3 and P2X2/3 receptor agonist α,β-methyleneATP lithium salt (Gever et al., 2006) (α,β-meATP: 10, 30, 100, 300 e 900 μg/knee, i.a., Oliveira-Fusaro et al., 2014 *in press*); the selective P2X3 and P2X2/3 receptor antagonist 5-([(3-Phenoxybenzyl)[(1S)-1,2,3,4-tetrahydro-1-naphthalenyl] amino]carbonyl)-1,2,4-benzenetricarboxylic acid (Jarvis et al., 2002) (A-317491: 540 μg/knee, i.a., Teixeira et al., 2014b *in press*); the selective bradykinin B_1 receptor antagonist des-Arg^9-[Leu^8]-BK - DALBK (Ferreira et al., 2008) (3.0 μg/knee, i.a., Teixeira et al., 2014a *in press*); the selective bradykinin B_2 receptor antagonist Bradyzide (Burgess et al., 2000) (1.5 μg/knee, i.a., Teixeira et al., 2014a *in press*); the selective β_1 receptor antagonist Atenolol (Allibardi et al., 1999) (6.0 μg/knee, i.a., Teixeira et al., 2014a *in press*); the selective β_2 receptor antagonist ICI 118,551 (Yalcin et al., 2009) (1.5 μg/knee, i.a., Teixeira et al., 2014a *in press*); the cyclooxygenase inhibitor Indomethacin (Summ and Evers, 2013) (100 μg/knee, i.a., Teixeira et al., 2014a *in press*) and the nonspecific selectin inhibitor Fucoidan (Ley et al., 1993) (25 mg/kg, i.v., Teixeira et al., 2014a *in*

press). The drugs were obtained from Sigma-Aldrich (MO, USA). All drugs were dissolved in sterile saline (0.9% NaCl).

Gait disturbance - Rat knee-joint incapacitation test

We used the rat knee-joint incapacitation test, as described previously (Tonussi and Ferreira, 1992). Briefly, 3 hours after α,β-meATP injection into their right knee joints, rats were put to walk on a steel rotary cylinder (30 cm wide x 50 cm diameter), covered with a fine-mesh non-oxidizable wire screen, which rotates at 3 rpm. Specially, designed metal gaiters were wrapped around both hind paws. After placement of the gaiters, rats were placed to walk in the cylinder and the right paw was then connected via a simple circuit to microcomputer data input/output port. The paw elevation time (PET) is the total time that the rat walks failing to touch the cylinder surface with the injected hindpaw, during a 60 sec period, which is directly proportional to the gait disturbance. Incapacitation was quantified as an increase in the PET, 3 hours after carrageenan injection into the right knee joint. To minimize variations in PET, all rats were introduced to the experimental environment and trained on the apparatus to habituation into the equipment before the testing sessions. To confirm the local effect of some test agents, they were injected into the contralateral rat's knee joint and the test was performed on the ipsilateral knee joint. The rat knee-joint incapacitation test provides automated measurements, which are independent of the subjectivity of the observer (Tonussi and Ferreira, 1992).

Synovial lavage fluid

Under deep anaesthesia (induced by the intraperitoneal injection of 80 mg/kg ketamine and 20 mg/kg xylazine) the rats were then killed by cervical dislocation, the skin overlying the

knee was excised, the patellar ligament was dissected and a 30-gauge needle connected to a 100 μL Hamilton syringe was inserted through the joint capsule. The knee joint cavity was washed twice by injecting and immediately aspirating 100 μL of phosphate-buffered saline solution (PBS) containing 4 mM EDTA (Ekundi-Valentim et al., 2010).

ELISA procedure

An adaptation of ELISA (Enzyme-Linked Immunosorbent Assay) (Safieh-Garabedian et al., 1995) was used to determine whether α,β-meATP was able to increase the local concentration of TNF-α, IL-1β, IL-6 and CINC-1 in the rat's knee joint. Briefly, the synovial lavage fluid was homogenized in solution of phosphate buffered saline (PBS) containing 0.4 M NaCl, 0.05% Tween 20, 0.5% bovine serum albumine (BSA), 0.1 mM phenyl-methylsulfonyl fluoride, 0.1 mM benzotonic chloride, 10 mM EDTA, and 20 KL/mL aprotinine (Sigma, USA). The samples were centrifuged at 10,000 rpm for 15 minutes at 4ºC and the supernatants were stored at -70ºC for posterior use to evaluate the protein levels of TNF-α, IL-1β, IL-6 and CINC-1 in the rat's knee joint. The cytokines were quantified by the following kits: TNF-α: Rat TNF-α/ TNFSF1A DuoSet ELISA Kit (R&D Systems, catalog number DY510); IL-1β: Rat IL-1β/IL-1F2 DuoSet ELISA Kit (R&D Systems, catalog number DY501), IL-6: Rat IL-6 DuoSet ELISA Kit (R&D Systems, catalog number: DY506) and CINC-1: Rat CXCL1/CINC-1 DuoSet ELISA Kit (R&D Systems, catalog number DY515). All procedures followed the instructions of the manufacturer R&D Systems. All procedures were repeated twice to guarantee the authenticity of the results. In the present study, the levels of cytokines (TNF-α, IL-1β, IL-6 e CINC-1) were assessed three hours after α,β-meATP administration.

Measurement of myeloperoxidase activity

Myeloperoxidase is one of the enzymes released from neutrophils and directly associated to tissue injury. Although monocytes/macrophages and fibroblasts also contain myeloperoxidase, neutrophils show the highest intracellular levels of this enzyme, that represents up to 5% of neutrophil proteins (Klebanoff, 1991). Therefore, the measurement of myeloperoxidase's activity was used as a marker of neutrophil migration (Klebanoff, 1991) in the knee joint of rats after application of the stimulus. Three hours after α,β-meATP injection in the knee joint, synovial lavage fluid was collected and homogenized in 500µL of buffer 1 (0.1 M NaCl, 0.02 M $NaPO_4$,1.015 M Na EDTA, pH 5.4) followed by centrifugation at 3000 rpm for 15 min. The pellet was resuspended in 500 µL of buffer 1 and subjected to hypotonic lyses by the addition of 500 µL of 0.2% NaCl followed 30 seconds later by addition of 500 µL of 1.6% NaCl in 5% glucose. After a further centrifugation, the pellet was resuspended in 0.05 M $NaPO_4$ buffer (pH 5.4) containing 0.5% hexadecyl-trimethylammonium bromide (HTAB). After that, the samples were snap-frozen in liquid nitrogen and thawed, three times, and centrifuged at 10,000 rpm for 15 min.

The myeloperoxidase kinetic-colorimetric assay was conducted, as previously described (Bradley et al., 1982, Perin-Martins et al., 2013). Fifty microliters of each sample (supernatant) and 0.08 M $NaPO_4$ were dropped into wells of a 96-well microplate. 25 µl of 3,3′,3,3′-tetramethylbenzidine (TMB) was added in each well and the reaction was initiated by the addition of 100 µL of H_2O_2. The reaction was stopped 5 min later by the addition of 50 µL of 4M H_2SO_4. The optical density was read at 450 nm using an Asys UVM340 (3 readings at intervals of 30 seconds). Results were calculated by comparing the optical density of rat's knee joint synovial lavage fluid supernatant with a standard curve of neutrophil (>95% purity). All

procedures were repeated twice to guarantee the authenticity of the results. The results were presented as number of neutrophils/knee.

Statistical analysis

To determine if there were significant differences ($p<0.05$) between treatment groups, One-way ANOVA or T-test was performed. If there was a significant between-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. For data shown in Fig. 1B a Two-way repeated measures ANOVA with one between subjects factor (i.e., treatment) and one within-subjects factor (i.e., time) were used to determine whether there were significant ($p<0.05$) differences among the groups. If there was a significant between-subjects main effect of treatment group, post hoc contrasts using the Bonferroni test were performed to determine the basis of the significant difference. Data are expressed in figures as means ± S.E.M.

Results

α,β-meATP-induced articular hyperalgesia in the rat's knee joint

To investigate whether the P2X3 and P2X2/3 receptors activation induces articular hyperalgesia, the P2X3 and P2X2/3 receptors agonist α,β-meATP was administrated in the rat's knee joint. The intra-articular administration of α,β-meATP (10, 30, 100, 300 and 900 µg/knee) induced articular hyperalgesia in a dose-response manner. The dose of 300 µg/knee (submaximal dose) was used in all subsequent experiments (Fig. 1A, $P<0.05$, two-way ANOVA post hoc Bonferroni test). As showed in figure 1B, the α,β-meATP-induced articular hyperalgesia (300

μg/knee) reached its maximum effect 3 hours after its administration and was completely resolved 24 hours later when compared with 0.9% NaCl control group ($P<0.05$, two-way ANOVA post hoc Bonferroni test). The subsequent experiments was performed 3 hours after α,β-meATP administration.

To confirm that α,β-meATP-induced articular hyperalgesia was mediated by P2X3 and P2X2/3 receptors, the selective P2X3 and P2X2/3 receptors antagonist A-317491 was co-administered with α,β-meATP. A-317491 (540 μg/knee) reduced the α,β-meATP-induced articular hyperalgesia (Fig. 1C, $P<0.05$, one-way ANOVA post hoc Tukey test). To rule out the systemic effect of A-317491 (540 μg/knee), it was administrated in contralateral knee joint and did not affect the articular hyperalgesia induced by α,β-meATP administrated in ipsilateral knee joint (Fig. 1C, $P>0.05$, one-way ANOVA post hoc Tukey test). In addition, to confirm the selectivity of α,β-meATP (300 μg/knee) as a P2X3 and P2X2/3 agonist, it was co-administrated with the selective P2X7 receptors antagonist A-740003 (568 μg/knee), which did not affect the α,β-meATP-induced articular hyperalgesia (Fig. 1C, $P>0.05$, one-way ANOVA post hoc Tukey test).

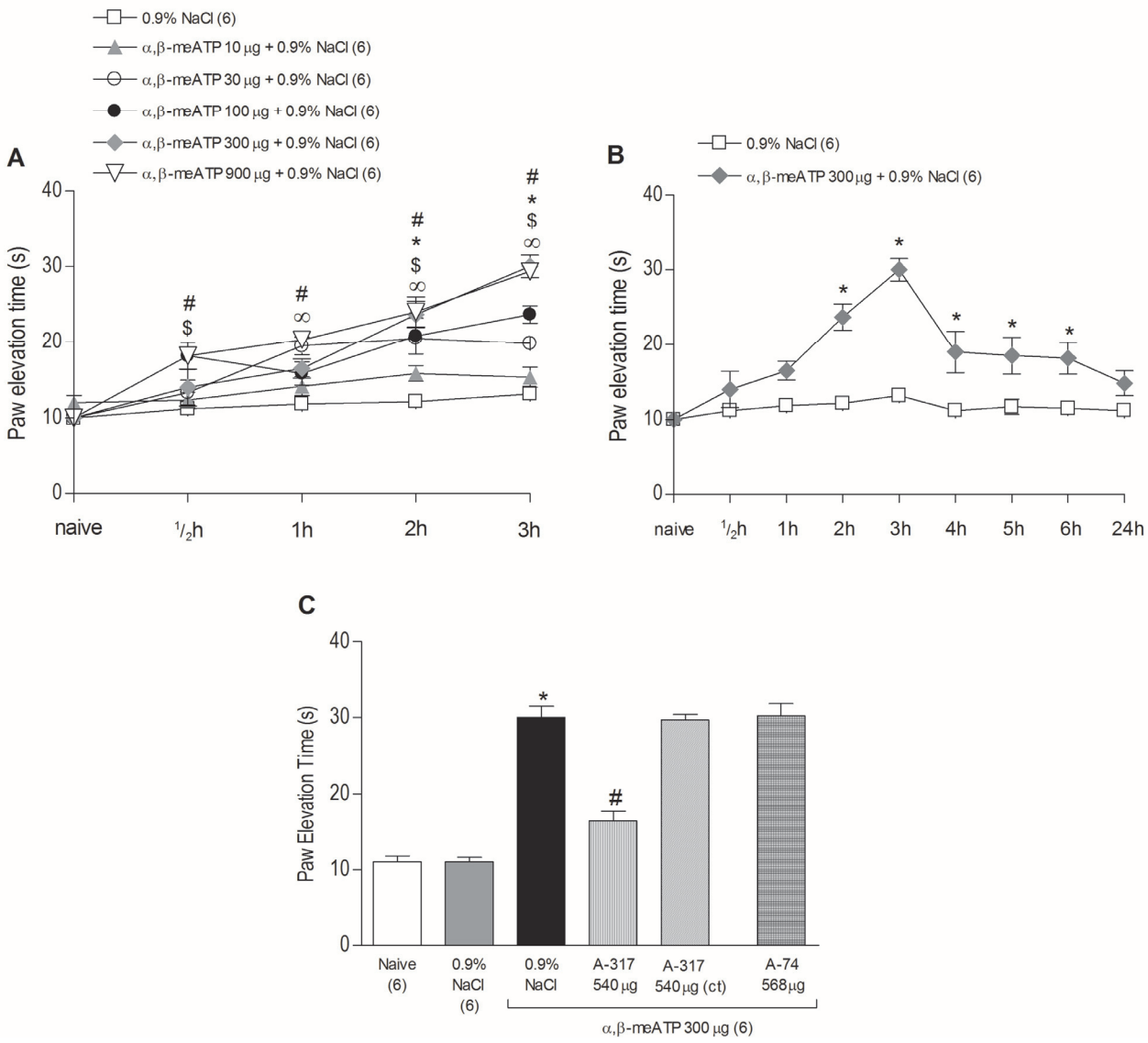

Figure 1 - Articular hyperalgesia induced by α,β-meATP administration in the rat knee joint. (A) Intra-articular administration of α,β-meATP (30, "∞"; 100, "$"; 300, "*" and 900 µg/knee, "#") in the rat knee joint induced a dose-dependent articular hyperalgesia when compared with 0.9% NaCl control group (P<0.05, Bonferroni test). **(B)** The α,β-meATP-induced articular hyperalgesia (300 µg/knee) in the rat's knee joint reached its maximum effect 3 hours after its administration. The symbol "*" indicates responses significantly greater than that induced by 0.9% NaCl group (P<0.05, Bonferroni test). **(C)** The α,β-meATP-induced articular hyperalgesia (300 µg/knee) was significantly reduced by the co-administration of A-317491 (A-317, 540 µg/knee, P<0.05, Tukey test) as indicated by the symbol "#", but not by the co-administration of A-740003 (A-74, 568 µg/knee, P>0.05, Tukey test). A-317491 (540 µg/knee)

applied in the contralateral knee joint (ct) did not affect the α,β-meATP-induced articular hyperalgesia. The symbol "*" indicates responses significantly greater than that induced by 0.9% NaCl or naive groups ($P<0.05$, Tukey test). In this and in subsequent figures the hyperalgesia was measured 3 hours after α,β-meATP administration and the numbers of rats used are shown in parentheses.

Blockade of local bradykinin B_1- or B_2-receptor reduced the articular hyperalgesia induced by α,β-meATP.

To verify whether α,β-meATP-induced articular hyperalgesia depends on bradykinin release, the bradykinin B_1- or B_2-receptor antagonist DALBK or Bradyzide, respectively, was co-administered with α,β-meATP. DALBK (3.0 μg/knee, Fig. 2A) or Bradyzide (0.5 μg/knee, Fig. 2B) reduced the α,β-meATP-induced articular hyperalgesia ($P<0.05$, one-way ANOVA post hoc Tukey test) and they did not affect the articular hyperalgesia when administered in the contralateral knee joint ($P>0.05$, one-way ANOVA post hoc Tukey test) confirming their local action. The co-administration of DALBK (3.0 μg/knee) or Bradyzide (0.5 μg/knee) with 0.9% NaCl did not change the paw elevation time (PET) when compared with 0.9% NaCl or naive control groups ($P>0.05$, one-way ANOVA post hoc Tukey test).

Blockade of local β_1- or β_2-adrenoceptors reduced the articular hyperalgesia induced by α,β-meATP

To verify whether α,β-meATP-induced articular hyperalgesia depends on sympathomimetics amines release, the β_1- or β_2-adrenoceptor antagonist Atenolol or ICI 118,551, respectively, was co-administered with α,β-meATP. As showed in figure 2, Atenolol (6.0 μg/knee, C) or ICI 118,551 (1.5 μg/knee, D) significantly reduced α,β-meATP-induced articular hyperalgesia ($P<0.05$, one-way ANOVA post hoc Tukey test) and they did not affect the articular

hyperalgesia when administered in the contralateral knee joint (P>0.05, one-way ANOVA post hoc Tukey test) confirming their local action. The co-administration of Atenolol (6.0 μg/knee) or ICI 118,551 (1.5 μg/knee) with 0.9% NaCl did not change the paw elevation time (PET) when compared with 0.9% NaCl or naive control groups (P>0.05, one-way ANOVA post hoc Tukey test).

Blockade of local prostaglandins synthesis reduced the articular hyperalgesia induced by α,β-meATP

To verify whether α,β-meATP-induced articular hyperalgesia depends on prostaglandins release, rats were treated with local administration of Indomethacin 30 minutes before α,β-meATP administration. Indomethacin (100 μg/knee, Fig. 2E) significantly reduced α,β-meATP-induced articular hyperalgesia (P<0.05, one-way ANOVA post hoc Tukey test) and did not affect the articular hyperalgesia when administered on the contralateral knee joint (P>0.05, one-way ANOVA post hoc Tukey test) confirming its local action. The co-administration of Indomethacin (100 μg/knee) with 0.9% NaCl did not change the paw elevation time (PET) when compared with 0.9% NaCl or naive control groups (P>0.05, one-way ANOVA post hoc Tukey test).

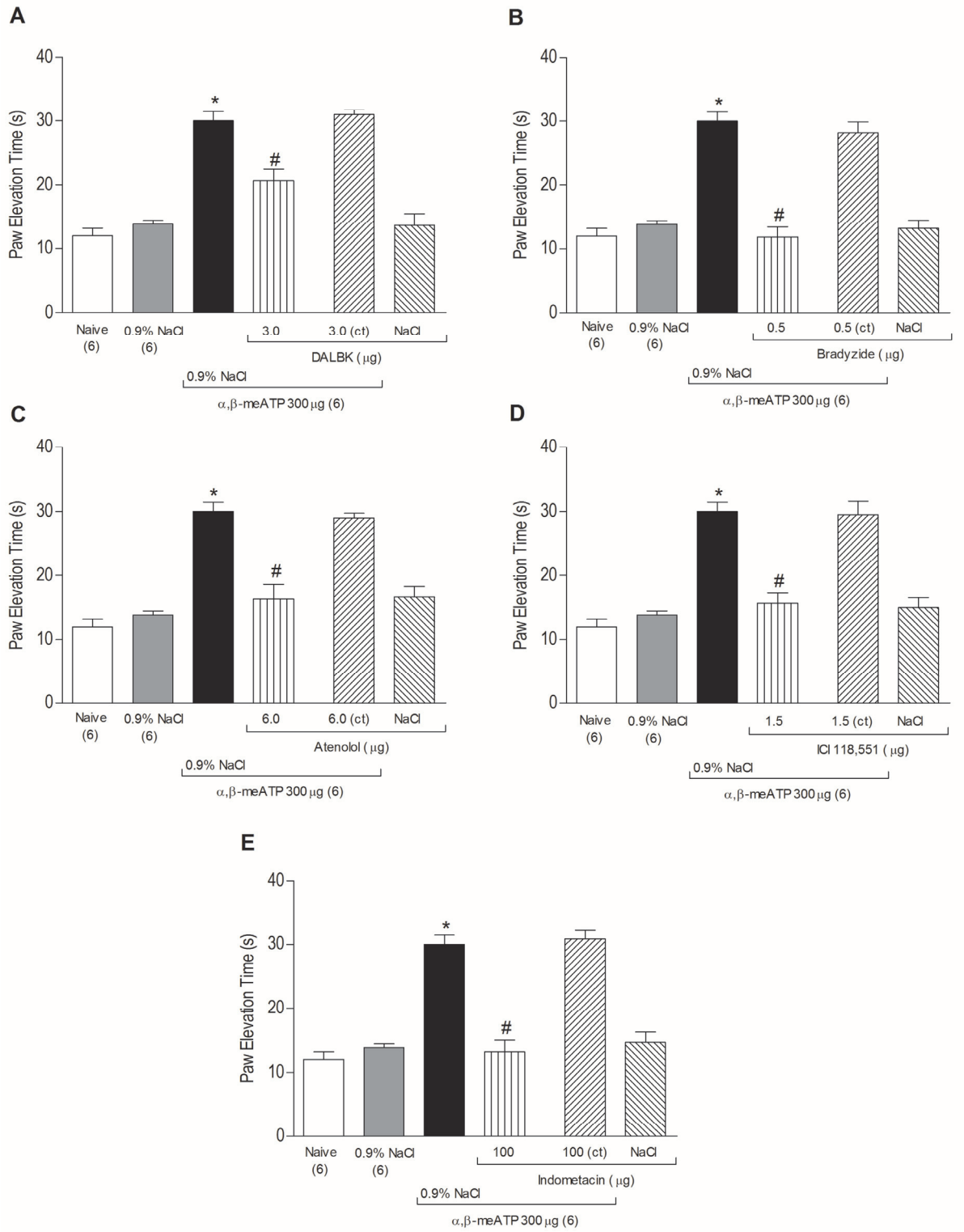

Figure 2 - Effect of B_1- or B_2-receptors antagonists, β_1- or β_2- adrenoceptors antagonists and the blockade of prostaglandins synthesis on the α,β-meATP-induced articular hyperalgesia in rat's knee joint. The co-administration of B_1 receptor antagonist DALBK (3.0 μg/knee, **A**),

B_2 receptor antagonist Bradyzide (0.5 µg/knee, **B**), β_1 adrenoceptor antagonist Atenolol (6.0 µg/knee, **C**), β_2 adrenoceptor antagonist ICI 118,551 (1.5 µg/knee, **D**) or cyclooxygenase inhibitor Indomethacin (100 µg/knee, 30 min pre-treatment, **E**) with α,β-meATP (300 µg/knee) significantly reduced the α,β-meATP-induced articular hyperalgesia, as indicated by the symbol "#" ($P<0.05$, Tukey test). DALBK (3.0 µg/knee, **A**), Bradyzide (0.5 µg/knee, **B**), Atenolol (6.0 µg/knee, **C**), ICI 118,551 (1.5 µg/knee, **D**) or Indomethacin (100 µg/knee, **E**) administrated in the contralateral knee joint (ct) or co-administrated with 0.9% NaCl did not affect the articular hyperalgesia induced by α,β-meATP or the paw elevation time, when compared with α,β-meATP alone or naive groups, respectively ($P>0.05$, Tukey test). The symbol "*" indicates responses significantly greater than that induced by 0.9% NaCl or naive groups ($P<0.05$, Tukey test).

Local administration of α,β-meATP increases the concentration of pro-inflammatory cytokines in rat's knee joint.

This set of experiments was performed to verify whether α,β-meATP induces local increase of concentration of TNF-α, IL-1β, IL-6 and CINC-1. Intra-articular administration of α,β-meATP (300 µg/knee) significantly increased the concentration of TNF-α (Fig. 3A), IL-1β (Fig. 3B), IL-6 (Fig. 3C) and CINC-1 (Fig. 3D) 3 hours later when compared with 0.9% NaCl administration or naive control groups ($P<0.05$, one-way ANOVA post hoc Tukey test). The co-administration of the selective P2X3 and P2X2/3 receptors antagonist A-317491 (540 µg/knee) with α,β-meATP reduced ($P<0.05$, one-way ANOVA post hoc Tukey test) the local concentration of TNF-α (Fig. 3A), IL-1β (Fig. 3B) and CINC-1 (Fig. 3D) but not IL-6 (Fig. 3C) ($P>0.05$, one-way ANOVA post hoc Tukey test). The intra-articular injection of 0.9% NaCl (50µL) alone did not affect the endogenous concentration of TNF-α, IL-1β, IL-6 and CINC-1 when compared with naive group ($P>0.05$, one-way ANOVA post hoc Tukey test).

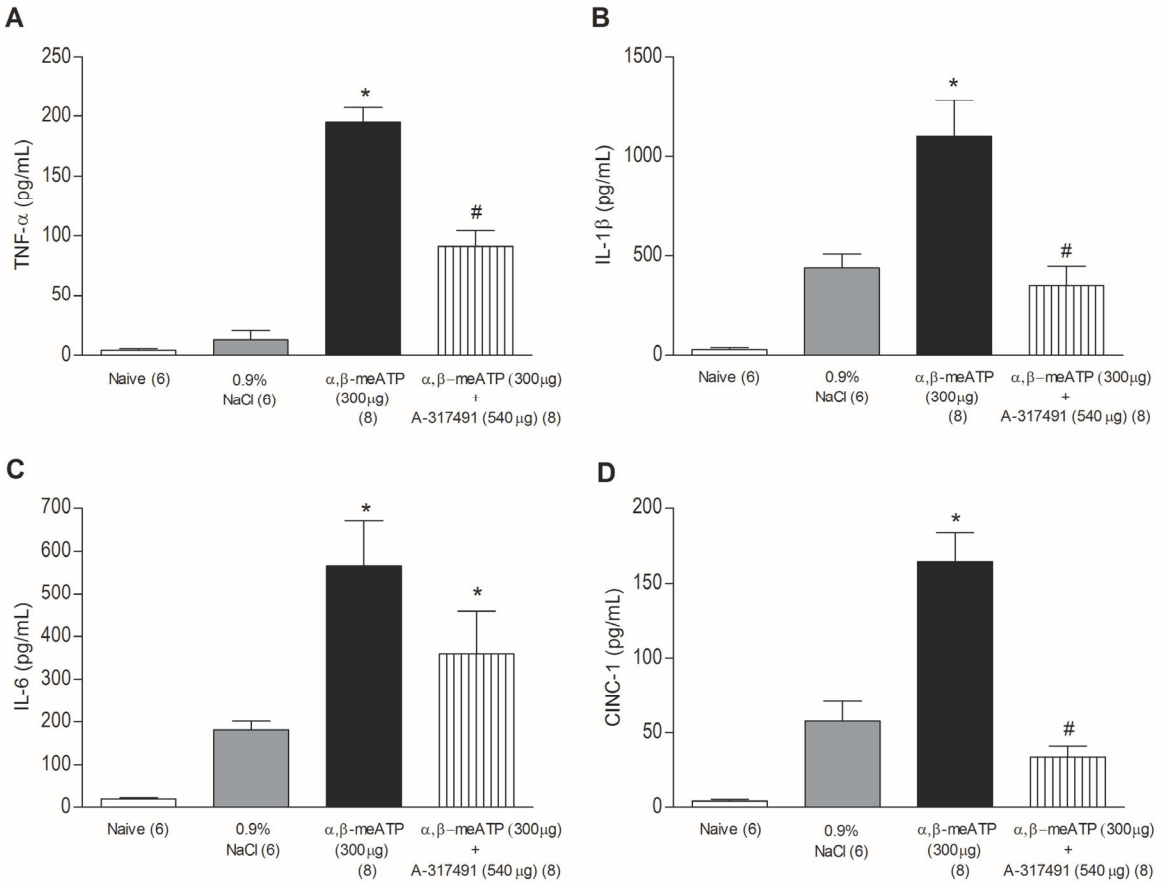

Figure 3 - Effect of α,β-meATP on the local concentration of pro-inflammatory cytokines in the rat's knee joint. The intra-articular administration of α,β-meATP (300 μg/knee) induced an increase of TNF-α (**A**), IL-1β (**B**), IL-6 (**C**) and CINC-1 (**D**) concentrations. The co-administration of A-317491 (540μg/knee) with α,β-meATP significantly reduced the α,β-meATP-induced increase of TNF-α (**A**), IL-1β (**B**) and CINC-1 (**D**) ($P<0.05$, Tukey test), but not IL-6 (**C**) ($P>0.05$, Tukey test) concentrations, as indicated by the symbol "#". The intra-articular injection of 0.9% NaCl did not affect the endogenous concentration of TNF-α, IL-1β, IL-6 and CINC-1 when compared with naive group ($P>0.05$, Tukey test). The symbol "*" indicated a cytokines concentrations significantly greater than that induced by 0.9% NaCl or by naive control groups ($P<0.05$, ANOVA, pos hoc Tukey test).

Neutrophil migration contributes to α,β-meATP-induced articular hyperalgesia

To verify whether local administration of α,β-meATP (300 μg/knee) induces neutrophil migration and if this is important to development of articular hyperalgesia, rats were treated with

Fucoidan (25 mg/kg, i.v.) 20 minutes before the α,β-meATP administration. As showed in figure 4A, the articular hyperalgesia measured 3 hours later α,β-meATP administration was reduced by the pre-treatment with Fucoidan ($P<0.05$, one-way ANOVA post hoc Tukey test). Intra-articular administration of α,β-meATP (300 µg/knee) increased the myeloperoxidase's activity when compared with 0.9% NaCl control group which was blocked by Fucoidan (Fig. 4B, $P<0.05$, one-way ANOVA post hoc Tukey test). The intra-articular injection of 0.9% NaCl alone did not affect the myeloperoxidase's activity when compared with naive rats ($P>0.05$, one-way ANOVA post hoc Tukey test).

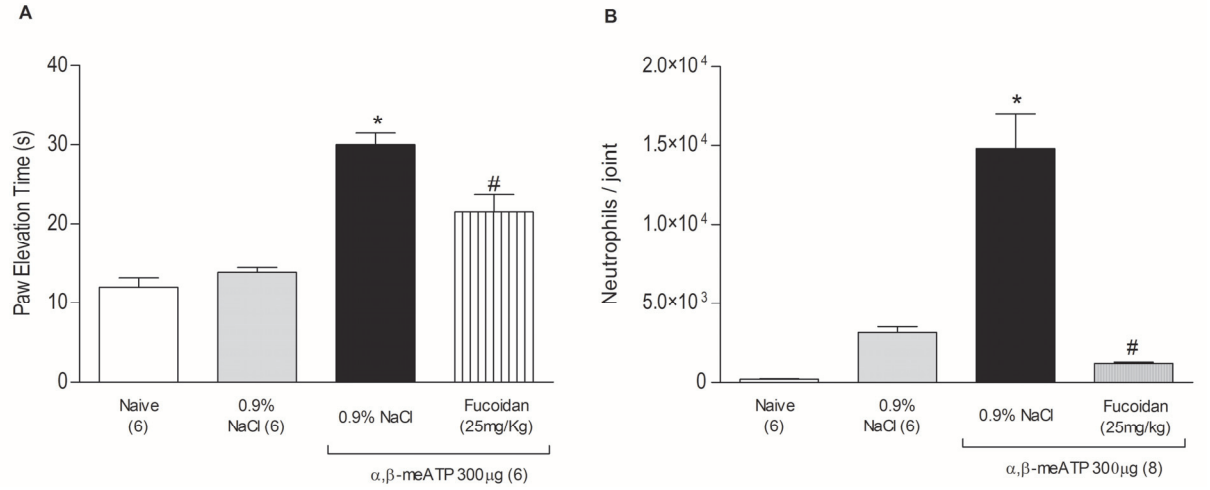

Figure 4 - Effect of Fucoidan on α,β-meATP-induced articular hiperalgesia and neutrophil migration in the rat's knee joint.

(A) The pre-treatment with Fucoidan (25 mg/kg, i.v.) significantly decreased α,β-meATP-induced articular hyperalgesia, as indicated by the symbol "#" ($P<0.05$, Tukey test). The symbol "*" indicates an articular hyperalgesia significantly greater than that induced by 0.9% NaCl or naive groups ($P<0.05$, Tukey test). **(B)** The intra-articular administration of α,β-meATP (300 µg/knee) induced an increase of myeloperoxidase's activity which was blocked by the pre-treatment with Fucoidan (25 mg/kg, i.v.), as indicated by the symbol "#" ($P<0.05$, Tukey test). The symbol "*" indicates a myeloperoxidase's activity significantly greater than that induced by 0.9% NaCl or naive groups ($P<0.05$, Tukey test).

Discussion

This study demonstrated that peripheral P2X3 and P2X2/3 receptors activation induce articular hyperalgesia, at least in part, by an indirect sensitization of the primary afferent nociceptors once it depends on release of bradykinin, prostaglandins, sympathomimetic amines, pro-inflammatory cytokines and on neutrophil migration.

Although α,β-meATP is a non-selective P2X3 receptor agonist once it also bind to P2X1 and P2X2/3 receptor, the involvement of P2X1 in hyperalgesia seems to be unlikely, since it has been demonstrated that IP5I, a potent and selective P2X1 receptor antagonist, is ineffective in reducing inflammatory pain (Honore et al., 2002) or mechanical hypersensitivity (Dai et al., 2004), in addition it has been shown that the P2X1 receptor does not contribute to hyperalgesia in the temporomandibular joint (Shinoda et al., 2005).

While in this study we assumed the involvement of both P2X3 and P2X2/3 receptor activation in articular hyperalgesia induced by α,β-meATP, the involvement of P2X2/3 in this mechanism is not clear. Studies demonstrate that nociceptive behaviors and thermal hyperalgesia produced by α,β-meATP may primarily reflect P2X3 receptor activation because the responses to α,β-meATP were completely eliminated in P2X3 receptor deficient animals (Cockayne et al., 2000, Souslova et al., 2000, Zhong et al., 2001). Moreover, oligodeoxynucleotide antisense against P2X3 receptor prevents inflammatory hyperalgesia at same magnitude than the selective P2X3 and P2X2/3 receptor antagonist A-317491 (Oliveira et al., 2009). Indeed, α,β-meATP is the most used agonist for P2X3 and P2X2/3 receptors available (Gever et al., 2006). In this study we also demonstrated that the articular hyperalgesia induced by α,β-meATP was prevented by the P2X3 and P2x2/3 receptor antagonist A-317491 (Jarvis et al., 2002), but was unaffected by the

selective P2X7 receptors antagonist A-740003 (Honore et al., 2006), strengthening previous data showing that α,β-meATP has not agonist activity in P2X7 receptors (Bianchi et al., 1999).

In agreement with the findings of this study, the activation of P2X3 and P2X2/3 receptors in knee joint induces articular hyperalgesia which depends on the subsequent activation of B_1 and B_2 receptors. Bradykinin is an inflammatory mediator rapidly generated after tissue injury (Ferreira et al., 1993a, Ferreira et al., 1993b) and modulates most of the events observed during the inflammatory processes, including increase of vascular permeability, cell migration, nociception and inflammatory hyperalgesia (Marceau et al., 1998, Calixto et al., 2000, Couture et al., 2001). Also, studies suggest that the mechanical hyperalgesia induced by bradykinin depends on release of endogenous ATP and P2X3 receptor activation (de Oliveira Fusaro et al., 2010).

Similarly to cutaneous tissue (Ferreira et al., 1993a), this study confirm that bradykinin plays a crucial role in the development of articular hyperalgesia in rat's knee joint. Considering that bradykinin is an inflammatory mediator released at the early phase of inflammatory hyperalgesia (Ferreira et al., 1993a, Ferreira et al., 1993b), data of this study suggest that the release of ATP and the subsequent P2X3 receptor activation is a inflammatory process upstream to release of bradykinin.

It has been described that bradykinin induces hyperalgesia by two distinct pathways that ultimately result in the local prostaglandins synthesis and sympathomimetic amines release (Ferreira et al., 1993a, Ferreira et al., 1993b), which directly sensitize the primary afferent nociceptor (Gold et al., 1996, Khasar et al., 1999, Rush and Waxman, 2004). Data of this study also showed that β-adrenoceptors antagonists or the cyclooxygenase inhibitor indomethacin reduced the α,β-meATP-induced articular hyperalgesia. These results demonstrated that articular hyperalgesia induced local activation of P2X3 and P2X2/3 receptors ultimately depends on the

production of prostaglandins and release of sympathomimetic amines, which in turn act directly on primary afferent nociceptors.

The synovium is innervated by postganglionic sympathetic fibers which are between half and two-thirds of the nerve fibers in the synovium (Hildebrand et al., 1991). Noradrenaline is co-released with ATP from sympathetic nerves, and both agents contribute to sympathetically mediated autonomic responses (Burnstock, 1995). Thus, the current data are consistent with earlier studies which demonstrated strong synergistic effect between the noradrenergic system (sympathetic postganglionic neurons) and P2X3 receptor in neuropathic and thermal pain (Waldron and Sawynok, 2004, Meisner et al., 2007, Meisner et al., 2008).

It is interesting to point out that, although the activation of P2X3 and P2X2/3 receptors in knee joint induces articular hyperalgesia by two independent pathways, one mediated by prostaglandins and other by sympathomimetic amines, the blockage of just one pathway completely prevented the articular hyperalgesia induced by α,β-meATP. These data suggest that the articular hyperalgesia induced by P2X3 and P2X2/3 receptor activation in knee joint tissue may involve the activation of more than one type of receptor and consequently more than one signaling pathway activation in primary afferent nociceptor.

Although P2X3 and P2X2/3 receptors are predominantly distributed on terminals of primary afferent neurons (Bradbury et al., 1998, Burnstock and Knight, 2004, Gever et al., 2006), they are also expressed in chondrocytes (Varani et al., 2008) and during joint inflammation occurs an increase of P2X3 receptor expression in the chondrocytes of rat's knee joint articular cartilage (Teixeira et al., 2014b *in press*). Studies *in vitro* demonstrated that α,β-meATP increases the synthesis of prostaglandin E_2 (PGE_2) by chondrocytes via P2X3 and P2X2/3 receptor

activation (Varani et al., 2008). This data suggest that, at least PGE_2 is released by chondrocytes as a result of P2X3 and P2X2/3 receptors receptor activation.

Therefore, our data suggest that extracellular ATP, in addition to directly activation of P2X3 and P2X2/3 receptors in primary afferent nociceptor in the rat knee joint, as previously demonstrated (Dowd et al., 1998), it can also activates these receptors in articular chondrocytes that releases inflammatory mediators, which in turn, ultimately sensitize the primary afferent nociceptors.

Because the prostaglandins synthesis and sympathomimetic amines release usually depends on prior pro-inflammatory cytokines release (Cunha et al., 1991, Cunha et al., 1992, Ferreira et al., 1993a), we also investigated whether P2X3 and P2X2/3 receptor activation induces release of TNF-α, IL-1β, IL-6 and CINC-1. The current results demonstrated that P2X3 and P2X2/3 receptor activation increases the concentration of TNF-α, IL-1β, IL-6 and CINC-1 in the rat's knee joint, suggesting that P2X3 and P2X2/3 activation initiates the release of pro-inflammatory cytokines during articular inflammation. Indeed, pro-inflammatory cytokines participate in the development and maintenance of articular hyperalgesia (Williams et al., 1992, Lewthwaite et al., 1995, Ohtani et al., 2012).

Different resident cells can release pro-inflammatory cytokines in knee joint tissue, such as synoviocytes (Firestein, 2003), however chondrocytes should be a key candidate to release these cytokines by P2X3 and P2X2/3 receptor activation. In fact, it has been demonstrated *in vitro* that stimulated chondrocytes can release TNF-α, IL-1β, IL-6, IL-8 and IL-11 (Guerne et al., 1990, Lotz et al., 1992, Aida et al., 2006). It is important to note, however, that the activation of P2X3 receptor on primary afferent nociceptor induces release of ATP (Kirkpatrick and Burnstock, 1994) which in turn, can activate P2X7 receptor. This purinergic receptor is expressed

in inflammatory cells and synovial fibroblast (Surprenant et al., 1996, Collo et al., 1997, Mancino et al., 2001, Caporali et al., 2008). Therefore, P2X7 receptor activation by ATP release can collaborate to increase of pro-inflammatory cytokines induced by α,β-meATP.

Neutrophil migration to synovial fluid is another important sign during joint inflammation and it correspond to about 90% of the leukocytic infiltrate in synovial fluid of patients with arthritis (Mohr, 1995). It has been demonstrated that neutrophils play an important role in the arthropathies pathogenesis, because their interaction with resident cells and local inflammatory mediators amplify the inflammatory response contributing to the chronic and acute inflammation (Kasama et al., 2005). Based on that, we also investigated whether P2X3 and P2X2/3 receptors activation induces neutrophil migration to rat's knee joint, contributing to the articular hyperalgesia.

According with the data of this study, the activation of P2X3 and P2X2/3 receptor induces neutrophil migration to the rat's knee joint, because the pre-treatment with fucoidan, which inhibit neutrophil rolling (Ley et al., 1993, Cunha et al., 2008), prevented articular hyperalgesia and neutrophil migration induced by administration of α,β-meATP in knee joint. Therefore, the neutrophil migration induced by P2X3 and P2X2/3 receptors activation probably results from the release of the chemokine CINC-1, which induces chemotaxis and leukocytes activation (Romano et al., 1997, Moon et al., 2010). Although, the release of prostaglandins by P2X3 and P2X2/3 receptors activation could be resulted from the chondrocytes activation (Varani et al., 2008), neutrophils may contribute to the release of prostaglandins (Cunha et al., 2008) and consequently to development of articular hyperalgesia.

In conclusion, the findings of the present study suggest that P2X3 and P2X2/3 receptors activation induces articular inflammatory hyperalgesia by an indirect mechanism that involves

release of bradykinin and pro-inflammatory cytokines, neutrophil migration with and the subsequently release of prostaglandins and sympathomimetic amines which ultimately sensitize primary afferent nociceptors in joint tissue. The data of this study also suggest that P2X3 and P2X2/3 receptors activation has an important participation on inflammation of the knee joint and could be an interesting target to control arthropathies.

Acknowledgements

This work was supported by Grants from Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP, Brazil (2009/16854-3 and 2010/05381-4).

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V. CAPÍTULO 04

Peripheral mechanisms involved in the articular hyperalgesia induced by P2X7 receptor activation in the rat's knee joint

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Abstract

Recently we have demonstrated that endogenous ATP, via P2X7 receptor activation is essential to development of carrageenan-induced articular hyperalgesia in the rats knee joint. In this study, we hypothesized that P2X7 receptor activation induces articular hyperalgesia in the rat's knee joint and, if so, whether depends on release of inflammatory mediators such as bradykinin, prostaglandins, sympathomimetic amines, pro-inflammatory cytokines and neutrophil migration. We found that BzATP, a P2X7 receptor agonist, induced a dose-dependent articular hyperalgesia that was blocked by the selective P2X7 receptor antagonist A-740003, but not by the selective P2X3 and P2X2/3 receptors antagonist A-317491. These findings confirm that although BzATP also acts at P2X3 receptor, BzATP-induced articular hyperalgesia was mediated by P2X7 receptor activation. Co-administration of selective antagonists for bradykinin B_1- (DALBK) or B_2-receptors (Bradyzide), β_1- (Atenolol) or β_2-adrenoceptors (ICI 118,551) and the local pre-treatment with cyclooxygenase inhibitor (Indomethacin) significantly reduced BzATP-induced articular hyperalgesia in the rat's knee joint. BzATP also induced the pro-inflammatory cytokines release TNF-α, IL-1β, IL-6 and CINC-1, an effect that was significantly reduced by A-740003. Moreover, BzATP induced the neutrophil migration to the knee joint that was significantly reduced by the nonspecific selectin inhibitor (Fucoidan). On the other hand, the pre-treatment with Fucoidan did not reduced BzATP-induced articular hyperalgesia in the rat's knee joint. Taken together, these findings suggest that peripheral P2X7 receptor activation induces articular hyperalgesia by an indirect sensitization of the primary afferent nociceptor of the rat's knee joint which depends on release of bradykinin, prostaglandins, sympathomimetic amines and pro-inflammatory cytokines.

Keywords: BzATP, P2X7 receptor, articular hyperalgesia, knee joint, inflammatory mediators, pro-inflammatory cytokines.

Introduction

The P2X7 purinergic receptor subtype belongs to the family of ATP-sensitive ionotropic P2X receptors (P2X1-P2X7) (Buell et al., 1996, North, 2002) and have been implicated in processes of pain and hyperalgesia in several tissues, including articular tissues such as the temporomandibular joint (TMJ), ankle and knee joints of rodents (Labasi et al., 2002, Broom et al., 2008, Teixeira et al., 2010).

Osteoarthritis (OA) is a common disease of aged population and one of most important causes of pain and physical disability, affecting the quality of life of patients. Affects the whole joint including cartilage, synovial membrane, muscle and bones and its occurrence is most common in the knees, hips and hands (Woolf and Pfleger, 2003, Heidari, 2011). Although the cause of pain in OA patients is not yet clear, evidences suggest the involvement of ATP in the development of hyperalgesia during OA (Dowd et al., 1998, Seino et al., 2006, Kumahashi et al., 2011, Teixeira et al., 2014b *in press*). Indeed, extracellular ATP is frequently found in synovial fluid of patients with arthropathies (Ryan et al., 1991, Park et al., 1996, Kumahashi et al., 2011).

During inflammation, ATP released from injured cells (Filippini et al., 1990, Dubyak and el-Moatassim, 1993, Beigi et al., 1999, Sikora et al., 1999, Mizumoto et al., 2003 Campwala and Fountain, 2013) can activates the P2X7 receptor, which is predominantly found on macrophages, mast cells, erythrocytes, monocytes, fibroblasts, peripheral macrophages, T and B lymphocytes and in type B synoviocytes (fibroblasts like-synoviocytes - FLS cells (Chiozzi et al., 1997, Collo

et al., 1997, Kim et al., 2001, Greig et al., 2003, Caporali et al., 2008), where it can triggers cellular responses such as membrane permeabilization, caspases activation, pro-inflammatory cytokine release and cell proliferation (Panenka et al., 2001, Chakfe et al., 2002, North, 2002, Verhoef et al., 2003, Kahlenberg and Dubyak, 2004), contributing to inflammatory hyperalgesia.

In addition to ATP, others inflammatory mediators, such as bradykinin, prostaglandins, sympathomimetic amines and pro-inflammatory cytokine are released after tissue injury and are essential to induce and maintain the inflammatory hyperalgesia (Cunha et al., 1992, Ferreira et al., 1993a, Loram et al., 2007 Luiz et al., 2010, Petho and Reeh, 2012, Villarreal et al., 2013). Also, neutrophil migration is essential to the development of inflammatory hyperalgesia (Jain et al., 2001, Tambeli et al., 2006, Oliveira et al., 2007, Cunha et al., 2008, Guerrero et al., 2008, Carreira et al., 2013). We have showed that endogenous ATP via P2X7 receptor activation is essential to development of articular hyperalgesia, pro-inflammatory cytokines release, and neutrophil migration induced by carrageenan in the rat's knee joint (Teixeira et al., 2014b *in press*).

Therefore, the aim of this study was to verify whether the administration of P2X7 receptor agonist BzATP in the rat's knee joint induces articular hyperalgesia and, if so, whether depends on release of inflammatory mediators involved in hyperalgesia, such as bradykinin, prostaglandins, sympathomimetic amines, pro-inflammatory cytokines and neutrophil migration.

Materials and Methods

Animals

Male Wistar rats (200-250g) obtained from the Multidisciplinary Center for Biological Research (CEMIB) - University of Campinas, were used in this study. The animals were housed in plastic cages with soft bedding (five/cage) on a 12:12 light cycle (lights on at 06:00 A.M.) with food and water available *ad libitum*. They were maintained in a temperature-controlled room (±23°C) and handled for at least one week prior to the experiments. Each animal was used once and the number of animals per group was kept to a minimum. Experimental protocols were approved by the Committee on Animal Research of the University of Campinas (protocol number: 2049-1) and conformed to IASP guidelines for the study of the pain in animals (Zimmermann, 1983).

General Procedures

Testing sessions took place during light phase (between 09:00 AM and 5:00 PM) in a quiet room maintained at 23ºC (Rosland, 1991). During the tests the animals had no access to water or food. The animals were habituated for 1 hour prior to the experiment to minimize stress.

Drugs administration

Drugs or their vehicle were intra-articularly administrated in the right rats knee joint by a 26-gauge needle that was connected to a catheter of polyethylene and also to a Hamilton syringe (50 µL). The volume of injection was 50 µL.

BzATP-induced knee joint inflammation

Under brief inhalation of isoflurane anesthesia, rats were subjected to intra-articular (i.a.) injection of BzATP dissolved in 25 μL sterile 0.9% saline solution into their right knee joint, in the same manner as demonstrated in carrageenan-induced articular hyperalgesia (Tonussi and Ferreira, 1992). The injection site was shaved and treated with an antiseptic solution of iodine alcohol. The other drugs were also injected into the knee joint in the same manner that the BzATP and the control animals received vehicle.

Drugs and doses

The following drugs were used: the P2X7 receptor agonist 2′(3′)-O-(4-Benzoylbenzoyl)adenosine 5′-triphosphate Triethylammonium salt (Jacobson et al., 2002) (BzATP: 25, 75, 225 and 675 μg/knee, (Jarvis et al., 2001); the selective P2X7 receptor antagonist [*N*-(1-{[(Cyanoimino) (5-quinolinylamino) methyl] amino}-2,2-dimethylpropyl)- 2-(3,4-dimethoxyphenyl)acetamide] (Honore et al., 2006) (A-740003: 568 μg/knee, i.a., Teixeira et al., 2014b *in press*); the selective bradykinin B_1 receptor antagonist des-Arg^9-[Leu^8]-BK - DALBK (Ferreira et al., 2008) (3.0 μg/knee, i.a., Teixeira et al., 2014a *in press*); the selective bradykinin B_2 receptor antagonist Bradyzide (Burgess et al., 2000) (1.5 μg/knee, i.a. Teixeira et al., 2014a *in press*); the selective β_1 receptor antagonist Atenolol (Allibardi et al., 1999) (6.0 μg/knee, i.a., Teixeira et al., 2014a *in press*); the selective β_2 receptor antagonist ICI 118,551 (Yalcin et al., 2009) (1.5 μg/knee, i.a., Teixeira et al., 2014a *in press*); the cyclooxygenase inhibitor Indomethacin (Summ and Evers, 2013) (100 μg/knee, i.a., Teixeira et al., 2014a *in press*) and the nonspecific selectin inhibitor Fucoidan (Ley et al., 1993) (25 mg/kg, i.v., Teixeira et al., 2014a *in press*). A-740003 was obtained from Tocris Bioscience (Ellisville, MO) and all

other drugs were obtained from Sigma-Aldrich (MO, USA). A-740003 was dissolved in 25 μL of dimethyl sulfoxide (DMSO, 50%) and propylene glycol (50%) and all other drugs were dissolved in sterile saline (0.9% NaCl).

Gait disturbance - Rat knee-joint incapacitation test

We used the rat knee-joint incapacitation test, as described previously (Tonussi and Ferreira, 1992). Briefly, 3 hours after BzATP injection into their right knee joints, rats were put to walk on a steel rotary cylinder (30 cm wide x 50 cm diameter), covered with a fine-mesh non-oxidizable wire screen, which rotates at 3 rpm. Specially, designed metal gaiters were wrapped around both hind paws. After placement of the gaiters, rats were placed to walk in the cylinder and the right paw was then connected via a simple circuit to microcomputer data input/output port. The paw elevation time (PET) is the total time that the rat walks failing to touch the cylinder surface with the injected hindpaw, during a 60 sec period, which is directly proportional to the gait disturbance. Incapacitation was quantified as an increase in the PET, 3 hours after carrageenan injection into the right knee joint. To minimize variations in PET, all rats were introduced to the experimental environment and trained on the apparatus to habituation into the equipment before the testing sessions. To confirm the local effect of some test agents, they were injected into the contralateral rat's knee joint and the test was performed on the ipsilateral knee joint. The rat knee-joint incapacitation test provides automated measurements, which are independent of the subjectivity of the observer (Tonussi and Ferreira, 1992).

Synovial lavage fluid

Under deep anaesthesia (induced by the intraperitoneal injection of 80 mg/kg ketamine and 20 mg/kg xylazine) the rats were then killed by cervical dislocation, the skin overlying the knee was excised, the patellar ligament was dissected and a 30-gauge needle connected to a 100 μL Hamilton syringe was inserted through the joint capsule. The knee joint cavity was washed twice by injecting and immediately aspirating 100 μL of phosphate-buffered saline solution (PBS) containing 4 mM EDTA (Ekundi-Valentim et al., 2010).

ELISA procedure

An adaptation of ELISA (Enzyme-Linked Immunosorbent Assay) (Safieh-Garabedian et al., 1995) was used to determine whether BzATP was able to increase the local concentration of TNF-α, IL-1β, IL-6 and CINC-1 in the rat's knee joint. Briefly, the synovial lavage fluid was homogenized in solution of phosphate-buffered saline (PBS) containing 0.4 M NaCl, 0.05% Tween 20, 0.5% bovine serum albumine (BSA), 0.1 mM phenyl-methylsulfonyl fluoride, 0.1 mM benzotonic chloride, 10 mM EDTA, and 20 KL/mL aprotinine (Sigma, USA). The samples were centrifuged at 10000 rpm for 15 minutes at 4ºC and the supernatants were stored at -70ºC for posterior use to evaluate the protein levels of TNF-α, IL-1β, IL-6 and CINC-1 in the rats knee joint. The cytokines were quantified by the following kits: TNF-α: Rat TNF-α/ TNFSF1A DuoSet ELISA Kit (R&D Systems, catalog number DY510); IL-1β: Rat IL-1β/IL-1F2 DuoSet ELISA Kit (R&D Systems, catalog number DY501), IL-6: Rat IL-6 DuoSet ELISA Kit (R&D Systems, catalog number: DY506) and CINC-1: Rat CXCL1/CINC-1 DuoSet ELISA Kit (R&D Systems, catalog number DY515). All procedures followed the instructions of the manufacturer R&D Systems. All procedures were repeated twice to guarantee the authenticity of the results. In

the present study, the levels of cytokines (TNF-α, IL-1β, IL-6 e CINC-1) were assessed three hours after BzATP administration.

Measurement of myeloperoxidase activity

Myeloperoxidase is one of the enzymes released from neutrophils and directly associated to tissue injury. Although monocytes/macrophages and fibroblasts also contain myeloperoxidase, neutrophils show the highest intracellular levels of this enzyme, that represents up to 5% of neutrophil proteins (Klebanoff, 1991). Therefore, the measurement of myeloperoxidase activity was used as a marker of neutrophil migration (Klebanoff, 1991) in the knee joint of rats after application of the stimulus. Three hours after BzATP injection in the knee joint, synovial lavage fluid was collected and homogenized in 500µL of buffer 1 (0.1 M NaCl, 0.02 M $NaPO_4$,1.015 M Na EDTA, pH 5.4) followed by centrifugation at 3000 rpm for 15 min. The pellet was resuspended in 500 µL of buffer 1 and subjected to hypotonic lyses by the addition of 500 µL of 0.2% NaCl followed 30 seconds later by addition of 500 µL of 1.6% NaCl in 5% glucose. After a further centrifugation, the pellet was resuspended in 0.05 M $NaPO_4$ buffer (pH 5.4) containing 0.5% hexadecyl-trimethylammonium bromide (HTAB). After that, the samples were snap-frozen in liquid nitrogen and thawed, three times, and centrifuged at 10,000 rpm for 15 min.

The myeloperoxidase kinetic-colorimetric assay was conducted, as previously described (Bradley et al., 1982, Perin-Martins et al., 2013). Fifty microliters of each sample (supernatant) and 0.08 M $NaPO_4$ were dropped into wells of a 96-well microplate. 25 µl of 3,3′,3,3′-tetramethylbenzidine (TMB) was added in each well and the reaction was initiated by the addition of 100 µL of H_2O_2. The reaction was stopped 5 min later by the addition of 50 µL of 4M H_2SO_4. The optical density was read at 450 nm using an Asys UVM340 (3 readings at

intervals of 30 seconds). Results were calculated by comparing the optical density of rats knee joint synovial lavage fluid supernatant with a standard curve of neutrophil (>95% purity). The results were presented as number of neutrophils/knee.

Statistical analysis

To determine if there were significant differences ($p<0.05$) between treatment groups, One-way ANOVA or T-test was performed. If there was a significant between-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. For data shown in Fig. 1B a Two-way repeated measures ANOVA with one between subjects factor (i.e., treatment) and one within-subjects factor (i.e., time) were used to determine whether there were significant ($p<0.05$) differences among the groups. If there was a significant between-subjects main effect of treatment group, post hoc contrasts using the Bonferroni test were performed to determine the basis of the significant difference. Data are expressed in figures as means ± S.E.M.

Results

BzATP-induced articular hyperalgesia in the rats knee joint

To investigate whether the P2X7 receptor activation induces articular hyperalgesia, the P2X7 receptor agonist BzATP was administrated in the rats knee joint. As showed in the figure 1A, the intra-articular administration of BzATP (25, 75, 225 and 675 μg/knee) induced articular hyperalgesia in a dose-response manner. The dose of 225 μg/knee (submaximal dose) was used in all the subsequent experiments ($P<0.05$, two-way ANOVA post hoc Bonferroni test). As showed in figure 1B, the BzATP-induced articular hyperalgesia (225 μg/knee) reached its maximum effect 3 hours after its administration and was completely resolved 6 hours later when compared with 0.9% NaCl control group ($P<0.05$, two-way ANOVA post hoc Bonferroni test). The subsequent experiments were performed 3 hours after BzATP administration.

To confirm that BzATP-induced articular hyperalgesia was mediated by P2X7 receptors, the selective P2X7 receptors antagonist A-740003 was co-administered with BzATP. A-740003 (568 μg/knee) reduced the BzATP-induced articular hyperalgesia (Fig. 1C, $P<0.05$, one-way ANOVA post hoc Tukey test). To rule out the systemic effect of A-740003 (568 μg/knee), it was administrated in contralateral knee joint and did not affect the articular hyperalgesia induced by BzATP administrated in ipsilateral knee joint (Fig. 1C, $P>0.05$, one-way ANOVA post hoc Tukey test). In addition, to confirm the selectivity of BzATP (225 μg/knee) as a P2X7 agonist, it was co-administrated with the selective the selective P2X3 and P2X2/3 receptors antagonist A-317491 (540 μg/knee) which did not affect the BzATP-induced articular hyperalgesia (Fig. 1C, $P>0.05$, one-way ANOVA post hoc Tukey test).

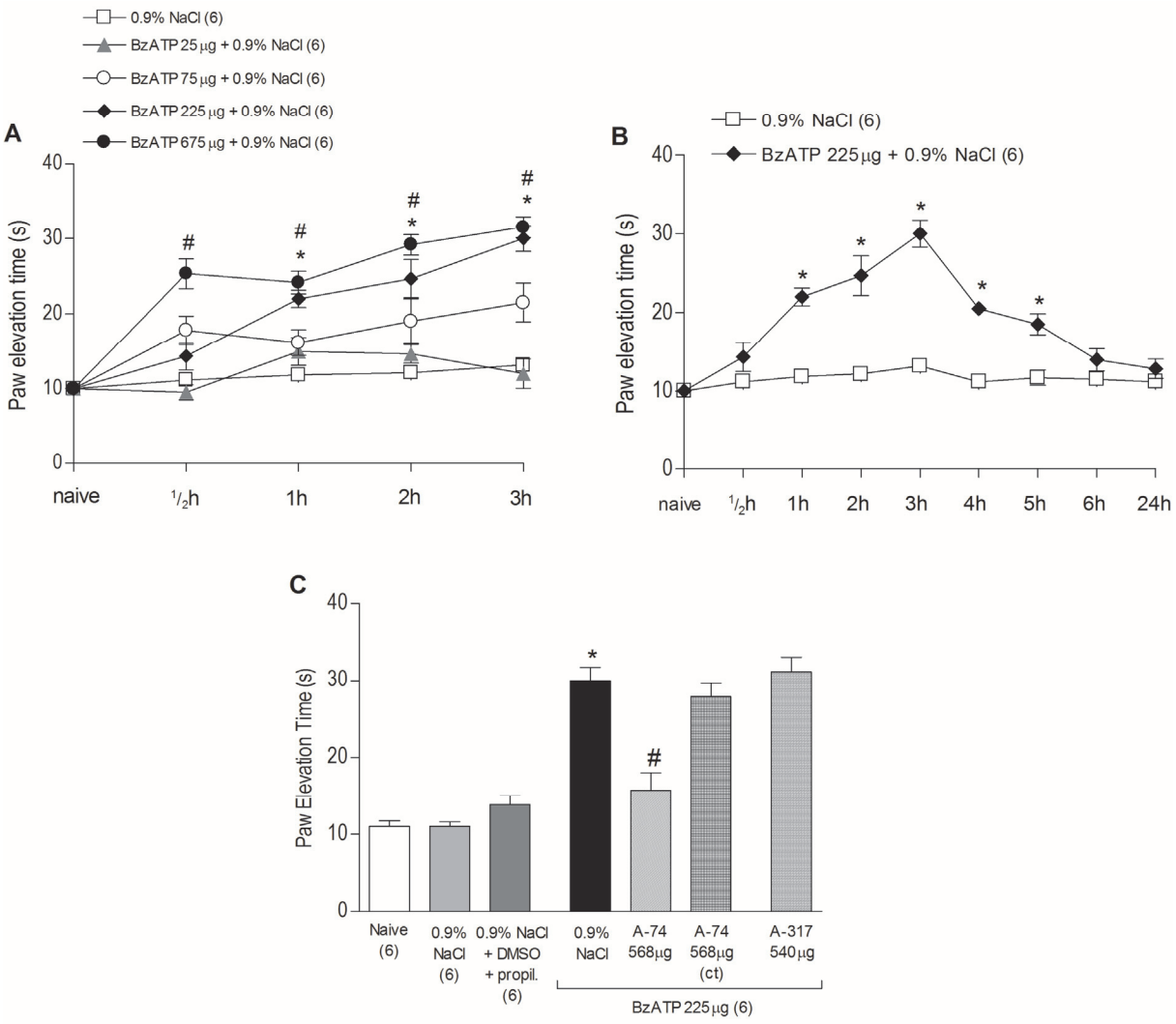

Figure 1 - Articular hyperalgesia induced by BzATP administration in the rat knee joint. **(A)** Intra-articular administration of BzATP (225 µg/knee, "*" and 675 µg/knee, "#") in the rats knee joint induced a dose-dependent articular hyperalgesia when compared with 0.9% NaCl control group ($P<0.05$, Tukey test). **(B)** The BzATP-induced articular hyperalgesia (225µg/knee) in the rat's knee joint reached its maximum effect 3 hours after its administration. The symbol "*" indicates responses significantly greater than that induced by 0.9% NaCl group ($P<0.05$, Bonferroni test). **(C)** The BzATP-induced articular hyperalgesia (225 µg/knee) was significantly reduced by the co-administration of A-740003 (A-74, 568 µg/knee, $P<0.05$, Tukey test) as indicated by the symbol "#", but not by the co-administration of A-317491 (A-317, 540 µg/knee, $P>0.05$, Tukey test). A-740003 (568 µg/knee) administrated in the contralateral knee joint (ct) did not affect the BzATP-induced articular hyperalgesia. The symbol "*" indicates responses

significantly greater than that induced by 0.9% NaCl or naive groups ($P<0.05$, Tukey test). In this and in subsequent figures the hyperalgesia was measured 3 hours after BzATP administration and the numbers of rats used are shown in parentheses.

Blockade of local bradykinin B_1- or B_2-receptors reduced the articular hyperalgesia induced by BzATP.

To verify whether BzATP-induced articular hyperalgesia depends on bradykinin release, the bradykinin B_1- or B_2-receptor antagonist DALBK or Bradyzide, respectively, was co-administered with BzATP. DALBK (3.0 µg/knee, Fig. 2A) or Bradyzide (0.5 µg/knee, Fig. 2B) reduced the BzATP-induced articular hyperalgesia ($P<0.05$, one-way ANOVA post hoc Tukey test) and they did not affect the articular hyperalgesia when administered on the contralateral knee joint ($P>0.05$, one-way ANOVA post hoc Tukey test) confirming their local action. The co-administration of DALBK (3.0 µg/knee) or Bradyzide (0.5 µg/knee) with 0.9% NaCl did not change the paw elevation time (PET) when compared with 0.9% NaCl or naive control groups ($P>0.05$, one-way ANOVA post hoc Tukey test).

Blockade of local β_1- or β_2-adrenoceptors reduced the articular hyperalgesia induced by BzATP.

To verify whether BzATP-induced articular hyperalgesia depends on sympathomimetics amines release, the β_1- or β_2-adrenoceptor antagonist Atenolol or ICI 118,551, respectively, was co-administered with BzATP. Atenolol (6.0 µg/knee, Fig. 2C) and ICI 118,551 (1.5 µg/knee, Fig. 2D) significantly reduced BzATP-induced articular hyperalgesia ($P<0.05$, one-way ANOVA post hoc Tukey test) and they did not affect the articular hyperalgesia when administered on the contralateral knee joint ($P>0.05$, one-way ANOVA post hoc Tukey test) confirming their local action. Co-administration of Atenolol (6.0 µg/knee) or ICI 118,551 (1.5 µg/knee) with 0.9%

NaCl did not change the paw elevation time (PET) when compared with 0.9% NaCl group or naive control groups (P>0.05, one-way ANOVA post hoc Tukey test).

Blockade of local prostaglandins synthesis reduced the articular hyperalgesia induced by BzATP.

To verify whether BzATP-induced articular hyperalgesia depends on prostaglandins release, rats were treated with local administration of Indomethacin 30 minutes before BzATP administration. Indomethacin (100 μg/knee, Fig. 2E) significantly reduced BzATP-induced articular hyperalgesia (P<0.05, one-way ANOVA post hoc Tukey test) and did not affect the articular hyperalgesia when administered on the contralateral knee joint (P>0.05, one-way ANOVA post hoc Tukey test) confirming its local action. The co-administration of Indomethacin (100 μg/knee) with 0.9% NaCl did not change the paw elevation time (PET) when compared with 0.9% NaCl or naive control groups (P>0.05, one-way ANOVA post hoc Tukey test).

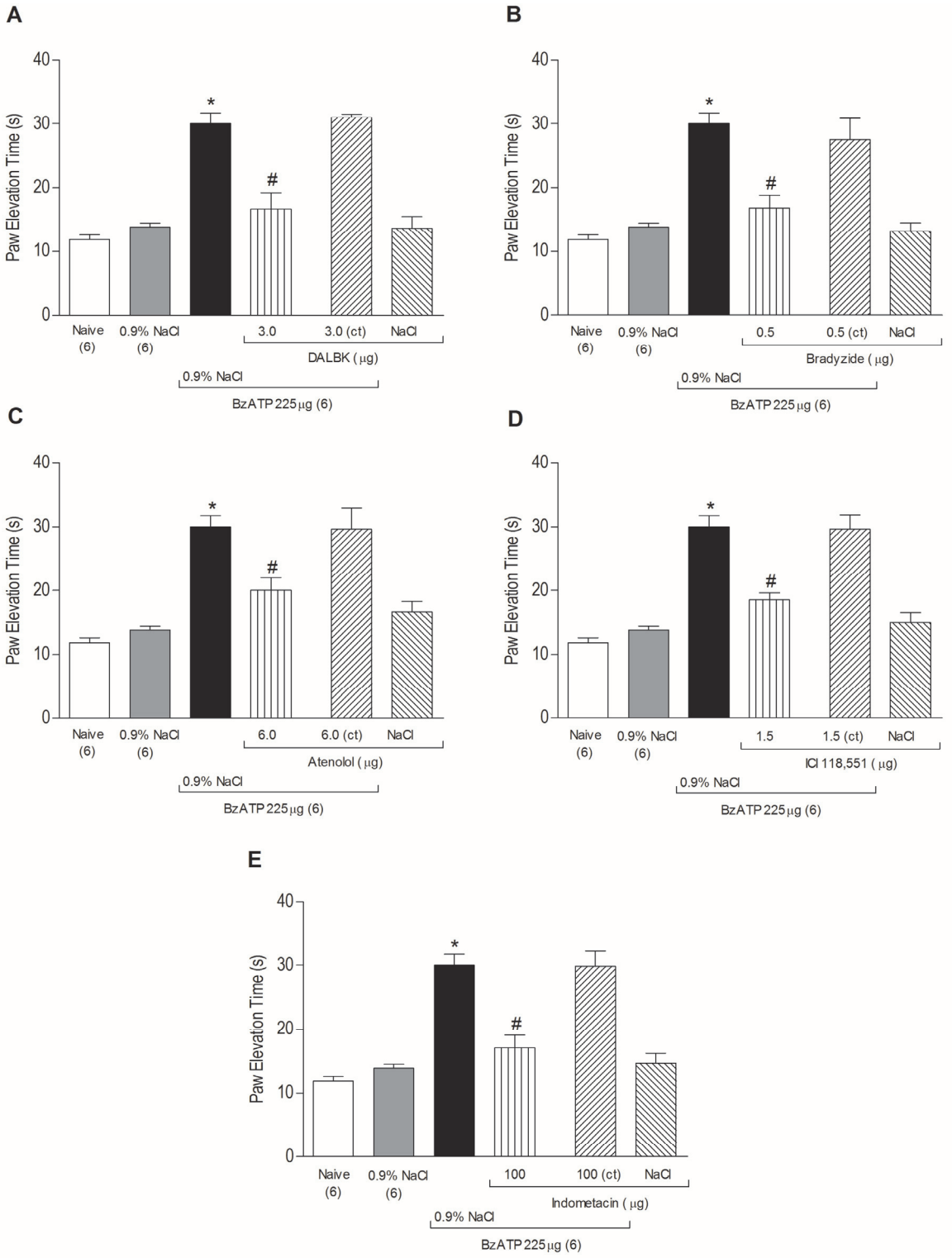

Figure 2 - Effect of B_1- or B_2-receptors antagonists, β_1- or β_2- adrenoceptors antagonists and the blockade of prostaglandins synthesis on the BzATP-induced articular hyperalgesia in rat's knee joint. The co-administration of B_1 receptor antagonist DALBK (3.0 μg/knee, **A**), B_2

receptor antagonist Bradyzide (0.5 μg/knee, **B**), β_1 adrenoceptor antagonist Atenolol (6.0 μg/knee, **C**), β_2 adrenoceptor antagonist ICI 118,551 (1.5 μg/knee, **D**) or cyclooxygenase inhibitor Indomethacin (100 μg/knee, 30 min pre-treatment, **E**) with BzATP (225 μg/knee) significantly reduced the BzATP-induced articular hyperalgesia, as indicated by the symbol "#" ($P<0.05$, Tukey test). DALBK (3.0 μg/knee, **A**), Bradyzide (0.5 μg/knee, **B**), Atenolol (6.0 μg/knee, **C**), ICI 118,551 (1.5 μg/knee, **D**) or Indomethacin (100 μg/knee, **E**) administrated in the contralateral knee joint (ct) or co-administrated with 0.9% NaCl did not affect the articular hyperalgesia induced by BzATP or the paw elevation time, when compared with BzATP alone or naive groups, respectively ($P>0.05$, Tukey test). The symbol "*" indicates responses significantly greater than that induced by 0.9% NaCl or naive groups ($P<0.05$, Tukey test).

Local administration of BzATP increases the concentration of pro-inflammatory cytokines in rats knee joint

This set of experiments was performed to verify whether BzATP induces the increase of concentration of TNF-α, IL-1β, IL-6 and CINC-1. Intra-articular administration of BzATP (225 μg/knee) significantly increased the concentration of TNF-α (Fig. 3A), IL-1β (Fig. 3B), IL-6 (Fig. 3C) and CINC-1 (Fig. 3D) 3 hours later when compared with 0.9% NaCl administration or naive control groups ($P<0.05$, one-way ANOVA post hoc Tukey test). The co-administration of the selective P2X7 receptor antagonist A-740003 (568 μg/knee) with BzATP reduced the local concentration of TNF-α (Fig. 3A), IL-1β (Fig. 3B), IL-6 (Fig. 3C) and CINC-1 (Fig. 3D) ($P<0.05$, one-way ANOVA post hoc Tukey test). The intra-articular injection of 0.9% NaCl (50 μL) alone did not affect the endogenous concentration of TNF-α, IL-1β, IL-6 and CINC-1 when compared with naive group ($P>0.05$, one-way ANOVA post hoc Tukey test).

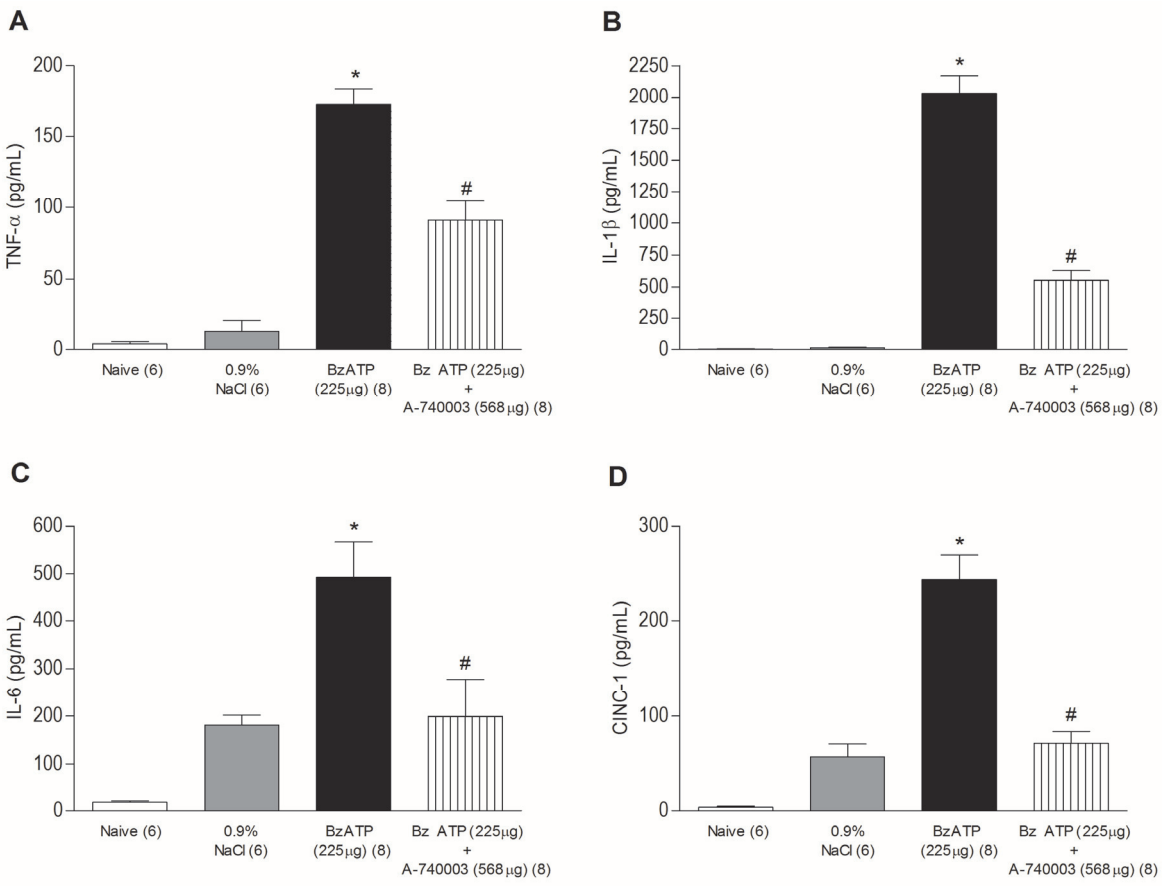

Figure 3 – Effect of BzATP on the local concentration of pro-inflammatory cytokines in the rat's knee joint. The intra-articular administration of BzATP (225 µg/knee) induced an increase of TNF-α (**A**), IL-1β (**B**), IL-6 (**C**) and CINC-1 (**D)** concentrations. The co-administration of A-740003 (568 µg/knee) with BzATP significantly reduced the BzATP-induced increase of TNF-α (**A**), IL-1β (**B**), IL-6 (**C**) and CINC-1 (**D**) concentrations, as indicated by the symbol "#" ($P<0.05$, Tukey test). The intra-articular injection of 0.9% NaCl did not affect the endogenous concentration of TNF-α, IL-1β, IL-6 and CINC-1 when compared with naive group ($P>0.05$, Tukey test). The symbol "*" indicated a cytokines concentrations significantly greater than that induced by 0.9% NaCl and by naive groups ($p<0.05$, ANOVA, pos hoc Tukey test).

Involvement of neutrophil migration on BzATP-induced articular hyperalgesia

To verify whether local administration of BzATP (225 µg/knee) induces neutrophil migration and if this is important to development of articular hyperalgesia, rats were treated with Fucoidan (25 mg/kg, i.v.) 20 minutes before BzATP administration. As showed in figure 4A, the articular hyperalgesia measured 3 hours later BzATP administration was not reduced by the pre-treatment with Fucoidan ($P>0.05$, one-way ANOVA post hoc Tukey test). The intra-articular administration of BzATP (225 µg/knee) increased the myeloperoxidase's activity when compared with 0.9% NaCl control group which was blocked by Fucoidan (Fig. 4B, $P<0.05$, one-way ANOVA post hoc Tukey test). The intra-articular injection of 0.9% NaCl alone did not affect the myeloperoxidase's activity when compared with naive rats ($P>0.05$, one-way ANOVA post hoc Tukey test).

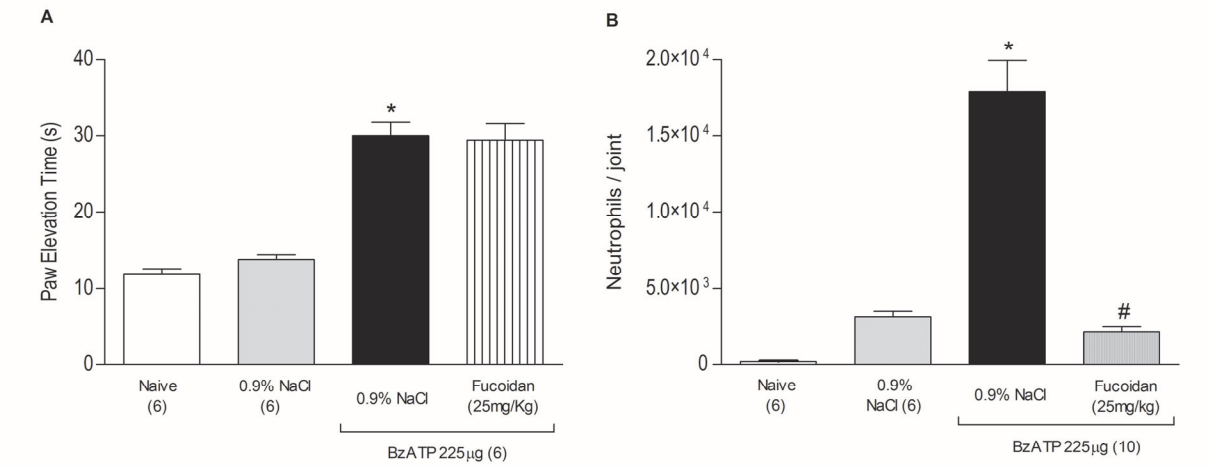

Figure 4 - Effect of Fucoidan on BzATP-induced articular hiperalgesia and neutrophil migration in rat knee joint. (A) The pre-treatment with Fucoidan (25mg/kg, i.v.) did not decreased BzATP-induced articular hyperalgesia ($P>0.05$, Tukey test). **(B)** The intra-articular administration of BzATP (225µg/knee) induced an increase of myeloperoxidase's activity which was blocked by the pre-treatment with Fucoidan (25mg/kg, i.v.), as indicated by the symbol "#" ($P<0.05$, Tukey test). The symbol "*" indicates a neutrophil migration significantly greater than that induced by 0.9% NaCl group ($P<0.05$, Tukey test).

Discussion

Recently, we have demonstrated that activation of P2X7 receptor by endogenous ATP release in inflamed articular tissue is essential to the development of the articular inflammatory hyperalgesia induced by carrageenan mediated by an indirect sensitization of the primary afferent nociceptors which depends on the previous pro-inflammatory cytokines release and neutrophil migration (Teixeira et al., 2014b *in press*). In the current study, we have showed that peripheral P2X7 receptor activation by its agonist induces articular hyperalgesia by an indirect sensitization of the primary afferent nociceptors, once it depends on inflammatory mediators release: bradykinin, prostaglandins, sympathomimetic amines and pro-inflammatory cytokines.

Although BzATP is a non-selective P2X7 receptor agonist once it also bind to P2X1 and P2X3 receptor (Bianchi et al., 1999, Jacobson et al., 2002), the involvement of P2X1 seems to be unlikely, since it has been demonstrated that IP5I, a potent and selective P2X1 receptor antagonist, is ineffective in reducing inflammatory pain (Honore et al., 2002) or mechanical hypersensitivity (Dai et al., 2004). Morevover, it has been shown that the P2X1 receptor does not contribute to hyperalgesia in temporomandibular joint (Shinoda et al., 2005).

In addition, BzATP is the most potent agonist for P2X7 receptor available (Jacobson et al., 2002). In this study we demonstrated that the articular hyperalgesia induced by BzATP was prevented by the P2X7 receptor antagonist A-740003 (Honore et al., 2006) but was unaffected by the selective P2X3 and P2X2/3 receptors antagonist, A-317491 (Jarvis et al., 2002), suggesting that BzATP-induced articular hyperalgesia was mediated by P2X7 receptor activation. Our data are consistent with previous studies showing that BzATP induces nociceptive and hyperalgesic behaviors by P2X7 receptor activation (Teixeira et al., 2010, Ito et al., 2013, Teixeira et al.,

2014a). Because the activation of spinal P2X7 receptor contributes to mechanical hyperalgesia (Clark et al., 2010), the finding that the administration of A-740003 in the contralateral knee joint did not affect BzATP-induced articular hyperalgesia confirms that only local P2X7 receptors of the peripheral tissue were targeted.

Agreeing with the results of this study, P2X7 receptor activation in knee joint can induces the release of bradykinin, an inflammatory mediator which has been pointed out as a key target in arthropathies models (Valenti et al., 2010, Valenti et al., 2012, Gomis et al., 2013). It has been described that the bradykinin is rapidly generated after tissue injury and modulates most of the events observed during the inflammatory processes, including increase of vascular permeability and vasodilatation, producing local heating and oedema, leukocyte recruitment, excitation and sensitization of sensory nerves, evoking pain and hyperalgesia (Marceau et al., 1998, Calixto et al., 2000, Couture et al., 2001, Pawlak et al., 2008). In addition, in the arthritic joints bradykinin has also been involved in endothelial cell proliferation, cartilage matrix homeostasis and bone resorption, potentially affecting, therefore, the synovial angiogenesis and cartilage destruction (Colman, 2006, Brechter and Lerner, 2007, Meini and Maggi, 2008).

Our results showed that bradykinin also plays a crucial role in the development of articular hyperalgesia in rats knee joint, because the bradykinin B_1 and B_2-receptors antagonists greatly reduced BzATP-induced articular hyperalgesia. This result is similar to that occurs in subcutaneous tissue (Teixeira et al., 2014a *in press*). Considering that bradykinin is an inflammatory mediator released during earlier phase of inflammation (Ferreira et al., 1993a, Ferreira et al., 1993b), it is plausible to suggest that P2X7 receptor activation in the knee joint initiates the articular inflammatory process.

Bradykinin induces hyperalgesia by two distinct pathways that result in the local production of prostaglandins and release of sympathomimetic amines (Ferreira et al., 1993a, Ferreira et al., 1993b), which directly sensitize the primary afferent nociceptor (Gold et al., 1996, Khasar et al., 1999, Rush and Waxman, 2004). The synovium of knee joint is innervated by both postganglionic sympathetic fibers and afferent C-fibers. Sympathetic postganglionic fibers constitute between half and two-thirds of the nerve fibers in the synovium (Hildebrand et al., 1991). The results of this study demonstrate that the articular hyperalgesia induced by local activation of P2X7 receptor depends on both pathways: the production of prostaglandins and release of sympathomimetic amines, which act directly on primary afferent nociceptors. Specifically, both β-adrenoceptor antagonists and inhibitor of cyclooxygenase indomethacin reduced the BzATP-induced articular hyperalgesia. These results showed that the blockade of one pathway completely reverses the BzATP-induced articular hyperalgesia.

The data of this study demonstrated that local P2X7 receptor activation in the rat's knee joint induces release of TNF-α, IL-1β, IL-6 and CINC-1. These findings are consistent with recent data which demonstrated that P2X7 receptor activation in the subcutaneous tissue also triggered pro-inflammatory cytokines release (Teixeira et al., 2014a *in press*).

It has been systematically demonstrated that P2X7 receptor is selectively expressed in peripheral macrophages, mast cells, lymphocytes, fibroblasts, erythrocytes, monocytes and FLS cells (Surprenant et al., 1996, Collo et al., 1997, Mancino et al., 2001, Caporali et al., 2008) and these cells produce and secrete pro-inflammatory cytokines (Guerne et al., 1989, Dubravec et al., 1990, Mekori and Metcalfe, 2000, Hayashida et al., 2001, Shakoory et al., 2004, Mor et al., 2005). Thus, our results suggest that the pro-inflammatory cytokines release in knee joint

mediated by activation of P2X7 receptors expressed in resident cells, especially peripheral macrophages and FLS cells.

In fact, it has been demonstrated a role of P2X7 receptor activation in IL-6 release by FLS cells (Caporali et al., 2008). Moreover, ATP induces the release of IL-1β, TNF-α and IL-6 via P2X7 receptor activation (Ferrari et al., 1997, Hide et al., 2000, Solle et al., 2001, Colomar et al., 2003, Chessell et al., 2005, Gourine et al., 2005, Mingam et al., 2008). Several studies have been demonstrated the ability of P2X7 receptor activation increases IL-1β. Experiments performed *in vitro* show that the activation of P2X7 receptor stimulates caspase-1, triggering IL-1β release in LPS stimulation models (Ferrari et al., 1996, Sanz and Di Virgilio, 2000, Kahlenberg and Dubyak, 2004, Ferrari et al., 2006). Also, the activation of P2X7 receptors by the BzATP did not stimulate the IL-1β production by macrophages obtained from P2X7 knockout mice when compared with wild type (Basso et al., 2009).

Therefore, our results suggested that P2X7 activation-induced articular hyperalgesia depends on the prior release of pro-inflammatory cytokines TNF-α, IL-1β, IL-6 and CINC-1, with subsequent prostaglandins synthesis and sympathomimetic amines release in the rat's knee joint, which in turn directly sensitize the primary afferent nociceptor.

Another important sign involved in inflammatory hyperalgesia is the migration of neutrophils to the site of inflammation. Neutrophils are present in higher quantity, about 90% of the leukocytic infiltrate in synovial fluid of patients with arthritis (Mohr, 1995) and play an important role in the arthropathies pathogenesis, because they interact with resident cells and local inflammatory mediators. This interaction amplify the inflammatory response, contributing directly to the chronic and acute inflammation and release of nociceptive mediators (Kasama et al., 2005). Neutrophils induce the release of proteolytic enzymes which contribute tissue damage

(Edwards and Hallett, 1997). Studies from our laboratory have showed that ATP released in the site of inflammation via P2X7 receptor activation induces articular hyperalgesia that is mediated by neutrophil migration. Thus, in this study we also investigated whether the P2X7 receptor activation induces neutrophil migration to the rats knee joint, contributing to P2X7 activation-induced articular hyperalgesia.

Surprisingly, our results demonstrate that neutrophils do not contribute to the development of BzATP-mediated articular hyperalgesia in the rat's knee joint, because the pre-treatment with Fucoidan does not reduces the BzATP-induced articular hyperalgesia. Fucoidan was used as a pharmacological tool to investigate this issue, as it binds to L- and P-selectins and consequently, inhibits neutrophil rolling (Ley et al., 1993, Cunha et al., 2008). This data indicates a difference between the pathophysiology of articular inflammatory hyperalgesia and subcutaneous tissue inflammatory hyperalgesia, because data from our laboratory demonstrated that in the rats paw subcutaneous tissue BzATP-induced mechanical hyperalgesia depends on neutrophils migration (Teixeira et al., 2014a *in press*).

By the other hand, our results showed that BzATP induces neutrophil migration to the rat's knee joint, which was blocked by Fucoidan. We can hypothesize that the neutrophil migration induced by BzATP probably results from its ability to induce the pro-inflammatory cytokines release, mainly CINC-1 which induces chemotaxis and leukocytes recruitment (Romano et al., 1997, Ramos et al., 2003, Moon et al., 2010).

In conclusion, the findings of the present study suggest that peripheral mechanisms involved in the articular hyperalgesia induced by P2X7 receptors activation in the rat knee joint involve previous release of bradykinin, prostaglandins, sympathomimetic amines and pro-

inflammatory cytokines. Therefore, P2X7 receptor antagonist may be an interesting target to control articular inflammatory hyperalgesia.

Acknowledgements

This work was supported by Grants from Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP, Brazil (2009/16854-3 and 2010/05381-4).

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VI. CAPÍTULO 05

Involvement of peripheral P2X3, P2X2/3 and P2X7 receptors in the articular hyperalgesia induced by bradykinin, PGE_2 and dopamine

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Abstract

Activation of P2X3, P2X2/3 and P2X7 receptors by endogenous ATP plays an essential role in the development of carrageenan-induced hyperalgesia in the rat's knee joint through the previous release of pro-inflammatory cytokines and neutrophil migration. Moreover, P2X3, P2X2/3 and P2X7 activation by their agonists, induces articular hyperalgesia mediated by bradykinin, prostaglandins, sympathomimetic amines, pro-inflammatory cytokines and neutrophil migration in the rat's knee joint. In this study, we asked whether the activation of P2X3, P2X2/3 and P2X7 receptors, contributes to the articular hyperalgesia induced by the inflammatory mediators belonging to carrageenan inflammatory cascade, such as bradykinin, tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), chemokine-induced chemoattractant-1 (CINC-1), prostaglandin E_2 (PGE_2) and dopamine. We found that the co-administration of the selective P2X3 and P2X2/3 receptors antagonist A-317491 or of the selective P2X7 receptor antagonist A-740003 significantly reduced the articular hyperalgesia induced by bradykinin, and dopamine, but not by TNF-α, IL-1β and CINC-1. The co-administration of the selective P2X3 and P2X2/3 receptors antagonist A-317491 also significantly reduced the articular hyperalgesia induced by PGE_2. These results indicate the activation of P2X3, P2X2/3 and P2X7 receptors contributes to bradykinin- and dopamine-induced hyperalgesia and that the activation of P2X3 and P2X2/3, but not P2X7 receptors, contributes to PGE_2-induced hyperalgesia. Further, they also indicate that these purinergic receptors contribute to the hyperalgesia induced by bradykinin, dopamine and PGE_2, through a previous release of endogenous ATP induced by these inflammatory mediators.

Keywords: ATP, P2X3 and P2X2/3 receptors, P2X7 receptor, articular inflammatory hyperalgesia, rat knee joint, inflammatory mediators.

Introduction

Extracellular ATP released from damaged cells such as macrophages, platelets, keratinocytes, neutrophils and dying cells (Filippini et al., 1990, Dubyak and el-Moatassim, 1993, Beigi et al., 1999, Sikora et al., 1999, Mizumoto et al., 2003, Campwala and Fountain, 2013) plays an important role in the development of inflammatory pain by activating P2X receptors. The P2X3, P2X2/3 and P2X7 purinergic receptors subtypes belongs to the family of ATP-sensitive ionotropic P2X receptors (P2X1-P2X7) (Buell et al., 1996, North, 2002) and are involved in pain and hiperalgesia mechanisms in several tissues, including articular tissues (Labasi et al., 2002, Shinoda et al., 2005, Seino et al., 2006, Broom et al., 2008, Teixeira et al., 2010, Teixeira et al., 2014a *in press*, Teixeira et al., 2014b *in press*).

The P2X3 and P2X2/3 receptors are localized on terminals of primary sensory afferents fibers where they mediate sensory neurotransmission (Bradbury et al., 1998, Burnstock and Knight, 2004, Gever et al., 2006), on endothelial cells of thymus (Glass et al., 2000), urothelial cells (Sun and Chai, 2004), human epidermal keratinocytes (Inoue et al., 2005) and chondrocytes (Varani et al., 2008, Teixeira et al., 2014a *in press*). The P2X7 receptors are found predominantly in cells of immunological origin such as mast cells, erythrocytes, monocytes, fibroblasts, peripheral macrophages, T and B lymphocytes (Chiozzi et al., 1997, Collo et al., 1997, Kim et al., 2001, Mancino et al., 2001, North, 2002, Greig et al., 2003, Surprenant and North, 2009) and in type B synoviocytes (fibroblasts like-synoviocytes - FLS cells, Caporali et al., 2008).

In addition to ATP, many inflammatory mediators such as bradykinin, prostaglandins, sympathomimetic amines and the pro-inflammatory cytokines are released after tissue injury induce and/or maintain the inflammatory hyperalgesia (Cunha et al., 1991, Cunha et al., 1992,

Ferreira et al., 1993a, Loram et al., 2007, Luiz et al., 2010, Petho and Reeh, 2012, Villarreal et al., 2013). All of these inflammatory mediators play an important role in pain development in the arthropathies (Attur et al., 1998, Kasama et al., 2005, Colman, 2006, Brechter and Lerner, 2007, Meini and Maggi, 2008, Moon et al., 2010, Tanaka et al., 2013).

We have recently demonstrated that peripheral P2X3, P2X2/3 and P2X7 receptors activation by endogenous ATP, not only contributes, but it is essential to the development of carrageenan-induced articular hyperalgesia in the rat's knee joint through an indirect sensitization of the primary afferent nociceptors, dependent on the previous release of pro-inflammatory cytokines and neutrophil migration (Teixeira et al., 2014a *in press*, Teixeira et al., 2014b *in press*). Further, we have also demonstrated that the activation of P2X3, P2X2/3 and P2X7 receptors by their agonists, induces articular hyperalgesia mediated by bradykinin, prostaglandins, sympathomimetic amines, pro-inflammatory cytokines and neutrophil migration in the rat's knee joint (Teixeira et al., 2014c *in press*, Teixeira et al., 2014d *in press*). However, it is not known whether the P2X3, P2X2/3 and P2X7 receptors contribute to the articular hyperalgesia induced by inflammatory mediators belonging to carrageenan cascade (Ferreira et al., 1993a, Ferreira et al., 1993b).

In this study, we asked whether the activation of P2X3, P2X2/3 and P2X7 receptors, contributes to the articular hyperalgesia induced by the inflammatory mediators bradykinin, TNF-α, IL-1β, IL-6, CINC-1, PGE_2 and dopamine. For this study, we explored the ability of the selective P2X3, P2X2/3 and P2X7 receptors antagonists, A-317491 and A-740003, respectively, to reduce the articular hyperalgesia induced by each one of these inflammatory mediators.

Materials and Methods

Animals

Male Wistar rats (200-250g) obtained from the Multidisciplinary Center for Biological Research (CEMIB) - University of Campinas, were used in this study. The animals were housed in plastic cages with soft bedding (five/cage) on a 12:12 light cycle (lights on at 06:00 A.M.) with food and water available *ad libitum*. They were maintained in a temperature-controlled room (±23°C) and handled for at least one week prior to the experiments. Each animal was used once and the number of animals per group was kept to a minimum. Experimental protocols were approved by the Committee on Animal Research of the University of Campinas (protocol number: 2049-1) and conformed to IASP guidelines for the study of the pain in animals (Zimmermann, 1983).

General Procedures

Testing sessions took place during light phase (between 09:00 AM and 5:00 PM) in a quiet room maintained at 23ºC (Rosland, 1991). During the tests the animals had no access to water or food. The animals were habituated for 1 hour prior to the experiment to minimize stress.

Drugs administration

Drugs or their vehicle were intra-articularly administrated in the right rat's knee joint by a 26-gauge needle that was connected to a catheter of polyethylene and also to a Hamilton syringe (50 μL). The volume of injection was 50 μL.

Knee joint inflammation induced by inflammatory mediators

Under brief inhalation of isoflurane anesthesia, rats were subjected to intra-articular (i.a.) injection of inflammatory mediators (bradykinin, TNF-α, IL-1β, IL-6, CINC-1, PGE_2 and dopamine) dissolved in 25μL sterile 0.9% saline or PBS solution into their right knee joints (Tonussi and Ferreira, 1992). The injection site was shaved and treated with an antiseptic solution of iodine alcohol. The other drugs were also injected into the knee joint in the same manner that the inflammatory mediators and the control animals received vehicle.

Drugs and doses

The following drugs were used: bradykinin (1.5, 4.5, 13.5 and 40.5 μg/knee, i.a., de Oliveira Fusaro et al., 2010); tumor necrosis factor alpha (TNFα: 0.8, 2.4 and 7.2 pg/knee, i.a, de Oliveira Fusaro et al., 2010); interleukin-1 beta (IL-1β: 0.5, 1.5 and 4.5 pg/knee, i.a., Cunha et al., 2008); interleukin-6 (IL-6: 0.1, 0.3 and 0.9 ng/knee, i.a., de Oliveira Fusaro et al., 2010); chemokine-induced chemoattractant-1 (CINC-1: 1.0, 3.0 and 9.0 pg/knee, i.a., de Oliveira Fusaro et al., 2010); prostaglandin E_2 (PGE_2: 100, 300 and 900 ng/knee, i.a., de Oliveira Fusaro et al., 2010); dopamine (10, 30 and 90 μg/knee, i.a., de Oliveira Fusaro et al., 2010); the selective P2X3 and P2X2/3 receptor antagonist 5-([(3-Phenoxybenzyl)[(1S)-1,2,3,4-tetrahydro-1-naphthalenyl] amino]carbonyl)-1,2,4-benzenetricarboxylic acid (Jarvis et al., 2002) (A-317491: 540 μg/knee, i.a., Teixeira et al., 2014a *in press*); the selective P2X7 receptor antagonist [*N*-(1-{[(Cyanoimino) (5-quinolinylamino) methyl] amino}-2,2-dimethylpropyl)- 2-(3,4-dimethoxyphenyl)acetamide] (Honore et al., 2006) (A-740003: 568 μg/knee, i.a., Teixeira et al., 2014b *in press*). TNF-α, IL-1β, IL-6 and CINC-1 were obtained from R&D Systems (Minneapolis, USA) and dissolved in phosphate-buffered saline (PBS, Sigma Chemicals, St. Louis, Missouri, USA). A-740003 was

obtained from Tocris Bioscience (Ellisville, MO) and dissolved in 25μL of dimethyl sulfoxide (DMSO, 50%) and propylene glycol (50%). All other drugs were obtained from Sigma-Aldrich (MO, USA) and dissolved in sterile 0.9% saline (0.9% NaCl).

Gait disturbance - Rat knee-joint incapacitation test

We used the rat knee-joint incapacitation test, as described previously (Tonussi and Ferreira, 1992). Briefly, after drugs injection into their right knee joints, rats were put to walk on a steel rotary cylinder (30 cm wide x 50 cm diameter), covered with a fine-mesh non-oxidizable wire screen, which rotates at 3 rpm. Specially, designed metal gaiters were wrapped around both hind paws. After placement of the gaiters, rats were placed to walk in the cylinder and the right paw was then connected via a simple circuit to microcomputer data input/output port. The paw elevation time (PET) is the total time that the rat walks failing to touch the cylinder surface with the injected hindpaw, during a 60 sec period, which is directly proportional to the gait disturbance. Incapacitation was quantified as an increase in the PET, 3 hours after carrageenan injection into the right knee joint. To minimize variations in PET, all rats were introduced to the experimental environment and trained on the apparatus to habituation into the equipment before the testing sessions. To confirm the local effect of some test agents, they were injected into the contralateral rat's knee joint and the test was performed on the ipsilateral knee joint. The rat knee-joint incapacitation test provides automated measurements, which are independent of the subjectivity of the observer (Tonussi and Ferreira, 1992).

Statistical analysis

To determine if there were significant differences ($p<0.05$) between treatment groups, One-way ANOVA was performed. If there was a significant between-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 expressed in figures as means ± S.E.M.

Results

Bradykinin, TNF-α, IL-1β, CINC-1, PGE_2 or dopamine-induced articular hyperalgesia in the rat's knee joint

The intra-articular administration of bradykinin (13.5 and 40.5 μg/knee, Fig. 1A and 2A), TNF-α (2.4 pg/knee, Fig. 1B and 2B), IL-1β (1.5 and 4.5 pg/knee, Fig. 1C and 2C), CINC-1 (3.0 and 9.0 pg/knee, Fig. 1D and 2D), PGE_2 (300 and 900 ng/knee, Fig. 1E and 2E) or dopamine (30 and 90 μg/knee, Fig. 1F and 2F) into the rat's knee joint induced a dose-dependent articular hyperalgesia 3 hours after their administration when compared with vehicle administration and naive rats ($P<0.05$, one-way ANOVA post hoc Tukey test). Intra-articular administration of IL-6 (0.1, 0.3 and 0.9 ng/knee Fig. 3) into the rat's knee joint did not induce articular hyperalgesia ($P>0.05$, one-way ANOVA post hoc Tukey test).

Blockade of P2X3 and P2X2/3 receptors prevented bradykinin, PGE_2 and dopamine-induced hyperalgesia in the rat's knee joint

To verify whether P2X3 and P2X2/3 receptors activation mediates the articular hyperalgesia induced by bradykinin, TNF-α, IL-1β, CINC-1, PGE_2 or dopamine, the selective P2X3 and P2X2/3 receptors antagonist A-317491 was co-administered with each one of these inflammatory mediators. Co-administration of A-317491 (540 μg/knee) significantly reduced the articular hyperalgesia induced by bradykinin (13.5 μg/knee, Fig. 1A), PGE_2 (300 ng/knee, Fig. 1E) and dopamine (30 μg/knee, Fig. 1F) ($P<0.05$, one-way ANOVA post hoc Tukey test) but not the articular hyperalgesia induced by TNF-α (2.4 pg/knee, Fig. 1B), IL-1β (1.5 pg/knee, Fig. 1C) and CINC-1 (3.0 pg/knee, Fig. 1D) ($P>0.05$, one-way ANOVA post hoc Tukey test). The administration of A-317491 (540 μg/knee) in the contralateral knee joint did not affect the articular hyperalgesia induced by bradykinin, PGE_2 or dopamine, ruling out a possible systemic effect. The administration of A317491 (540 μg/knee) with 0.9% NaCl did not induce articular hyperalgesia by itself ($P>0.05$, one-way ANOVA post hoc Tukey test, Fig 1A, 1E and 1F). These findings suggest that P2X3 and P2X2/3 receptors mediate the articular hyperalgesia induced by bradykinin, PGE_2 and dopamine, but not by TNF-α, IL-1β, CINC-1.

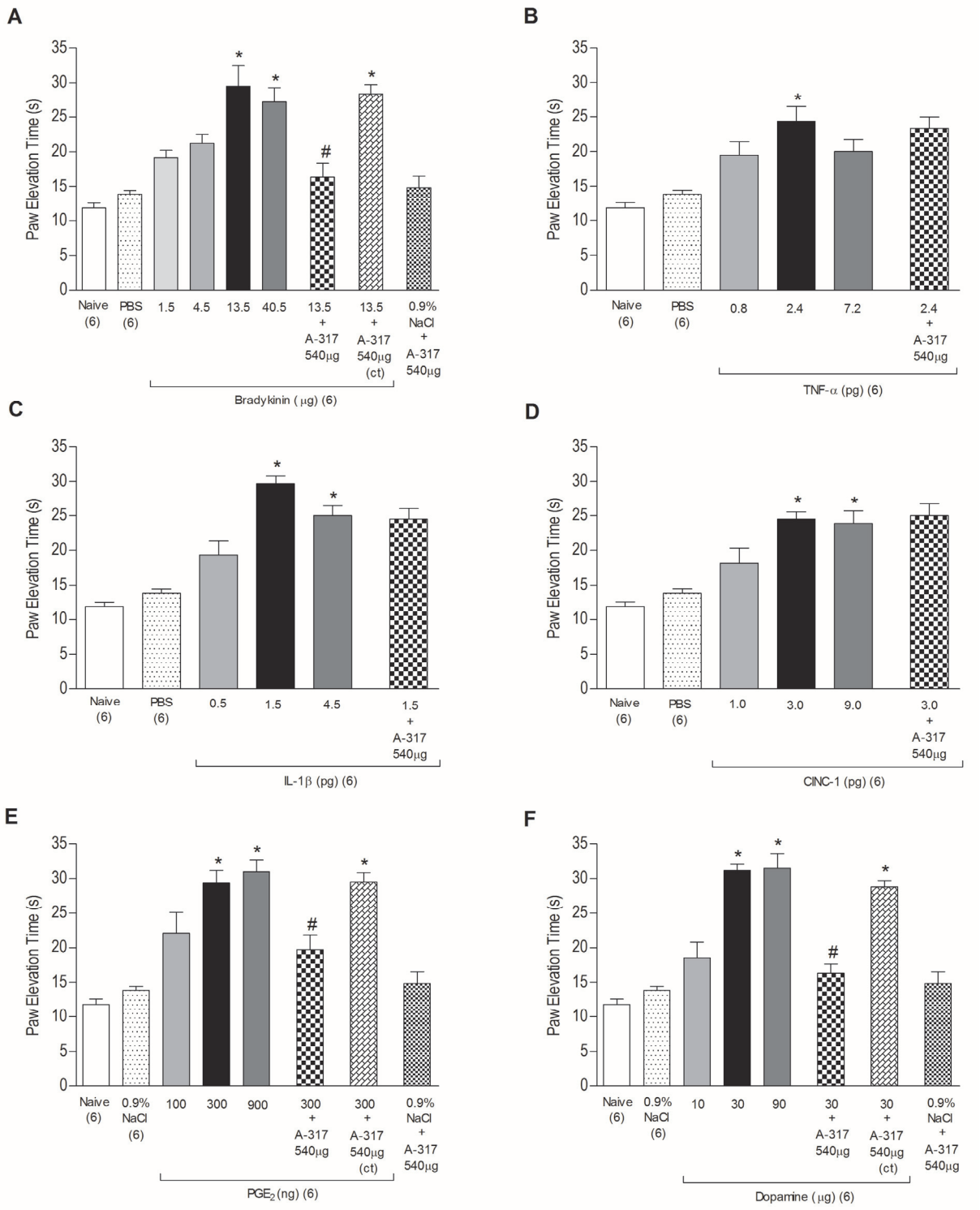

Figure 1 - Effect of P2X3 and P2X2/3 receptors antagonist on bradykinin, TNF-α, IL-1β, CINC-1, PGE_2 or dopamine-induced articular hyperalgesia in the rat's knee joint. The co-administration of A-317491 (A-317, 540 µg/knee) significantly reduced the hyperalgesic

response of Bradykinin (13.5 μg/knee, **A**), PGE_2 (300 ng/knee, **E**) and Dopamine (30 μg/knee, **F**) ($P<0.05$, Tukey test) but not the hyperalgesic response induced by TNF-α (2.4 pg/knee, **B**), IL-1β (1.5 pg/knee, **C**) and CINC-1 (3.0 pg/knee, **D**) ($P>0.05$, Tukey test). The intra-articular administration of A-317491 (A-317, 540 μg/knee) in the contralateral knee joint did not affect the articular hyperalgesia induced by Bradykinin, PGE_2 or Dopamine ($P>0.05$, Tukey test). The administration of A-317491 (A-317, 540 μg/knee) plus 0.9% NaCl had no effect ($P>0.05$, Tukey test). The symbol "*" indicates responses significantly higher than that induced by vehicle and naive groups ($P<0.05$, Tukey test). The symbol "#" indicates responses significantly lower than that induced by Bradykinin, PGE_2 and Dopamine ($P<0.05$, Tukey test). In this and in the subsequent figures, measurements were taken 3 hour after injections and the number of rats used is between parentheses.

Blockade of P2X7 receptors reduces bradykinin and dopamine-induced hyperalgesia in the rat's knee joint

To investigate whether P2X7 receptor activation mediates the articular hyperalgesia induced by bradykinin, TNF-α, IL-1β, CINC-1, PGE_2 or dopamine, the selective P2X7 receptor antagonist A-740003 was co-administered with each one of these inflammatory mediators. Co-administration of A-740003 (568 μg/knee) significantly reduced the articular hyperalgesia induced by bradykinin (13.5 μg/knee, Fig. 2A) and dopamine (30 μg/knee, Fig. 2F) ($P<0.05$, one-way ANOVA post hoc Tukey test) but not the articular hyperalgesia induced by TNF-α (2.4 pg/knee, Fig. 2B), IL-1β (1.5 pg/knee, Fig. 2C), CINC-1 (3.0 pg/knee, Fig. 2D) and PGE_2 (300 ng/knee, Fig. 2E) ($P>0.05$, one-way ANOVA post hoc Tukey test). The administration of A-740003 (568 μg/knee) in the contralateral rat's knee joint did not affect the articular hyperalgesia induced by bradykinin or dopamine, ruling out a systemic effect. The administration of A-740003 (568 μg/knee) with 0.9% NaCl into the knee joint, did not induce articular hyperalgesia by itself ($P>0.05$, one-way ANOVA post hoc Tukey test, Fig 2A and 2F). These findings suggest

that P2X7 receptor mediates the articular hyperalgesia induced by bradykinin and dopamine, but not by TNF-α, IL-1β, CINC-1 and PGE_2.

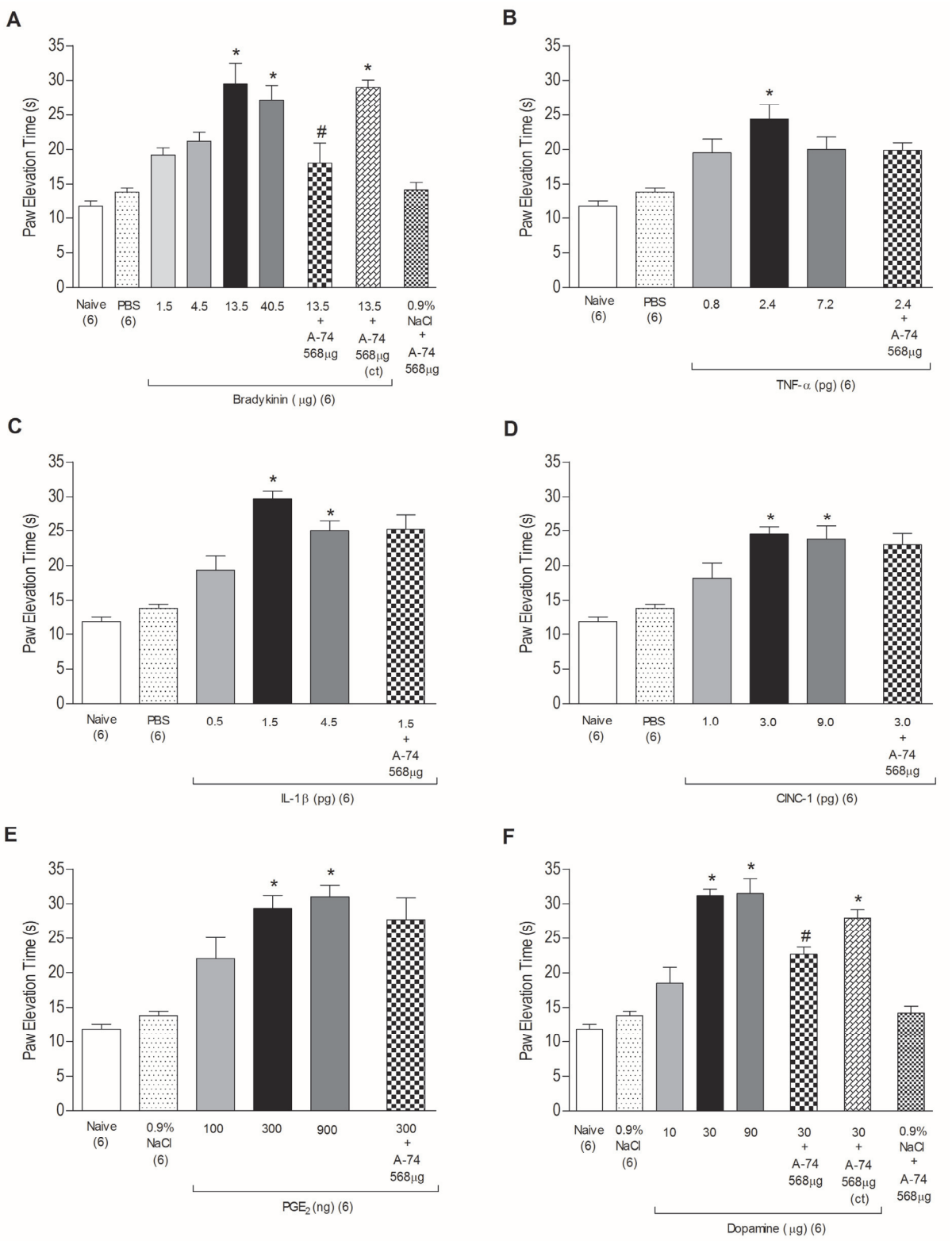

Figure 2 - Effect of P2X7 receptor antagonist on bradykinin, TNF-α, IL-1β, CINC-1, PGE_2 or dopamine-induced articular hyperalgesia in the rat's knee joint.

Co-administration of A-740003 (A-74, 568 μg/knee) significantly reduced the hyperalgesic response of Bradykinin (13.5 μg/knee, **A**) and Dopamine (30 μg/knee, **F**) ($P<0.05$, Tukey test) but not the hyperalgesic response of TNF-α (2.4 pg/knee, **B**), IL-1β (1.5 pg/knee, **C**), CINC-1 (3.0 pg/knee, **D**) and PGE_2 (300 ng/knee, **E**) ($P>0.05$, Tukey test). The intra-articular administration of A-740003 (A-74, 568 μg/knee) in the contralateral knee joint did not affect the articular hyperalgesia induced by Bradykinin or Dopamine ($P>0.05$, Tukey test). The administration of A-740003 (A-74, 568 μg/knee) plus 0.9% NaCl had no effect($P>0.05$, Tukey test). The symbol "*" indicates responses significantly higher than that induced by vehicle and naive groups ($P<0.05$, Tukey test). The symbol "#" indicates responses significantly lower than that induced by Bradykinin or Dopamine ($P<0.05$, Tukey test).

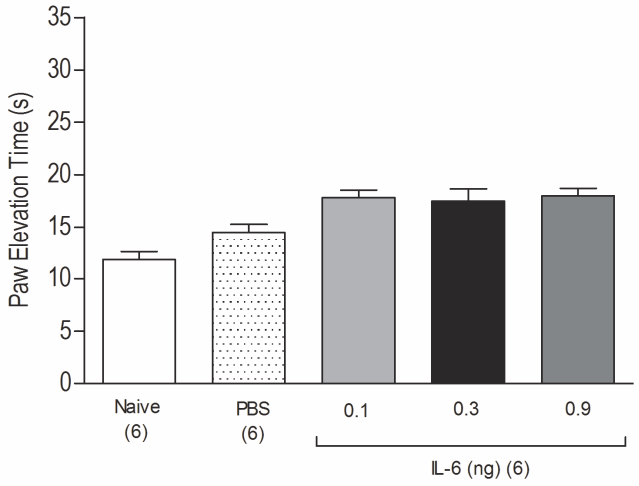

Figure 3 - Effect of IL-6 administration into the rat's knee joint. The intra-articular administration of IL-6 (0.1, 0.3 and 0.9ng/knee) did not induce articular hyperalgesia when compared with naive and vehicle (PBS) administered rats ($P>0.05$, Tukey test).

Discussion

This study shows that in the knee joint of rats, the activation of P2X3, P2X2/3 and P2X7 receptors contributes to the hyperalgesic response of bradykinin and dopamine through a previous release of endogenous ATP induced by these inflammatory mediators. However, the activation of P2X3 and P2X2/3, but not P2X7 receptor, contributes to PGE_2-induced hyperalgesia.

The evidences are that the selective P2X3 and P2X2/3 receptor antagonist A-317491 (Jarvis et al., 2002) prevented the articular hyperalgesia induced by bradykinin, PGE_2 and dopamine, and that the selective P2X7 receptor antagonist A-740003 (Honore et al., 2006) prevented the articular hyperalgesia induced by bradykinin and dopamine. However, neither A-317491 nor A-740003 significantly affected the hyperalgesia induced by TNF-α, IL-1β, and CINC-1. Because the activation of spinal P2X3, P2X2/3 and P2X7 receptors contribute to the inflammatory hyperalgesia (McGaraughty et al., 2003, Clark et al., 2010), the lack of effect of the administration of A-740003 or A-317491 in the contralateral knee joint, confirms that only P2X3, P2X2/3 and P2X7 receptors of the articular tissue were targeted.

In fact, P2X3 and P2X2/3 receptors expressed on terminals of primary sensory afferents fibers (Bradbury et al., 1998, Dowd et al., 1998, Burnstock and Knight, 2004, Gever et al., 2006) and chondrocytes (Varani et al., 2008) and P2X7 receptors expressed on macrophages, mast cells, monocytes, fibroblasts, T and B lymphocytes and FLS cells (Chiozzi et al., 1997, Collo et al., 1997, Kim et al., 2001, Mancino et al., 2001, North, 2002, Greig et al., 2003, Caporali et al., 2008, Surprenant and North, 2009), may have been targeted to contribute to the articular hyperalgesia induced by bradykinin, PGE_2 and dopamine.

These findings indicate that bradykinin induces the endogenous release of ATP, which in turn, activates P2X3, P2X2/3 and P2X7 receptors located in the knee joint, as previously shown in the subcutaneous tissue and in cell culture (Chopra et al., 2005, Zhao et al., 2007, de Oliveira Fusaro et al., 2010, Pinheiro et al., 2013).

After tissue injury, bradykinin is rapidly generated and modulates the increase of vascular permeability and vasodilatation, producing local heating and oedema, leukocyte recruitment, excitation and sensitization of sensory nerves, evoking pain and hyperalgesia (Marceau et al., 1998, Calixto et al., 2000, Couture et al., 2001, Pawlak et al., 2008). In the arthritic joints, bradykinin has been involved in endothelial cell proliferation, cartilage matrix homeostasis and bone resorption, thus potentially affecting the synovial angiogenesis and cartilage destruction (Colman, 2006, Brechter and Lerner, 2007, Meini and Maggi, 2008). Considering that bradykinin is an inflammatory mediator released at the early phase of inflammatory hyperalgesia (Ferreira et al., 1993a, Ferreira et al., 1993b), our results suggest that the activation of P2X3, P2X2/3 and P2X7 receptors by endogenous ATP may play a role in the beginning of the development of the inflammatory hyperalgesia in the knee joint, reinforcing our previous results that the activation of peripheral P2X3, P2X2/3 and P2X7 receptors by endogenous ATP, is essential to the development, but not to the maintenance of the carrageenan-induced articular hyperalgesia (Teixeira et al., 2014a *in press*, Teixeira et al., 2014b *in press*).

Bradykinin induces hyperalgesia by two distinct pathways that result in the local production of prostaglandins and release of sympathomimetic amines (Ferreira et al., 1993a, Ferreira et al., 1993b), which directly sensitize the primary afferent nociceptor (Gold et al., 1996, Khasar et al., 1999, Rush and Waxman, 2004). The activation of peripheral P2X3, P2X2/3 and P2X7 receptors by endogenous ATP in the knee joint, plays an important role not only in the

early, but also in the late phase of the inflammatory hyperalgesia. This is because the blockade of P2X3, P2X2/3 and P2X7 receptors prevented the articular hyperalgesia induced by final inflammatory mediators such as PGE_2 and dopamine. Specifically, blockade of P2X3, P2X2/3 and P2X7 receptors prevented dopamine-induced hyperalgesia, and blockade of P2X3 and P2X2/3, but not P2X7 receptor, prevented PGE_2-induced hyperalgesia. These findings suggest that like bradykinin, PGE_2 and dopamine also induce the release of ATP in the articular tissue, which in turn, activates P2X3, P2X2/3 and P2X7 receptors of the knee joint.

We have previously demonstrated that the blockade of P2X3 and P2X2/3 receptors in the subcutaneous tissue of the rat's paw prevents the mechanical hyperalgesia induced only by bradykinin (de Oliveira Fusaro et al., 2010). Others unpublished data from our group show that blockade of P2X7 receptor prevents the mechanical hyperalgesia induced by TNF-α, IL-6, CINC-1 and dopamine, but not that induced by bradykinin, PGE_2 and IL-1β in the subcutaneous tissue of the rat's paw. Taken together, these findings suggest a significant difference in the purinergic mechanisms involved in the inflammatory hyperalgesia in the articular and in the subcutaneous tissues.

In summary, this study shows that in the knee joint, the activation of P2X3, P2X2/3 and P2X7 receptors contributes to bradykinin- and dopamine-induced hyperalgesia and that the activation of P2X3 and P2X2/3, but not P2X7 receptors, contributes to PGE_2-induced hyperalgesia. Furthermore, these purinergic receptors contribute to the hyperalgesia induced by bradykinin, dopamine and PGE_2, through a previous release of endogenous ATP induced by these inflammatory mediators. These findings suggest that the P2X3, P2X2/3 and P2X7 receptors are important targets to control inflammatory pain in the arthropathies.

Acknowledgements

This work was supported by Grants from Fundação de Amparo à Pesquisa do Estado de São Paulo - FAPESP, Brazil (2009/16854-3 and 2010/05381-4).

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VII. CONCLUSÕES

Os resultados apresentados nesse estudo em ratos sugerem que:

- A ativação dos receptores purinérgicos P2X3, P2X2/3 e P2X7 pelo ATP endógeno é essencial para o desenvolvimento da hiperalgesia e inflamação articular induzida pela carragenina na articulação do joelho de ratos machos e fêmeas em estro (níveis baixos de hormônios ovarianos), sendo essas fêmeas mais sensíveis do que os machos aos efeitos anti-hiperalgésicos e anti-inflamatórios induzidos pelo bloqueio do receptor P2X7 na articulação do joelho.

- Durante o processo de inflamação articular ocorre o aumento da expressão dos receptores P2X3 nos condrócitos da cartilagem articular, podendo sugerir que o aumento da sua expressão e sua ativação pelo ATP extracelular pode contribuir com a liberação de mediadores inflamatórios e consequente ativação dos nociceptores aferente primários.

- O papel essencial dos receptores P2X3, P2X2/3 e P2X7 no desenvolvimento da hiperalgesia articular é mediado pela sensibilização indireta nos nociceptores aferentes primários, dependente da liberação de citocinas pró-inflamatórias e migração de neutrófilos.

- A ativação dos receptores P2X3, P2X2/3 e P2X7 induz hiperalgesia articular no joelho de ratos dependente da liberação de bradicinina, aminas simpatomiméticas, prostaglandinas e citocinas pró-inflamatórias.

- A hiperalgesia articular induzida pelos mediadores inflamatórios como a bradicinina, a PGE_2 e dopamina é mediada pela ativação de receptores P2X3 e P2X2/3, enquanto que a ativação de receptor P2X7 contribui para a hiperalgesia articular induzida pela bradicinina e dopamina.

Concluindo, nossos resultados podem sugerir que tanto os receptores purinérgicos P2X3 e P2X2/3 quanto os P2X7 são alvos farmacológicos interessantes para o controle da dor e inflamação das doenças inflamatórias articulares como a osteoartrite. Particularmente em relação

ao receptor P2X7, antagonistas seletivos podem ser usados para reduzir a dor nas artropatias especialmente em mulheres, que são mais afetadas por essa doença do que os homens.

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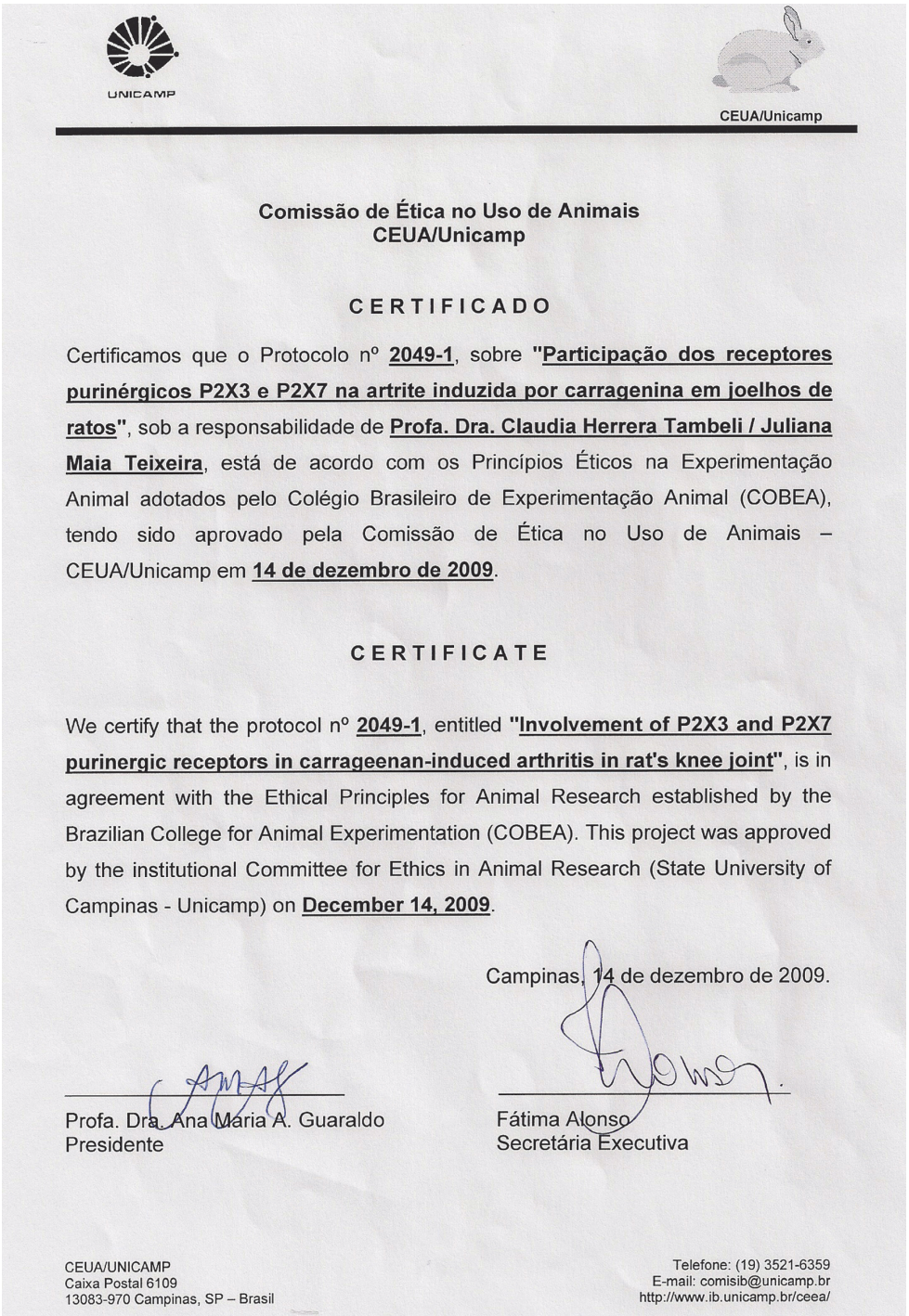
IX. ANEXOS

A- Certificado aprovação pelo Comitê de Ética em Pesquisa Animal da Universidade Estadual de Campinas:



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 Protocolo nº **2049-1**, sobre **"Participação dos receptores purinérgicos P2X3 e P2X7 na artrite induzida por carragenina em joelhos de ratos"**, sob a responsabilidade de **Profa. Dra. Claudia Herrera Tambeli / Juliana Maia Teixeira**, está de acordo com os Princípios Éticos na Experimentação Animal adotados pelo Colégio Brasileiro de Experimentação Animal (COBEA), tendo sido aprovado pela Comissão de Ética no Uso de Animais – CEUA/Unicamp em **14 de dezembro de 2009**.

C E R T I F I C A T E

We certify that the protocol nº **2049-1**, entitled **"Involvement of P2X3 and P2X7 purinergic receptors in carrageenan-induced arthritis in rat's knee joint"**, is in agreement with the Ethical Principles for Animal Research established by the Brazilian College for Animal Experimentation (COBEA). This project was approved by the institutional Committee for Ethics in Animal Research (State University of Campinas - Unicamp) on **December 14, 2009**.

Campinas, 14 de dezembro de 2009.

Profa. Dra. Ana Maria A. Guaraldo
Presidente

Fátima Alonso
Secretária Executiva

CEUA/UNICAMP
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B - Deferimento da Comissão de Ética no uso de Animais (CEUA/UNICAMP):

DECLARAÇÃO

Declaro para os devidos fins que o conteúdo de minha tese de Doutorado intitulada "Participação dos receptores purinérgicos P2X3 e P2X7 na hiperalgesia inflamatória articular em joelho de ratos e o estudo dos mecanismos periféricos envolvidos":

( ) não se enquadra no § 4º do Artigo 1º da Informação CCPG 002/13, referente a bioética e biossegurança.

Tem autorização da(s) seguinte(s) Comissão(ões):

( ) CIBio – Comissão Interna de Biossegurança , projeto No. _____, Instituição: _____.

(X) CEUA – Comissão de Ética no Uso de Animais , projeto No. 2049-1, Instituição: Universidade Estadual de Campinas.

( ) CEP - Comissão de Ética em Pesquisa, protocolo No. _____, Instituição: _____

* *Caso a Comissão seja externa ao IB/UNICAMP, anexar o comprovante de autorização dada ao trabalho. Se a autorização não tiver sido dada diretamente ao trabalho de tese ou dissertação, deverá ser anexado também um comprovante do vínculo do trabalho do aluno com o que constar no documento de autorização apresentado.*

Juliana Maia Teixeira
_____
Aluno: Juliana Maia Teixeira

_____
Orientador: Profa. Dra. Cláudia Herrera Tambeli

Para uso da Comissão ou Comitê pertinente:
(X) Deferido ( ) Indeferido

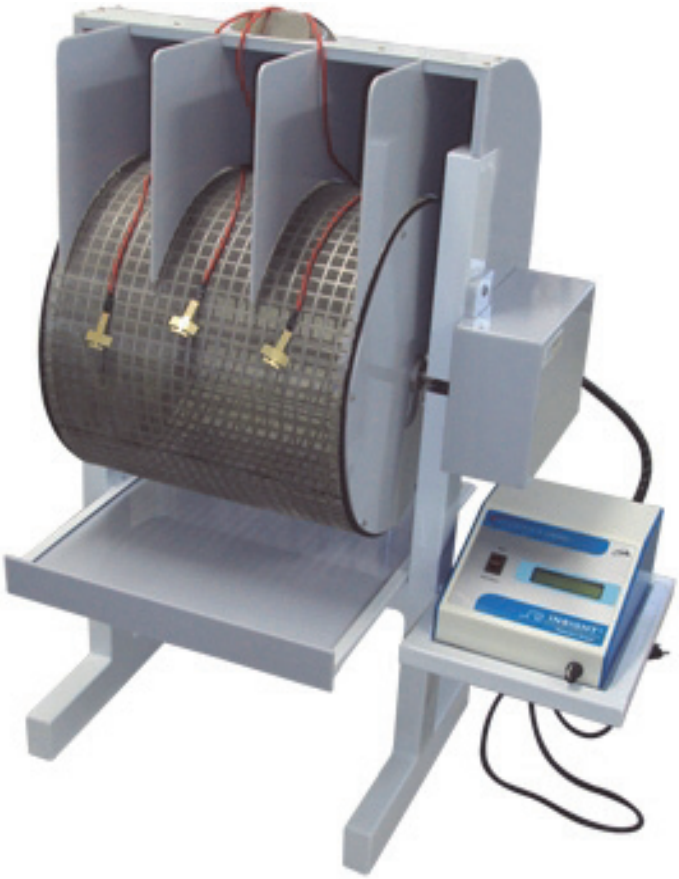
_____
Carimbo e assinatura

Prof. Dr. ALEXANDRE LEITE RODRIGUES DE OLIVEIRA
Presidente da Comissão de Ética no Uso de Animais CEUA/UNICAMP

Para uso da Comissão ou Comitê pertinente:
( ) Deferido ( ) Indeferido

_____
Carimbo e assinatura

C- Equipamento utilizado no Teste de Incapacitação Articular (IA):



Aparelho utilizado no Teste de Incapacitação Articular (IA): A foto apresenta o aparelho utilizado no teste de incapacitação articular (IA) (Modelo EFF 413 - Teste de Incapacitação Articular, Ribeirão Preto, São Paulo, Brasil), que consiste em um tambor rotativo de aço (30 cm de largura x 50 cm de diâmetro), coberto com uma malha de arame inoxidável, que gira a uma velocidade de 3 rpm.